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Effects of terpenes on fluidity and lipid extraction in phospholipid membranes

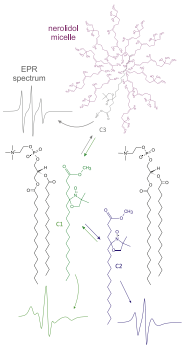
Sebastião Antonio Mendanha, Antonio Alonso *

Instituto de Física, Universidade Federal de Goiás, Goiânia, GO, Brazil

HIGHLIGHTS

- Terpenes increase the overall lipid dynamics in phospholipid membranes.
- Lipid extraction from a model membrane by terpenes is characterized.
- The thermodynamic profile associated with the terpene-membrane interactions is presented.

GRAPHICAL ABSTRACT



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ABSTRACT

Electron paramagnetic resonance (EPR) spectroscopy was used in a detailed study of the interactions of several terpenes with DPPC membranes. EPR spectra of a spin-label lipid allowed the identification of two well-resolved spectral components at temperatures below and above the main phase transition of the lipid bilayer. Terpenes caused only slight mobility increases in each of these spectral components; however, they substantially increased the population of the more mobile component. In addition, the terpenes reduced the temperature of the main phase transition by more than 8 °C and caused the extraction of the spin-labeled lipid. Nerolidol, which had the highest octanol–water partition coefficient, generated the highest amount of spin label extraction. Acting as spacers, terpenes should cause major reorganization in cell membranes, leading to an increase in the overall molecular dynamics of the membrane. At higher concentrations, terpenes may cause lipid extraction and thus leakage of the cytoplasmic content.

1. Introduction

Terpenes are volatile substances that are primarily extracted from vegetable oils, and their molecular components include only carbon, hydrogen and oxygen atoms. Terpenes are composed of isoprene units arranged in either linear chains or rings, and they function mainly as chemoattractants or chemorepellents [1,2]. Many dietary terpenes have shown antitumor activity and are effective in the prevention and chemotherapy of cancer [2–8]. Furthermore, essential oils, or the terpenes contained therein, have shown activity against bacteria, fungi, parasites and viruses [7,8], including the antifungal activity of the monoterpene terpinen-4-ol [9] and the antileishmanial activity of limonene [10] and nerolidol [11].

The electron paramagnetic resonance (EPR) spectroscopy of spin labels has been employed to investigate the mechanisms by which monoterpenes act as enhancers of skin permeation [12–15]. The intercellular lipid matrix of the outermost skin layer, the stratum corneum,

* Corresponding author. Fax: +55 62 3521 1014.
E-mail address: alonso@ufg.br (A. Alonso).

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which is the major permeability barrier of the skin, becomes more fluid in the presence of the monoterpenes L-menthol and 1,8-cineole [12,13], and several monoterpenes increase the membrane partition coefficients of the small water-soluble spin labels TEMPO and DTBN in stratum corneum membranes [14,15]. Recently, EPR spectroscopy has been used to show that terpenes can cause pronounced increases in the plasma membrane fluidity of *Leishmania amazonensis* promastigotes at terpene concentrations similar to those that inhibit the growth of the parasite [16]. In addition, the terpenes nerolidol, (+)-limonene, α-terpineol and 1,8-cineole at concentrations that inhibited the growth of *Leishmania* parasites by 50% caused cell lysis of 4 to 9%, and this percentage rose to approximately 50% for concentrations that were double or triple the IC_{50} values [16]. The cytotoxicity of seven monoterpenes and the sesqui-terpene nerolidol in cultured fibroblast cells also correlates with the hemolytic potential of these terpenes [17].

With the main objective of understanding the interactions of terpenes with the lipid component of cell membranes, we measured the EPR spectra of spin labels in pure DPPC model membranes to study the effects of a sesquiterpene and three monoterpenes on lipid bilayers, and we primarily focused on the temperature dependence of the molecular dynamics and lipid extraction at three terpene concentrations.

2. Materials and methods

2.1. Chemicals

The terpenes 1,8-cineole, α-terpineol, (+)-limonene and nerolidol were purchased from Acros Organics (Geel, Belgium). The spin labels 5-doxyl-stearic acid methyl ester (5-DMS), 16-doxyl-stearic acid methyl ester (16-DMS), 5-doxyl-stearic acid (5-DSA) and 12-doxyl-stearic acid (12-DSA) were purchased from Sigma-Aldrich (St. Louis, USA), and the phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids Inc. (Alabaster, USA) (Fig. 1). The other reagents were purchased from Sigma-Aldrich or Merck SA (Rio de Janeiro, Brazil) at the highest available purity.

