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Diphenylhexatriene–Phosphatidylcholine Fluorescence in POPC Vesicles: Comparison of the Exponential-Series and the Maximum-Entropy Methods

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We have investigated the time-resolved fluorescence of diphenylhexatriene (DPH) covalently linked to phosphatidylcholine (PC) in palmitoyloleoylglycerophosphocholine (POPC) vesicles with special consideration of the comparison of two methods for distributional lifetime analysis: the exponential-series method (ESM) and the maximum-entropy method (MEM). Generally, both methods were found to reveal equivalent results on high-quality data. Different are the shapes of the recovered distributions (symmetry and width) as well as the time effort for the numerical analysis.

KEY WORDS: Distributional analysis; diphenylhexatriene; fluorescence-labeled vesicles.

INTRODUCTION

Recently, unbiased numerical methods that reveal underlying lifetime distributions $\Phi(\tau)$ from nonexponential fluorescence decays have been established instead of, or additional to, the multiexponential approach. For single-photon counting data, two methods were established for the recovery of lifetime distributions: the exponential-series method [1] and the maximum-entropy method [2]. In both computational techniques the nonexponential δ-pulse fluorescence response $F(t) = \mathcal{L}\Phi(\tau)$ is approximated by a coarse discretization of the Laplace operator $\mathcal{L}$. By using terms of a series of fixed lifetimes τ_i, the corresponding discrete set of amplitudes Φ_i is evaluated from the convolution integral $H(t) = C\mathcal{L}\Phi(\tau)$ (C is the convolution with the lamp). In the ESM analysis [3] a Tikhonov regularization function is necessary to stabilize the ill-posed inverse problem, whereas in the MEM the Shannon–Jaynes entropy [4] is used to overcome artificial oscillations in the inversion of the fluorescence profile.

For chromophores that either can exist in multiple conformations or are integrated in a macromolecular matrix, distributed fluorescence deactivation pathways are assumed due to conformational changes or distinct conformations noninterconvertible on a fluorescence time scale [5]. These distributions led to the concept of distributed fluorescence lifetimes which reflect the accessibility of certain interactions of the chromophore with any quenching functional group available. Since our chromophore, diphenylhexatriene, is a complex molecule itself, which is, moreover, integrated in a lipid vesicle, the assumption of distributed fluorescence lifetimes is justified in the above sense.

The sensitivity provided by fluorescence spectroscopy regarding environmental as well as dynamical parameters makes it attractive for the investigation of

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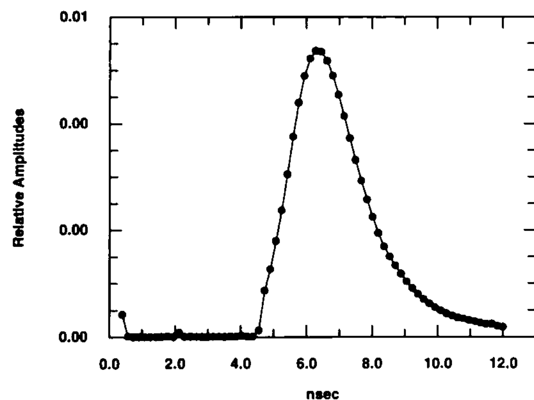

Fig. 1. ESM analysis of vesicles consisting of 4 m*M* DPH-PC in 400 m*M* POPC, recorded at room temperature at $\lambda_{ex}/\lambda_{em}$ = 360/460 nm. Seventy lifetimes were spaced over a linear time scale.

chromophore-labeled artificial membrane systems. In this study we compare the results of ESM and MEM obtained from the fluorescence decay of diphenylhexatriene (DPH) covalently linked to phosphatidylcholine (PC). A selected example of label-lipid ratio has been chosen for the comparison of ESM and MEM. Details of the study will be presented elsewhere (Prenner *et al.*, manuscript in preparation).

The availability of a wide range of fluorescence decay data made DPH-PC a useful label to compare two methods of mathematical/statistical data interpretation. Since the fluorescence decay of DPH is affected by the molecular composition [6] as well as by the dielectric constant of the medium [7], the lifetime heterogeneity (multiplicity of excited states, revealed by distributional analysis of the labeled lipid fluorescence decay) is proposed to reflect environmental heterogeneity when the chromophore is integrated in a (natural or artificial) membrane.

MATERIALS AND METHODS

Lipid Synthesis. DPH-PC was prepared according to Kalb *et al.* [8].

Vesicle Preparation. Unilamellar vesicles were prepared by the ethanol injection method [9] and stored overnight at 4°C in the dark.

Fluorescence Measurements. Steady-state fluorescence measurements were performed on a SLM 8000C fluorometer (SLM Instruments, Urbana, IL). Time-resolved fluorescence measurements were performed as described previously [10].

