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A comparison of the fluorescence properties of TMA-DPH as a probe for plasma membrane and for endocytic membrane

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Abstract

In earlier studies, the fluorescence probe 1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) was shown to interact with living cells by instantaneous incorporation into the plasma membrane, according to a water (probe not fluorescent)/membrane (probe highly fluorescent) partition equilibrium. This made it interesting both as a fluorescence anisotropy probe for plasma membrane fluidity determinations and as a quantitative tracer for endocytosis and intracellular membrane traffic. In order to ascertain the limiting concentrations for its use in these applications, we performed a systematic study of its fluorescence properties (intensity, lifetime, anisotropy) in the plasma membrane and in endocytic membranes of intact L929 mouse fibroblasts. Some of the experiments were repeated on mouse-bone-marrow-derived macrophages and on phospholipidic LUV to confirm the results. Rather unexpectedly, it was observed that: (i) the incorporation of TMA-DPH into the membranes, monitored by UV absorption measurements, remained proportional to the probe concentration over the wide range explored ($5 \cdot 10^{-7}$ M–$2.5 \cdot 10^{-5}$ M); (ii) however, concerning fluorescence, quenching effects occurred in the membranes above certain critical concentrations. These effects were shown to result from Förster-type resonance auto-transfer; (iii) strikingly, the critical concentrations were considerably higher in early-endocytic-vesicle membranes than in the bulk plasma membrane. It was established that membrane fluidity was involved and this was confirmed by the parallel study on phospholipidic vesicles. Potential applications of these properties as a novel approach for evaluating membrane fluidity are suggested.

Keywords: Plasma membrane; Endocytic membrane; Fluorescence probe; TMA-DPH

1. Introduction

1-(4-(Trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) was first synthetized by Cundall et al. [1], who compared some of its photophysical properties with those of other diphenylhexatriene (DPH) derivatives. These properties were re-examined in detail by Prendergast et al. [2,3]. They described TMA-DPH as a model molecular rotor in lipid bilayer membranes according to the 'wobbling in cone' theory of Kinosita [4,5]. TMA-DPH was considered to better match this theory than the parent DPH molecule, because of being anchored by its cationic moiety at the membrane/water interface. This aspect was confirmed by other authors [6–12]. Furthermore, and for the same reason, when allowed to interact with living cells, TMA-DPH was found to rapidly incorporate into the plasma membrane and to remain attached to its external leaflet [13–17], contrary to DPH which invaded all lipidic regions of cells [18,19]. This property combined with suitable fluorescence characteristics [2,6,20,21], made TMA-DPH an excellent fluorescence anisotropy probe for plasma membrane fluidity determinations in intact cells [22–45].

Unlike DPH, TMA-DPH displays an amphipathic character revealed by partition equilibrium between water (probe not fluorescent) and membranes (high quantum yield) [46,47]. Interestingly, this partition results in the fluorescence intensity being proportional to the cell membrane surface area. It thus provides a simple method for

Abbreviations: C_6-NBD-glucosylceramide, 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoylglucosylsphingosine; DMEM, Dulbecco's modified Eagle's medium; DPH, diphenylhexatriene; DPPC, dipalmitoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; FCS, foetal calf serum; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; PBS, phosphate-buffered saline; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene.

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evaluating membrane surfaces [15,48] or for monitoring membrane surface changes as, for instance, in stimulated exocytosis [16,49–53].

On the other hand, being attached to the plasma membrane in cells, TMA-DPH is bound to follow the membrane intracellular traffic (endo- and exo-cytosis), which is responsible for its progressive internalization. This was actually shown to be the sole cause for its intracellular presence, and, hence, TMA-DPH also behaves as an endocytic marker [54–59]. As such, it displays very original features which will be recalled in this paper. In addition, as it is confined to endocytic compartments, TMA-DPH does not interact with nuclei, which probably explains its remarkable innocuity for cells, even at concentrations higher than 10^{-5} M [55].

In brief, TMA-DPH offers a wide variety of applications for studying membrane dynamics in living cells.

