Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1768 (2007) 467–478
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Location and dynamics of acyl chain NBD-labeled phosphatidylcholine (NBD-PC) in DPPC bilayers. A molecular dynamics and time-resolved fluorescence anisotropy study

Luís M.S. Loura [a,b,*], J.P. Prates Ramalho [a,c]

[a] Centro de Química and Departamento de Química, Universidade de Évora, Rua Romão Ramalho, 59, 7000-671 Évora, Portugal
[b] Centro de Química-Física Molecular, Complexo I, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[c] Centro de Física Teórica e Computacional, Av. Prof. Gama Pinto, 2, 1649-003 Lisboa, Portugal

Received 21 July 2006; received in revised form 9 October 2006; accepted 18 October 2006
Available online 27 October 2006

Abstract

100-ns molecular dynamics simulations of fluid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers, both pure and containing 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) acyl-chain labeled fluorescent analogs (C6-NBD-PC and C12-NBD-PC), are described. These molecules are widely used as probes for lipid structure and dynamics. The results obtained here for pure DPPC agree with both experimental and theoretical published works. We verified that the NBD fluorophore of both derivatives loops to a transverse location closer to the interface than to the center of the bilayer. Whereas this was observed previously in experimental literature works, conflicting transverse locations were proposed for the NBD group. According to our results, the maximum of the transverse distribution of NBD is located around the glycerol backbone/carbonyl region, and the nitro group is the most external part of the fluorophore. Hydrogen bonds from the NH group of NBD (mostly to glycerol backbone lipid O atoms) and to the nitro O atoms of NBD (from water OH groups) are continuously observed. Rotation of NBD occurs with ~2.5–5 ns average correlation time for these probes, but very fast, unresolved reorientation motions occur in <20 ps, in agreement with time-resolved fluorescence anisotropy measurements. Finally, within the uncertainty of the analysis, both probes show lateral diffusion dynamics identical to DPPC.

Keywords: Fluorescence probes; NBD-labeled lipids; Molecular simulation; Membrane model system; Membrane penetration depth

1. Introduction

Fluorescence has been used as a major tool to study lipid bilayer structure for several decades now. Being natural lipids, with very few exceptions, nonfluorescent, fluorescence spectroscopy and microscopy techniques rely on the use of extrinsic membrane probes [1]. These can be lipophilic fluorophores of non-lipid nature (e.g., pyrene, diphenylhexatriene (DPH)), or design fluorophores linked to a lipid moiety.

Among the latter, a popular family is that of phospholipids labeled with the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) fluorophore in one of the acyl chains (see [2] for a review). NBD derivatives are commercially available for all major phospholipid classes, and have been used extensively as fluorescent analogues of native lipids in biological and model membranes to study a variety of processes ([2–4]). As a fluorophore, NBD possesses convenient photophysical properties, such as good fluorescence quantum yield, environment sensitivity, and suitability for fluorescence resonance energy transfer experiments, as a donor (namely to rhodamine-based probes e.g., [5,6]) or acceptor, e.g., to DPH probes [7], and in homotransfer studies of lipid aggregation.

However, as with any extrinsic probe, two major points of concern arise when using NBD-acyl-chain-labeled lipids to report on membrane structure: The first, which will be addressed in this article, is the behaviour of the probe molecules inside the bilayer: what region of the bilayer is the probe

* Corresponding author. Centro de Química-Física Molecular, Complexo I, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal. Fax: +351 218464455.
E-mail address: pclloura@alfa.ist.utl.pt (L.M.S. Loura).

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.10.011

sensitive to (that is, the probe transverse location) and its translational and rotational dynamics. The second point, which will be addressed elsewhere, is the magnitude of perturbation induced by the probe on the host lipid structure.

These questions are especially important given that the NBD moiety, if labeled at the end of an acyl chain, is prone to loop or "snorkel" to the water/lipid interface, due to the chromophore polarity and the acyl chain flexibility. This effect has been observed using both fluorescence and NMR spectroscopy, although its precise magnitude is not totally certain. By determining the rate constant for non-radiative excited-state decay, and taking into account its dependence on the local dielectric constant, Mazères et al. [3] proposed an external location, near the phosphate group. Fluorescence quenching data analyzed with the parallax method indicate an external location of 1-palmitoyl, 2-[12 -amino]dodecanoyl-*sn*-glycero-3-phosphocholine (C12-NBD-PC), about 1.9–2.0 nm from the center of 1,2-dioleyol-*sn*-glycero-3-phosphocholine bilayers (DOPC), and thus near the phosphate or even choline groups of the host lipids [8]. However, the same technique had previously yielded a much smaller value (1.22 nm, near the glycerol backbone/carbonyl lipid region) for C12-NBD-PC and its shorter *sn*-2 acyl chain relative 1-palmitoyl, 2-[6-NBD-amino]hexadecanoyl-*sn*-glycero-3-phosphocholine (C6-NBD-PC) in DOPC [9]. More recently, using NMR cross-relaxation rate measurements, Huster et al. [10] again obtained a location near the glycerol backbone/carbonyl region for both C12-NBD-PC and C6-NBD-PC, and a slightly more external location for the C12 derivative relative to the C6 counterpart was proposed (taking into account more efficient fluorescence quenching of the C12 probe by aqueous quencher dithionite).

