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Biophysical study of resin acid effects on phospholipid membrane structure and properties

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ABSTRACT

Hydrophobic resin acids (RAs) are synthesized by conifer trees as part of their defense mechanisms. One of the functions of RAs in plant defense is suggested to be the perturbation of the cellular membrane. However, there is a vast diversity of chemical structures within this class of molecules, and there are no clear correlations to the molecular mechanisms behind the RA's toxicity. In this study we unravel the molecular interactions of the three closely related RAs dehydroabietic acid, neoabietic acid, and the synthetic analogue dichlorodehydroabietic acid with dipalmitoylphosphatidylcholine (DPPC) model membranes and the polar lipid extract of soybeans. The complementarity of the biophysical techniques used (NMR, DLS, NR, DSC, Cryo-TEM) allowed correlating changes at the vesicle level with changes at the molecular level and the co-localization of RAs within DPPC monolayer. Effects on DPPC membranes are correlated with the physical chemical properties of the RA and their toxicity.

1. Introduction

Resin acids (RAs) belong to an important class of natural biologically active compounds that form part of the defense mechanisms of certain plants, e.g. conifer trees [1]. Today they are widely applied as natural insecticides and have potential use in new industrial applications due to their antimicrobial and antifungal properties [2].

RAs are tricyclic diterpenes of the labdane type that derive from the precursor geranylgeranyl diphosphate (GGPP) and consist of 20 carbon atoms carrying a carboxyl group. Their high chemical diversity arises from various functional groups, diastereoisomers and the number and position of double bonds [1]. In order to circumvent toxic effects, the synthesis of natural RAs, e.g. in pines, takes place in specialized epithelial cells after which they are concentrated and stored in structures such as cell free resin ducts [3]. Additionally, a wide range of synthetic and modified RAs exists today, such as dichlorodehydroabietic acid (Cl_2DAA) schematically shown in Fig. 1. Cl_2DAA is a waste product from bleaching processes in paper mills and pulp effluents and is highly toxic for aquatic organisms [4].

The toxicity of RAs against insects, fungi and other organisms is currently linked to their ability to integrate into the cellular membrane due to their hydrophobicity. The concentrated release of RAs toward an invading organism or upon tissue damage is suggested to locally induce a toxic RA to lipid ratio. In particular, toxicological studies showed that the RAs' toxicity correlates to the perturbation of the cellular membrane integrity. Electron paramagnetic resonance used for *in vitro* assays to study the cytotoxicity of terpenes on erythrocytes, showed a clear weakening of the cell membrane upon exposure to terpenes closely related to the RAs used in this study [5]. Biophysical studies also showed that terpenes, such as abietic acid from the oleo resin, increase both the permeability and fluidity of the cellular membrane suggesting a more disordered lipid packing in DPPC and DMPC bilayers [6,7].

It is noteworthy that small structural differences in terpenes (for example, a single bond in *cis* or *trans* conformation or additional functional groups) can lead to major differences in toxicity that may correlate to their degree of lipid membrane perturbance [8]. However, no systematic studies investigating the structural effects of RAs on model phospholipid monolayers and bilayers have been reported to date.

Here, we study the lipid membrane structure and morphology using both DPPC and soybean polar lipid extract in admixture with 3 RAs of similar chemical structure, namely dehydroabietic acid (DAA) and neoabietic acid (NA), both found in conifer resin, as well as their synthetic analogue Cl_2DAA. Toxicological studies concluded that among

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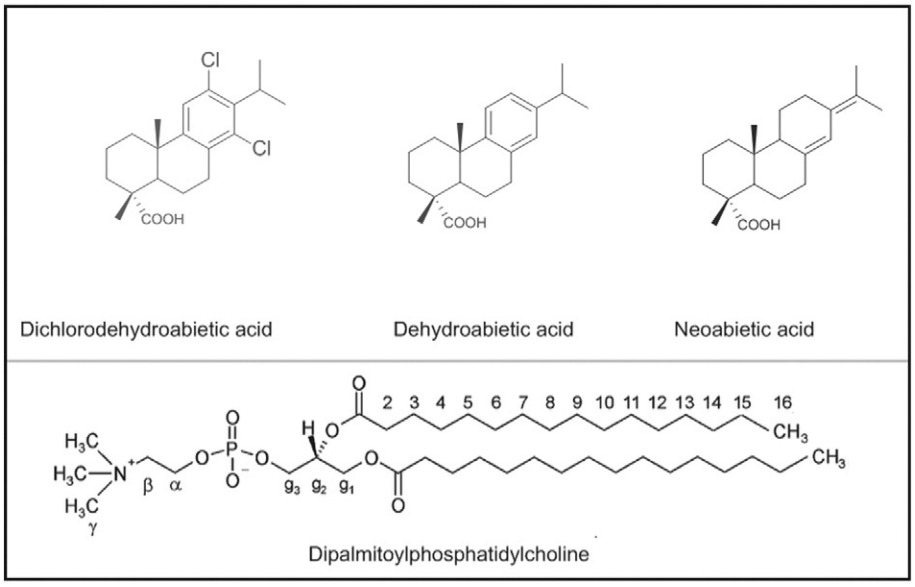