Therefore, it seemed useful to strictly delimit the conditions for its use, and especially, the concentration. Of course, monitoring early endocytosis would require high probe concentrations to enhance sensitivity. With this in mind, we undertook a systematic study of TMA-DPH fluorescence parameters (intensity, lifetime, anisotropy) as a function of the probe concentration. The experiments were performed on L929 cultured mouse fibroblasts, an easy to handle conventional cell line. Parallel measurements were carried out on plasma-and-endocytic membranes. The two were easily distinguished by washing (internal membranes) or not washing the cells (peripheral membrane). The study became much more attractive than expected when TMA-DPH fluorescence self-quenching effects were observed in the membranes and when these effects were found to differ considerably from one type of membrane to the other. Moreover, these quenching effects were found to depend strongly on membrane fluidity levels. In order to confirm the conclusions, complementary experiments were performed on mouse-bone-marrow-derived macrophages, and on phospholipidic large unilamellar vesicles (LUV).

2. Materials and methods

2.1. Cell cultures

Mouse fibroblasts from the L929 cell line were obtained from the American Tissue Collection, Rockville, MD, USA. They were cultured as monolayers in 75 cm^2 plastic culture flasks (Costar, D. Dutscher, France) using Dulbecco's modified Eagle's medium with 4.5 g/l glucose and 3.7 g/l sodium hydrogencarbonate (DMEM, Seromed, ATGC, France) supplemented with 10% heat-decomplemented fetal calf serum (FCS, Biolab, Euromedex, France), 1% 100 mM sodium pyruvate, 1% 200 mM L-glutamine and antibiotics (penicillin, 50 U/ml and streptomycin, 50 μg/ml, both from Seromed, ATGC, France), in a total volume of 30 ml per flask. The cultures were kept in a water-saturated atmosphere of 8% CO_2 in air at 37°C. For the experiments, the cells were recovered at confluency (4 days of culture) by mild trypsination (0.25% trypsin in PBS without Ca^{2+}, Mg^{2+}, Seromed, ATGC, France), and their viability (> 95%) was assessed by Trypan blue exclusion.

Mouse-bone-marrow-derived macrophages were obtained from balb C mouse femora stem cells, as described earlier [54], by differentiation and proliferation in the presence of macrophage colony stimulating factor contained in the L929-layer-supernatant. The cultures were performed in teflon bags (Heraeus, Hanau, Germany) to prevent adherence [60].

2.2. LUV preparation

The method described by Mayer et al. [61] was adapted as follows. Dipalmitoyl- and egg yolk phosphatidylcholine (DPPC, EYPC) multilamellar vesicles (MLV) were prepared first from a film deposited on a flask surface after rotative evaporation of a chloroformic phospholipid solution. The layer was hydrated with PBS for 30 min at 50°C (DPPC) or at room temperature (EYPC). 50°C is 10°C above the transition temperature for DPPC; EYPC has a low transition temperature. MLV were obtained by shaking the suspensions with a vortex for 2 min. LUV were then prepared by extrusion of the MLV suspensions through polycarbonate films (Nuclepore) at 50°C (DPPC) or at room temperature (EYPC). The size of the filter pores was first 0.2 μm (5 passages) and thereafter 0.1 μm (5 passages). The method provided homogenous LUV (0.11–0.12 μm diameter) as checked by light scattering measurements with and N4SD Coultronics nanosizer. For the experiments the suspensions had to be used within 48 h of preparation.

2.3. TMA-DPH labelling and sample preparation

Plasma membrane labelling

For the experiments, the cells were distributed in 3.5 cm diameter Petri dishes (typically $5 \cdot 10^5$ cells in DMEM-10% FCS) for adherence. The layers were then washed with PBS (without Ca^{2+}, Mg^{2+}) and incubated for 10 s at room temperature with TMA-DPH (Molecular Probes, Oregon, USA) at the desired concentration, in 2 ml PBS, from a $4 \cdot 10^{-3}$ M stock solution in dimethylformamide. 10 s were quite long enough to ensure that the probe reached the incorporation equilibrium in the membrane, with negligible internalization in the cells. The cells were recovered quantitatively with a cell lifter (Costar) and suspended homogeneously in the supernatant TMA-DPH solution. Under these conditions, the scraping operation left the cells undamaged; this was checked by comparing the fluorescence intensity of L929 cells recovered in this way with those after trypsination, and it was found to be identical for both methods. Scraping was preferred to trypsination as

being more rapid and because trypsination was inefficient for macrophages. It should be mentioned that scraping by means other than with cell lifter was not harmless. The prepared cell suspensions were used immediately for fluorescence measurements. An aliquot of each cell sample was taken for controlling cell density and viability by Trypan blue exclusion.