2.2. Preparation and spin labeling of DPPC membranes

DPPC and the spin label were dissolved in chloroform at 1 mg/mL and 5 μg/mL (DPPC:spin label molar ratio of ~100:1), respectively, to prepare a film composed of these lipids in the bottom of a glass tube. After an aliquot (50 μL) of the organic solvent was applied to the glass tube, the solvent was evaporated using a stream of gaseous nitrogen. The films were stored under vacuum for 12 h and subsequently hydrated at 55 °C with 200 μL of phosphate buffered saline (5 mM phosphate, 150 mM NaCl, pH 7.4) to form a multilamellar suspension of DPPC. Unilamellar vesicles were prepared using a mini-extruder purchased from Avanti Polar Lipids Inc. (Alabaster, USA) and polycarbonate filters with 0.1 μm diameter pores. Subsequently, the vesicle suspension was centrifuged (1000 ×*g* for 10 min) to increase the concentration to approximately 50 mM DPPC, and the final volume decreased to ~50 μL.

The terpenes were diluted in ethanol to a terpene:ethanol ratio of 1:2 (*v*/*v*), and aliquots of this solution were added to unilamellar vesicles of DPPC. The ethanol concentration necessary to deliver the highest terpene concentration used in the sample was 3% (*v*/*v*); this ethanol concentration does not affect DPPC membrane fluidity.

After homogenization, the membranes were introduced in capillary tubes with an internal diameter of 1 mm, which were sealed using a flame. Finally, the samples were preheated to 45 °C for 30 min before the EPR experiments.

2.3. EPR spectroscopy

EPR measurements were conducted using a Bruker ESP300 spectrometer (Rheinstetten, Germany), operating in the X-band (approximately 9.4 GHz) with an ER4102 ST resonant cavity and a Bruker system for temperature control. To improve the thermal stability of the samples, the capillaries containing DPPC vesicles were dipped in mineral oil inside the quartz tube used to position the sample within the resonant cavity. A thermocouple was inserted into the quartz tube and was in contact with the oil at a position slightly above the center of the cavity to increase the precision when measuring the temperature of the sample. The EPR measurements were performed using the following instrumental parameters: microwave power, 2 mW; modulation frequency, 100 kHz; amplitude of modulation, 1 G; magnetic field scan, 100 G; scan time, 168 s and detection time constant, 41 ms.

2.4. Spectral simulation

The simulation of the EPR spectra was performed using the nonlinear least-squares (NLLS) software developed by Freed JH et al. [18,19]. In this work, it was assumed that the rotational diffusion tensor R of the labeled molecule has axial symmetry with its principal molecular axis (z axis of the nitroxide radical) in the direction perpendicular to the plane of the lipid bilayer, and the axial rotational anisotropy was defined by the relationship $N = \log\left(R_{\parallel}/R_{\perp}\right) = 1$. The rate of rotational Brownian diffusion,

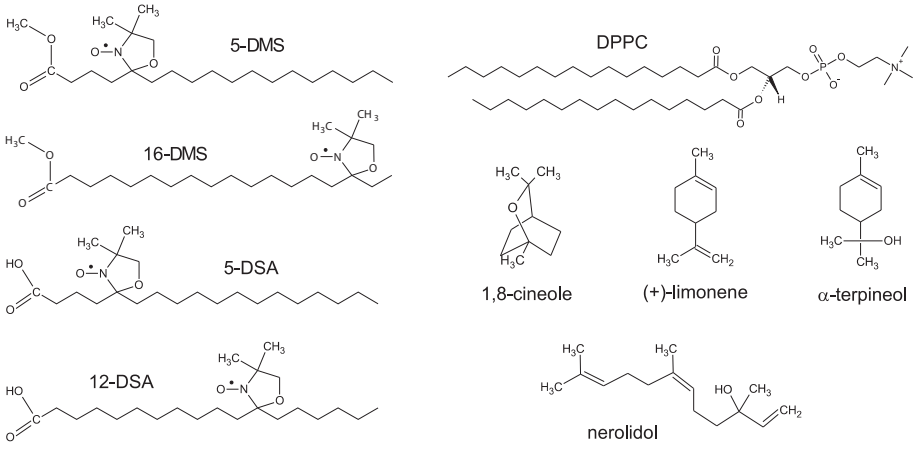

Fig. 1. Chemical structures of the lipid, spin labels and terpenes used in this work.