Transient Data Analysis. Details of the ESM analysis are given by Landl *et al.* [3]. MEM analysis essentially works with the same algorithms as the ESM. The main difference is that the substitution Eq. (2.4) in Ref. 3 is not performed and that the entropy term $\int\Phi(\tau)\ln[\Phi(\tau)/m(\tau)]d\tau$ is used to regularize the problem. The prior probability m was set to $m(\tau)\propto 1/\tau$, as proposed in Ref. 4. An exact description of the algorithm is given in Chapter 4 of Ref. 11.

RESULTS AND DISCUSSION

The steady-state excitation and the emission spectra of DPH-PC in POPC vesicles did not shift significantly upon variation of the emission and excitation wavelength, respectively. Excitation maxima were found at 346, 363, and 383 nm. The emission spectrum is the mirror image of the excitation spectrum (same electronic transition), with maxima at 407, 433, and 458 nm.

For evaluating the effect of the lipid matrix on the fluorescence decay of DPH-PC, a lipid-free 50 μ*M* solution of DPH-PC in DMSO/MeOH (1/19) has been recorded at $\lambda_{ex}/\lambda_{em}$ = 360/430 nm. Both ESM and MEM yielded a sharp unimodal distribution pattern centered at 3.83 ns, the MEM recovered distribution being very narrow. This points to a homogeneous environment of DPH-PC in solution and/or to fast interconverting conformations. Since DPH-PC is able to adapt intramolecularly to many conformations, we expect rapidly interconverting species rather than a homogeneous environment for DPH.

For the purpose of clarity we confine ourselves to presenting and discussing the data set recorded at $\lambda_{ex}/\lambda_{em}$ = 360/460 nm at the molar ratio label/lipid = 1/100 (details of the study will be presented elsewhere). In this case the label is very diluted and intermolecular fluorescence deactivation between label molecules is therefore possible only in clusters of DPH-PC.

In contrast to the decay recorded at the maximum of emission (430 nm), the distributional analysis of fluorescence data recorded at the red edge yielded a unimodal lifetime distribution (Figs. 1 and 2). The broad distribution found by ESM and by MEM is the result of the multitude of deactivation channels which DPH-PC is able to undergo. These deactivation pathways are attributed mainly to lipid-label interactions, because no significant shift in the lifetime center, when the fluorescence decays of the label/lipid ratios 1/20 (data not shown) and 1/100 are compared, was found. The short-lived component, which makes a minor contribution to

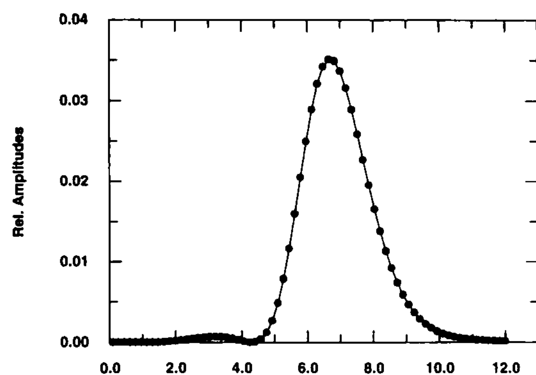

Fig. 2. MEM analysis of vesicles consisting of 4 m*M* DPH-PC in 400 m*M* POPC, recorded at room temperature at $\lambda_{ex}/\lambda_{em}$ = 360/460 nm. Seventy lifetimes were spaced over a linear time scale.

the DPH-PC decay in the blue edge and at the maximum of emission (independent on the concentration of the label in the vesicles), does not occur in this energy range.

ESM reveals a lifetime center at 6.65 ns (Fig. 1), which is in good agreement with the MEM value, centered at 6.28 ns (Fig. 2). The small difference between the respective centers in the two analysis methods should be a consequence of the different distributional shapes. We found the MEM recovery, in most cases, to be more asymmetrical than the ESM pattern, which may be of future interest when a certain photophysical model could be associated with the third moment of the distributions. However, we are not sure yet whether this is only a phenomenon inherent to the method, maybe due to the particular choice of the prior probability. Nevertheless, both methods were shown to work well and yield comparable results on high-quality data sets, i.e., the number of counts recorded in the peak channel maximum should exceed $2 \cdot 10^4$. Extensive work on synthetic data has shown that our ESM approach is less sensitive to the choice of a regularization parameter [3] compared to the MEM. This could be due to the particular ansatz used in the ESM program, which already has some smoothing effect [3]. Moreover, the ESM needs less CPU time (on a Micro VAX II). We conclude that, if both methods are available for the analysis of time-resolved fluorescence data, the advantage of ESM is the economic output of reliable data. However, MEM extracts information from high-quality data, which are not accessible the ESM. This is (i) the asymmetry of the lifetime distributions and (ii) the fact that, due to the principally more narrow distributions, contributions to the fluorescence decay with a low amplitude, which may be covered by the more broad ESM distributions, are found by MEM.

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