Intracellular labelling (endocytic compartments)

The cells were distributed in Petri dishes ($1.0 \cdot 10^6$ cells per dish) for adherence. The cell density was increased to enhance sensitivity. After washing, the layers were incubated at 37°C for the desired time with TMA-DPH, at the desired concentration, in DMEM-10% FCS, and then washed four times for 5 s with cold PBS. This ensured the complete removal of peripheral labelling thanks to TMA-DPH partition equilibrium, without affecting the intracellular labelling. After, this, the cells were recovered in 2 ml PBS by gentle scraping, as above. The cell suspension was immediately used for the fluorescence measurements, which could thus be performed less than 1 min after the end of incubation.

2.4. LUV labelling

LUV labelling with TMA-DPH was obtained by mixing variable volumes of one LUV suspension containing TMA-DPH at the highest concentration, and another TMA-DPH-free LUV suspension.

2.5. Differential UV absorption measurements

In order to determine the constant for the TMA-DPH water/plasma membrane partition equilibrium, the membrane-incorporated probe fraction was measured by UV absorption. To that end, the absorbance at 360 nm of TMA-DPH solutions at various concentrations in PBS was measured with a Cary 4 spectrophotometer, before and after 10 s contact with cell layers of a known density. The experiment was easier with layers, but identical results were obtained with suspensions (after low-speed centrifugation). The membrane-incorporated molar fraction is given by the relative difference in absorbance $\Delta A/A$.

2.6. Fluorescence measurements

Fluorescence intensity

With the cell or LUV samples prepared as described above, the fluorescence intensity measurements were performed at room temperature, under moderate stirring, using a Perkin-Elmer MPF66 spectrofluorimeter set at 360 nm for excitation and 435 nm for emission, with a 5 nm bandwidth. The background level measured on unlabelled controls was negligible.

Fluorescence lifetime

TMA-DPH fluorescence lifetime was determined on similarly prepared samples with an SLM 48000 phase and modulation spectrofluorimeter. Because of the rapid evolution of the system studied (progression of TMA-DPH internalization; cell viability in PBS buffer), the measurements were limited to average lifetimes at one single frequency, which allowed one determination per min. For diphenylhexatriene (DPH) derivatives, satisfactory approximations were obtained with 30 MHz for the excitation light frequency [62]. DPH in tetrahydrofuran was used as a reference ($\tau = 6.7$ ns) [63]. The measurements were made at room temperature, under gentle stirring.

Fluorescence anisotropy

Steady-state fluorescence anisotropy measurements (i.e., under continuous illumination) were performed at room temperature and with gentle stirring, on samples prepared as above, using an SLM 8000 spectropolarofluorimeter. The parameter measured was: $r = (I_{VV} - gI_{VH})/(I_{VV} + 2gI_{VH})$, where g is an instrumental correction factor accounting for the photomultiplier's balance, and I_{VV} and I_{VH} are the emission intensities with polarizers respectively parallel and perpendicular to the direction of the vertically polarized excitation light. The apparatus had a T configuration, allowing I_{VV} and I_{VH} to be obtained simultaneously and r values to be calculated every 5 s. Particular care was taken to operate on homogeneous unicellular suspensions to improve the precision and statistics. The results were corrected for background scattered light, which, at least for intracellular labelling, was not negligible with this apparatus, using unlabelled cell controls, as described earlier [14,46].

3. Results

3.1. Plasma-membrane-incorporated TMA-DPH fraction

Quantitative aspects of TMA-DPH partition had been studied earlier in phospholipidic vesicles [47], but not in cell membranes. The results of the differential UV absorption study on L929 cells are presented in Table 1. They show that the difference in absorbance ΔA, before and after interaction with the cells, is proportional to the probe concentration over the wide range covered ($5 \cdot 10^{-7}$ to $2.5 \cdot 10^{-5}$ M) and, thus, that the fraction incorporated $f = \Delta A/A$ is constant, for a given cell density. For instance, f was 4% for a typical density of $2.5 \cdot 10^5$ cells/ml in our experiments. It is noteworthy that the TMA-DPH molar extinction coefficient in PBS inferred from these measurements was $\varepsilon = 40\,000 \pm 2000$ M^{-1} cm^{-1}, which may be compared to the value of 30 000 reported by Prendergast et al. [2] for TMA-DPH in dimethylformamide. Table 1 also shows that varying the cell density resulted in a proportional variation in the incorporation.

Similar conclusions were obtained for bone-marrow-derived macrophages, with a membrane-incorporated fraction 1.8-times higher.