Fig. 1. Molecular structures of the three diterpene resin acids (RAs) and the main lipid used in this study. Top, from the left: dichlorodehydroabietic acid (Cl_2DAA), dehydroabietic acid (DAA) and neoabietic acid (NA). Bottom: the chemical structure of dipalmitoylphosphatidylcholine (DPPC) including the carbon numbering relevant for the PT ssNMR analysis.

natural compounds DAA has a higher antimicrobial toxicity than NA [9], while another study (that included Cl_2DAA and DAA) reported a significantly higher toxicity of chlorinated compounds over non-chlorinated compounds [10]. The chemical structures of the three RAs and the main phospholipid DPPC used in this study are shown in Fig. 1.

Specifically, we investigated the effect of RAs on the biophysical properties and structure of both lipid vesicles and lipid monolayers using a wide range of complementary biophysical techniques. Differential scanning calorimetry (DSC), polarization transfer solid-state nuclear magnetic resonance (PT ssNMR), dynamic light scattering (DLS) and cryogenic transmission electron microscopy (Cryo-TEM) were used to reveal the overall structural effects on DPPC bilayers. Neutron reflectometry (NR) and the Langmuir trough (LT) were used to investigate changes at the molecular level and the RAs´ localization within the lipid monolayer. The complementarity of these techniques allowed correlating changes at the vesicle level with changes at the molecular level of DPPC membranes in the presence of RAs. Additionally, the DLS measurements of vesicles composed of the polar soy lipid extract suggest that the effects observed for DPPC also apply to more biologically relevant lipid mixtures.

2. Experimental section

2.1. Materials

The lipids, DPPC, 1,2-dipalmitoyl-d62-*sn*-glycero-3-phosphocholine (d_{62}DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-*N*,*N*,*N*-trimethyl-d9 (d_9DPPC) and soybean polar lipid extract (composition in wt/wt%: phosphatidylcholine: 45.7, phosphatidylethanolamine: 22.1, phosphoinositol: 18.4, phosphatidic acid: 6.9 and unknown: 6.9) [11] were purchased from Avanti Polar Lipids, Inc. (AL, USA) with a purity over 99%. Tris-buffered saline (TBS) solution containing 50 mM Tris and 150 mM NaCl at pH of 7.4 was used in all preparations. Cl_2DAA, DAA and NA were purchased from Orchid, Cellmark (Canada). All other chemicals were obtained from Sigma Aldrich (Brøndby, Denmark) unless otherwise indicated.

2.2. Vesicle preparation

Vesicles were prepared by manual extrusion. Briefly, lipids and RAs dissolved in chloroform were mixed at a molar ratio of 9:1 and spread onto the glass vial surface with a glass syringe (Hamilton, USA), followed by drying under a soft nitrogen stream and subjection for 1 h under a vacuum to remove residual organic solvent. Lipid films were used immediately or stored at −20 °C. The lipid film was rehydrated and extruded in buffer well above the melting temperature (T_m), which is reported to be 41 °C for DPPC [12] and −63 °C [13] for soybean polar lipids. The vesicle suspension was extruded eleven times through a 100 nm pore size polycarbonate filter membrane using a mini extruder (Avanti Polar Lipids, Inc.).

2.3. Dynamic light scattering (DLS)

DLS measurements were performed using an ALV/CGS-3 (ALV-GmbH, Germany) apparatus containing a Helium-Neon Laser (λ = 632.8 nm) to determine the hydrodynamic size of the vesicle population. The experiment was performed in TBS buffer at a final vesicle concentration of 0.1 mg lipid/mL. Measurements (at least three per sample) for each sample were taken out immediately after extrusion (h_0) and one day later (h_{24}). Data was collected at a detection angle of $\theta = 90°$ for 60 s. The data was analyzed with the ALV correlator Software 3.0 (ALV GmbH, Germany).

For the analysis, the normalized electric field correlation ($g_1(t)$), which describes the measured intensity fluctuations, is determined by using the distribution of exponential decays

$$g_1(t) = \int A(\tau)\, exp(-t/\tau) d\tau$$

where τ is the relaxation time and $A(\tau)$ its distribution. Therefore, the contribution of a particle to $A(\tau)$ is proportional to its scattering intensity and thus large particles bias strongly the distribution. Furthermore, the cooperative diffusion coefficient (D_c) relates to τ via the relationship

$$\tau = \left(D_c q^2\right)^{-1}$$

with q as the scattering vector ($q = 4\pi n/\lambda \sin(\theta/2)$). At an infinite dilution D_c will be reduced to the self-diffusion coefficient of the particle in solution and therefore links to the hydrodynamic radius which is calculated by the Stokes- Einstein equation

$$D = \frac{kT}{6\pi\eta R}$$