Molecular dynamics (MD) simulations can be used to obtain detailed atomic-scale information on phospholipid bilayers [11,12]. MD Simulations of fluorescent probes DPH [13,14] and pyrene [15] in lipid membranes were recently reported. Although surprisingly few in number, these works showed the suitability of MD for calculation of a variety of properties of fluorescence probes in the bilayer, as well as their effect on the organization of the latter.

In the present study, we used 100-ns MD simulations of C6-NBD-PC and C12-NBD-PC in fluid phase 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers, to determine the transverse location of the fluorophore, as well as its preferred orientation in the membrane and translational and rotational dynamics. The results are compared with both literature experimental and theoretical data, and, for rotational dynamics, also original time-resolved fluorescence polarization measurements were carried out. The main findings of this work are: (i) the NBD moiety loops in the direction of the water/lipid interface. It has a broad transverse distribution in the bilayer, with a maximum located around the glycerol backbone/carbonyl region; (ii) NBD has a wide orientation range in the bilayer, the NO_2 group being the region of the fluorophore closest to the water/lipid interface; (iii) the NBD NH group is involved in hydrogen bonding to phospholipid glycerol backbone O atoms, and, for the case of C6-NBD-PC, these H bonds are predominantly intramolecular (whereas for C12-NBD-PC they are exclusively intermolecular); (iv) the theoretical rotational dynamics of the NBD group agree with the experimental fluorescence anisotropy decays, both pointing to average rotation correlation times of ~5 ns and (v) lateral translation diffusion coefficients of the NBD-PC probes are identical to that of the host lipid DPPC.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, Fig. 1A), 1-palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-*sn*-glycero-3-phosphocholine (C6-NBD-PC, Fig. 1B) and 1-palmitoyl-2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoyl]-*sn*-glycero-3-phosphocholine (C12-NBD-PC, Fig. 1C) were obtained from Avanti Polar Lipids (Birmingham, AL). 4-(2-hydroxyethyl)-1-piperazine sulfonic acid (HEPES), KOH and KCl (all from Merck, Darmstadt, Germany) were used to prepare the buffer solution 20 mM HEPES–KOH (pH 7.4). All organic solvents were of spectroscopic grade and came from Merck (Darmstadt, Germany). Deionized water was used throughout. All above materials were used without further purification. The concentrations of stock solutions of the probes were determined spectrophotometrically using ε(NBD-PC, 465 nm, in C_2H_5OH)=2.2×10^4 M^{-1} cm^{-1} [16].

2.2. Lipid vesicle preparation

Large unilamellar vesicles (LUV, ~100 nm diameter), containing the desired mole ratio of DPPC and NBD-PC, were prepared by extrusion of lipid dispersions through 100-nm pore diameter polycarbonate membranes as previously described [17]. The resulting lipid dispersions were stored at room temperature and used within 24 h of preparation. The concentrations of phospholipid stock solutions were determined using phosphate analysis [18].

2.3. Instrumentation

Absorption spectroscopy was carried out with a Jasco V-560 spectrophotometer. When necessary, absorption spectra were corrected for turbidity using the method of Castanho et al. [19]. Steady-state fluorescence measurements were carried out with an SLM-Aminco 8100 Series 2 spectrofluorimeter in a right angle geometry with the cell holder thermostated at the required temperature (±0.05 °C) using a circulating water bath. The light source was a 450 W Xe arc lamp and the reference was a Rhodamine B quantum counter solution. Correction of emission spectra was performed using the correction software of the apparatus. 5×5 mm quartz cuvettes were always used.

Fluorescence decay measurements were carried out with a time-correlated single-photon counting system, which is described elsewhere [20]. Excitation and emission wavelengths were 335 nm and 540 nm, respectively. Timescales were chosen for each sample in order to observe the decay through 2–3 intensity decades. Instrumental response functions for deconvolution were generated from a scattering dispersion (silica, colloidal water suspension, Aldrich, Milwaukee, WI).

Time-dependent fluorescence anisotropy, $r(t)$, is determined according to

$$r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)} \quad (1)$$

where I_{ij} are the steady-state vertical and horizontal components of the fluorescence emission with excitation vertical (I_{VV} and I_{VH}) and horizontal (I_{HV} and I_{HH}) to the emission axis. The correction for the eventual polarization dependence of the detection efficiency is given by the G factor ($G=I_{HV}/I_{HH}$), which, in our system, is equal to unity. Therefore, for calculation of $r(t)$, the intensity decays of polarized light $I_{VV}(t)$ and $I_{VH}(t)$ were obtained separately with the same accumulation time, and Eq. (1) was used with $G=1.00$.

The anisotropy decay parameters (rotational correlation times, ϕ_i, amplitudes, β_i, fundamental anisotropy, r_0, and residual anisotropy, r_∞) were determined using a global analysis method fitting simultaneously to the