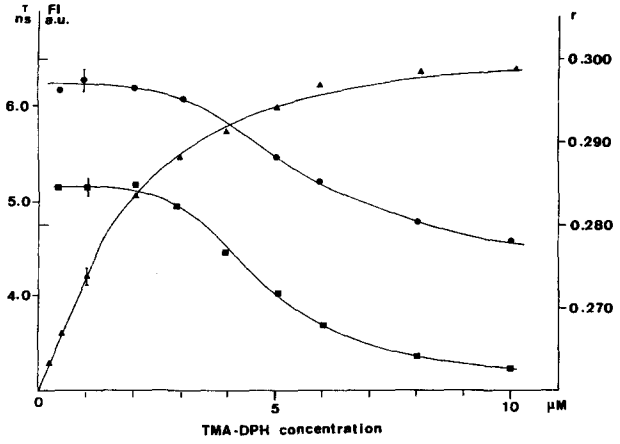

Fig. 1. TMA-DPH concentration dependance of fluorescence parameters: intensity (*FI*, ▲), lifetime (τ, ●) and anisotropy (r, ■), for the probe incorporated into L929 cell plasma membrane. $5 \cdot 10^5$ L929 adhered cells were incubated for 10 s at room temperature with various TMA-DPH concentrations in 2 ml PBS. After cell recovery by gentle scraping, measurements were performed at 435 nm (excitation 360 nm) with respectively a Perkin-Elmer 366 spectrofluorimeter, an SLM 48000 phase and modulation spectrofluorimeter and an SLM 8000 spectropolarofluorimeter. The results denote the average from four determinations, and error bars, typical S.D.

3.2. Concentration dependence of TMA-DPH fluorescence parameters for the probe incorporated into the cell plasma membrane

The results are presented in Fig. 1 for L929 cells. For a given cell density ($2.5 \cdot 10^5$ cells/ml), the fluorescence intensity, *FI*, varied proportionally to the TMA-DPH concentration up to 2 μM (the concentration reported is that of the TMA-DPH solution in PBS before interaction with the cells). For higher concentrations, the curve inflected abruptly, albeit without levelling off. For a given TMA-DPH concentration, *FI* increased proportionally to cell density, as observed earlier [46], over the range explored, $1.0 \cdot 10^5$ to $3 \cdot 10^6$ cells/ml (not represented).

Similar trends were observed for macrophages, with an inflexion of the fluorescence intensity curve at 3 μM.

The TMA-DPH fluorescence lifetime τ remained constant, 6.2 ± 0.2 ns, below the same critical concentration of 2 μM, but decreased significantly with higher concentrations. This decrease, however, slowed down above 5 μM.

The TMA-DPH fluorescence anisotropy, r, displayed a similar evolution to the fluorescence lifetime. It was first constant, 0.283 ± 0.003, again until 2 μM, and then decreased rapidly to 0.270 ± 0.003 with 5 μM, continuing to fall, but at a lower rate with higher concentrations.

The corresponding values for macrophages were $\tau = 6.3 \pm 0.2$ ns below 3 μM with a decrease parallel to that found in L929 cells for higher concentrations. Fluorescence anisotropy was 0.272 ± 0.003 below the critical concentration, and decreased first to 0.262 ± 0.003 for 6 μM, and then at a lower rate, as in L929 cells.

3.3. Concentration dependence of TMA-DPH fluorescence parameters for the probe internalized by endocytosis

An extensive endocytosis kinetic study with variable TMA-DPH concentration would have led to a very intricate discussion. Of course, TMA-DPH concentration does change during the evolution of endocytosis, owing to dilution in the successive compartments; besides, membrane fluidity changes too because vesicles lose their clathrin coat [58,64]. Consequently, we preferred to confine the experiments to one short incubation time (2 min) at 37°C in DMEM-10% FCS, so as to limit the location of the probe after cell washing in early endocytic vesicles. For the needs of the discussion, one exception was made, namely for the fluorescence lifetimes, which were also

Table 1
Determination of the membrane-incorporated TMA-DPH in L929 cells by differential absorption measurements

	Cell density	[TMA-DPH] during incubation (μM)							
	(10^5/ml)	0.5	1.0	2.0	5.0	8.0	10.0	15.0	25.0
ΔA	10	0.003	0.007	0.012	0.032	0.052	0.064	0.094	0.150
f%	10	15	17.5	15	16	16	16	16	15
ΔA	5			0.006	0.014		0.032	0.048	0.082
f%	5			7.5	7.0		8.0	8.0	8.0
ΔA	2.5			0.003	0.008		0.018		0.040
f%	2.5			4.0	4.0		4.5		4.0

L929 cells (respectively 2.0, 1.0 and $0.5 \cdot 10^6$) were allowed to adhere in Petri dishes, carefully washed with PBS, so as to eliminate any non adherent-material, and incubated for 10 s at room temperature with TMA-DPH at various concentrations, in 2 ml PBS. 10 s were quite long enough to ensure partition equilibrium was reached with the plasma membrane, with negligible internalization. We checked that adherence to the support did not modify the partition kinetics or rates. The incorporated fraction, f, was obtained from the relative difference in absorbance at 360 nm $\Delta A/A$ between the cell supernatant and a control solution. A molar extinction coefficient $\varepsilon = 40\,000 \pm 2000\ M^{-1}\ cm^{-1}$, determined in this study, was used for TMA-DPH in PBS. Measurements were performed with a Cary 4 spectrophotometer. The reported values denote the average from six separate determinations, with a typical S.D. of 0.001.

studied after 5 min incubation. The results are gathered in Fig. 2 for L929 cells. They differ considerably from those observed in the plasma membrane. The fluorescence intensity increased proportionally to TMA-DPH concentration up to 10 μM, as against 2 μM in the plasma membrane. The inflexion of the fluorescence lifetime curve (2 min incubation) was also shifted to 10 μM and the further decrease was less pronounced than in the plasma membrane. For the 5 min incubation, the data lay between those for the 2 min incubation and those found in the plasma membrane. Fluorescence anisotropy values were much higher than in the plasma membrane (0.330 ± 0.003) below the critical concentration of 10 μM, as against 0.283 $\pm$ 0.003 in the plasma membrane. Similar trends were again observed in macrophages (2 min incubation). The critical concentration was 10 μM, as in L929 cells, thus, less shifted, and the decrease in fluorescence lifetime above this concentration was rather more pronounced than in L929 cells (from 6.3 ± 0.4 ns for 10 μM to 5.4 ± 0.3 ns for 50 μM).

3.4. TMA-DPH fluorescence properties in phospholipidic LUV

The data for DPPC and EYPC LUV are shown in Fig. 3. They indicate that, under identical conditions (the same phospholipid and the same probe concentration), the fluorescence intensity is higher in EYPC than in DPPC vesicles, and that the linearity of this parameter with the probe concentration falls at a lower concentration (2.5 μM) for EYPC than for DPPC (5 μM). It should be mentioned that vesicles with identical phospholipidic polar heads were selected in order to prevent trivial incorporation differences for TMA-DPH.

The fluorescence anisotropy in the linear region was markedly different for the two vesicle types: $r = 0.175$ for EYPC and 0.277 for DPPC, without any significant difference in fluorescence lifetime ($\tau = 5.8 \pm 0.2$ ns).

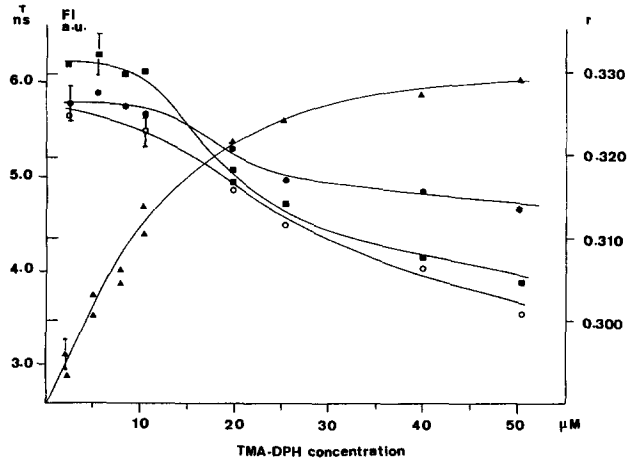

Fig. 2. Intracellular TMA-DPH fluorescence parameters measured after 1 min endocytosis in L929 cells, as a function of probe concentration. $1.0 \cdot 10^6$ L929 cells were allowed to adhere in Petri dishes and incubated at 37°C for 2 min with TMA-DPH, at various concentrations, in DMEM-10% FCS. After thorough washing with cold PBS (see Section 2), the cells were resuspended in 2 ml PBS after gentle scraping, and the fluorescence parameters, intensity (*FI*, ▲), lifetime (τ, ●; incubation 5 min, ○), anisotropy (r, ■) were measured immediately, as for the probe incorporated into the plasma membrane. The results represent the average from five determinations, and error bars, typical S.D.

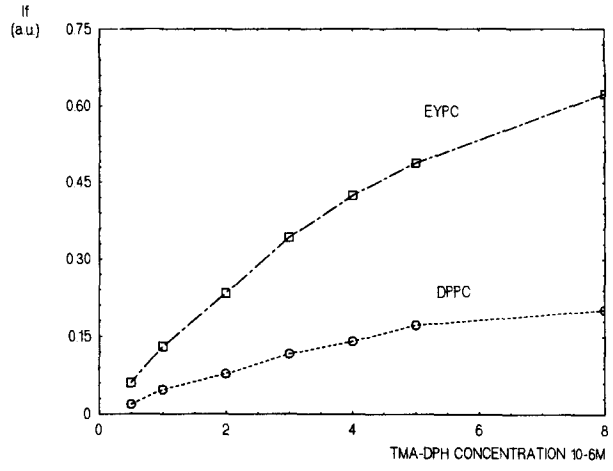

Fig. 3. DPPC and EYPC LUV were prepared and labelled with TMA-DPH, as described in Section 2. The fluorescence intensity (*If*) was measured as a function of the probe concentration, under identical conditions for EYPC (□) and DPPC (○), so values are directly comparable. Fluorescence anisotropy measured at $2 \cdot 10^{-6}$ M was 0.175 ± 0.002 for EYPC and 0.277 ± 0.002 for DPPC. The experiment was repeated three times with, no significant difference.

4. Discussion

4.1. Quantitative aspects of TMA-DPH partition equilibrium

In earlier studies [46], the TMA-DPH partition equilibrium was inferred from the proportion of fluorescence intensity to the amount of membrane, for a given probe concentration. From a kinetic point of view, it could also be established that the equilibrium took place instantaneously in either way. These properties were confirmed here with TMA-DPH titrations by UV absorption (Table 1). The data from Table 1 enable us to evaluate the equilibrium constant, K, according to:

$$K = [\text{TMA-DPH}]_{PM}/[\text{TMA-DPH}]_{aq} = f/\left(1 - f\frac{V}{v}\right)$$

(V being the volume of the external medium and v, that of the plasma membrane external leaflet in the sample and f, the TMA-DPH-membrane-incorporated fraction). Introducing cell dimensions: r, the cell radius, d, the membrane leaflet thickness, and n, the cell density, this expression becomes:

$$K = f/\left(1 - f\left(\frac{1}{4\pi r^2 dn}\right)\right)$$

Taking d as 2.5 nm, and r as 6.5 μm (L929) and 8.2 μm (macrophages) (from measurements performed in the laboratory) the values of K are respectively $1.1 \cdot 10^5$ for L929 cell and $1.2 \cdot 10^5$ for macrophages. These values are probably overestimated since, even though they are spherical shaped in suspension, the cells display more or less ruffles at their external membrane, which increases their surface. Nevertheless, K values denote a high affinity for cell membranes. Indeed, they allow the TMA-DPH to membrane lipids ratio to be approximated according to:

$$R = \frac{cf}{4\pi r^2 dn} \rho\alpha/M$$

where, in addition to the symbols used above, ρ is the volumic mass of the membrane (≈ 1000 g l^{-1}), α, the proportion of lipids (≈ 0.5, w/w), and M, the average lipid molecular mass (≈ 550 g, including cholesterol). For a typical TMA-DPH concentration of 1 μM, R is thus found to be ≈ 0.1 in L929 cells, which is often considered as a limit to the use of a probe. However, the following arguments indicate that from a biological point of view, TMA-DPH could be used as a probe at higher concentrations: (i) the TMA-DPH titrations by UV absorption (Table 1) show that the probe incorporation into the membrane remains linear. No change in the incorporation kinetics is observed either. This is probably because TMA-DPH is a small rod shaped molecule likely to insert into the spaces between membrane lipids without modifying their arrays; (ii) endocytosis kinetics monitored with TMA-DPH did not depend on its concentration, and endocytosis yields remained proportional to the probe concentration over the range studied; (iii) microscopic observations failed to reveal any morphological changes ($\times 100$ objective) at the highest TMA-DPH concentrations; (iv) L929 cells were cultured for several days in the presence of $4 \cdot 10^{-5}$ M TMA-DPH without any apparent change in the cell growth or functioning. Still, as reported in this work, high TMA-DPH concentrations do induce modifications in the fluorescence properties, which certainly limit its probe capacities.

4.2. Evolution of the fluorescence parameters for TMA-DPH incorporated into the plasma membrane, as a function of concentration

The question immediately raised by the fluorescence intensity curves is the marked inflexion at this outstanding critical concentration of 2 μM (3 μM for macrophages). Other authors [65] have obtained similar curves previously for TMA-DPH in human platelets. They explained the inflexion by saturation effects. We cannot agree with this interpretation, at least under the present systems, since our results (absorbance data) clearly show that the incorporation remains proportional to the probe concentration far above the critical concentration. An interpretation based on the occurrence of screening effects, i.e., reduced fluorescence intensity due to the fluorophore itself absorbing excitation light, could be ruled out too, since, using a 2 mm instead of a 10 mm optical path quartz cuvette did not affect the curve trends. Besides fluorescence intensities were duly corrected for such effects according to [66]. A more plausible explanation might be TMA-DPH self-quenching due to the high probe to lipid ratio (see above section). More precisely, as TMA-DPH fluorescence spectra are unchanged (not shown) the mechanism could be fluorescence resonant energy transfer (FRET) [67–71]. Within this framework, auto-transfer between TMA-DPH molecules would cause fluorescence quenching, increasing the probability of capture in non fluorescent traps [72,73]. The conditions for FRET to occur are in fact met; (i) TMA-DPH emission and excitation spectra display an overlapping zone (Fig. 4); (ii) the critical Förster's FRET intermolecular distance is given by [69]

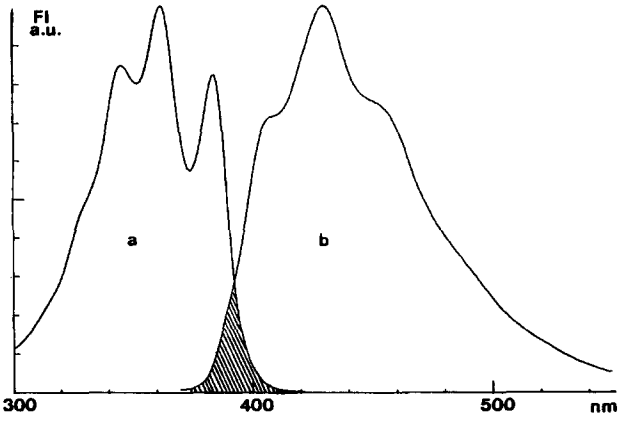

Fig. 4. TMA-DPH ($1.0 \cdot 10^{-6}$ M) excitation (a) and emission (b) spectra, in small unilamellar phosphatidylserine vesicles, obtained with an SLM 48000 spectrofluorimeter. The spectra exhibit an overlapping region, consistent with the occurrence of fluorescence resonance energy transfer.

$$R_0^6\,(\mathrm{cm}^6) = (8.79 \cdot 10^{-25})\, K^2 \eta^{-4} \Phi_0 J$$

where the overlapping integral J was computed form TMA-DPH spectra: $J = 7.7 \cdot 10^{-16}$ cm^2, the refraction index η was taken as 1.4 for lipids, the TMA-DPH unquenched quantum yield was estimated at 0.69 by comparison with the known data for DPH [74], and the orientation factor K^2 was considered to be 1 (parallel molecules) [69]. This led to $R_0 = 22$ Å, while the intermolecular TMA-DPH distance at the critical concentration of 2 μM was easily calculated from the probe/lipid ratio (see above) and found to be 17 Å.

In addition, FRET might account for the particular shape of the fluorescence lifetime curves (Fig. 1). As a matter of fact, when the probe concentration is increased above the critical concentration, more and more couples of TMA-DPH molecules may be involved in the transfer process. However, when every probe molecule has a neighbour close enough for the transfer to occur, then additional incorporation does not continue to increase the

transfer probability at the same rate, which would explain the slowing down observed in the decrease in lifetime. Finally, the fluorescence anisotropy data in Fig. 1 are also consistent with this mechanism. Indeed, fluorescence depolarization may be explained by energy transfer from primarily excited molecules to other molecules of the same kind but with a slightly different orientation of their transition dipoles [71]. The fall-off in the decrease of fluorescence anisotropy could then be interpreted like that observed for fluorescence lifetime.

4.3. Evolution of TMA-DPH fluorescence parameters in the membrane internalized by endocytosis

The main difference between the TMA-DPH fluorescence properties in the membrane of early endocytic vesicles and in the bulk plasma membrane of the studied cell systems, is that the inflexion of the fluorescence curves is shifted towards much higher probe concentrations. A first possible explanation for this phenomenon could be phase lipid separation [75,76], i.e., the membrane lipidic composition not being the same in the endocytic regions as in the rest of the plasma membrane. This would result, for instance, in lower TMA-DPH incorporation in the former. However, this interpretation, despite its attractiveness, should be rejected. It has in fact been shown by Belcher et al. [77] that in rat hepatocytes the membrane lipidic composition of the endosomal fraction is the same as in the plasma membrane. This has been corroborated by our results on TMA-DPH fluorescence anisotropy [58] in L929 cells, showing that membrane fluidity was identical in the plasma membrane, in the endocytic compartment, and even in early endocytic vesicles provided they had lost their clathrin coat. Furthermore, incorporation particularities for TMA-DPH in the plasma membrane endocytic regions may be ruled out on the following grounds: in a recent experiment [59] using in parallel TMA-DPH and C_6-NBD-glucosylceramide [78] another endocytic marker of a totally different nature, the endocytic yields and kinetics were found to be exactly the same.

We here propose a more reliable interpretation based on membrane fluidity features. It has been observed [58] that in L929 cells early endocytic vesicles display a higher lipidic order than that of the plasma membrane, owing to interactions of their membrane with the clathrin coat. This property has been confirmed in isolated bovine-brain endocytic vesicles [64] and has been observed again in the present study: TMA-DPH fluorescence anisotropy was 0.330 in the early endocytic stage (incubation 2 min) (Fig. 2) against 0.283 in the plasma membrane. Incorporation particularities being ruled out, we suggest that the TMA-DPH fluorescence quenching effects themselves are less in the less fluid endocytic regions than in the bulk plasma membrane. Not that the transfer is less efficient, but because the chances of encountering non-radiative traps are decreased: either the traps move less easily in a rigid membrane, or their concentration diminishes. Of course, an interesting trap candidate could be water (we have checked that molecular oxygen does not affect TMA-DPH fluorescence properties). Experimentally, TMA-DPH fluorescence is totally quenched in the presence of water. Besides, the presence of permeant water, even in the hydrophobic core of the membrane, is well established [79–81]. Moreover, Perochon et al. [82] have shown that reducing membrane fluidity with cholesterol was accompanied by a decrease in permeability to water. When early endocytic vesicles loose their clathrin coat after a few minutes of internalization [83], the cause of the increase of membrane rigidity is suppressed and the vesicle membrane fluidity increases to match that of the bulk plasma membrane [58]. This should then result in more quenching effects, which is actually observed for a 5 min incubation (Fig. 2). Finally, the influence of membrane fluidity on TMA-DPH fluorescence quenching, also appears in the experiments with phosphatidylcholine LUV (Fig. 3). The inflexion of the curve is observed at a higher TMA-DPH concentration in DPPC vesicles ($r = 0.277$) than in the more fluid EYPC ($r = 0.175$).

5. Conclusion

In this study, we first examined the quantitative features of TMA-DPH incorporation into cell membranes. For L929 cells and macrophages, the constant for the partition equilibrium between the plasma/membrane and the external buffer, $K = [\text{TMA-DPH}]_{mb}/[\text{TMA-DPH}]_{aq}$, was found to be in the order of magnitude of 10^5, with little difference between the two cell types. This denoted a strong affinity of the probe for membranes and confirmed its relevancy in evaluating membrane surface areas. No saturation in the membrane was observed in the probe concentration range covered, but fluorescence quenching did occur at critical concentrations. These quenching effects were shown to result from Förster-type fluorescence resonant auto-transfer. Comparing these effects in the plasma membrane and in the membrane internalized by endocytosis revealed considerable differences and strongly suggested an influence of membrane fluidity: the higher the lipidic order, the lower the quenching effects. This, of course, might introduce a novel approach to evaluating membrane fluidity, by determining the critical TMA-DPH concentration at which quenching effects appear. The method could indicate the tightness of the lipidic packing in the membrane, i.e., the capacity of the membrane to be more or less permeant for quenching trap molecules, such as water.

Finally, one aim of this study was to define the limiting concentrations at which TMA-DPH could be used in endocytosis. The results unfortunately indicate that, because of the complex quenching effects, the concentration of the probe (in the external medium) should be kept below 2 μM, at least for quantitative assays.

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