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Probing the position of resveratrol in lipid bilayers: A neutron reflectivity study

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ABSTRACT

The effect of the natural antioxidant resveratrol on the structure of solid supported di-palmitoyl-phosphatidyl-choline (DPPC) bilayers in their fluid state was investigated by neutron reflectometry. Results reveal an accumulation of resveratrol (up to 25%, mol/mol) inside the headgroups and they exclude its presence in the hydrophobic core. The presence of resveratrol induces an increase of the average thickness and of the interfacial roughness of the headgroup layer. This may be due to a change of the tilt angle of the phosphocholine headgroups residing next to the resveratrol to a more upright orientation and leading to a reduction of the projected area per headgroup. This effect is propagated into the hydrophobic core, where the chain packing is modified despite the absence of resveratrol. When interacting with a DPPC/cholesterol membrane, resveratrol has a similar effect on the neighboring PC headgroups as in the cholesterol free membrane. The almost precise 1:1 insertion ratio (resveratrol:cholesterol) suggests that resveratrol is most probably inserted on top of the hydroxyl group of the cholesterol in between the PC headgroups. The ordering effect of cholesterol on the hydrophobic core is absent when both cholesterol and resveratrol are present. Most probably, the interaction of resveratrol with lipid membranes is non-specific.

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1. Introduction

Resveratrol is a polyphenolic molecule found in fruits such as grapes. It is known for its antioxidant properties [1]. These can probably be related to its ability to scavenge radicals formed in the hydrophobic region of the membrane [2]. While the beneficial effects are commonly admitted [3–5], the mode of action of resveratrol in the membrane is not fully understood. The few existing studies are contradictory about its mechanism of action as an antioxidant and especially on how the molecule may induce structural changes in the membrane [6,7]. The presence of resveratrol is likely to have an impact on the interfacial structure and on the stability of the membrane, including its fluidity.

The exact location of resveratrol within the cell membrane is also unclear. There are currently contradictory studies about its position after absorption in model lipid bilayers [8–12]. Some studies state that resveratrol is located in the chain region [9,12], whereas others propose that resveratrol stands at the headgroup region [8,10,11]. In both cases, the interaction of resveratrol with lipid bilayers is non-specific.

Neutron reflectometry from supported membranes is an appropriate tool to elucidate the modification of the structure of a lipid bilayer in the presence of resveratrol. By using the contrast variation technique [13,14] (see Section 2.3), the detailed structure of lipid bilayers can be probed with a fraction of nanometer resolution. In addition, neutron scattering is a *non-invasive* technique since it is not destructive and does not require the inclusion of foreign molecules in the system, such as dyes or heavy metals, which could interact directly with resveratrol or modify the structure or dynamics of the system.

In this paper, results from a neutron reflectometry study on the effect of resveratrol on the structure of supported di-palmitoyl-phosphatidyl-choline (DPPC) bilayers are presented. DPPC was chosen because phosphocholines with 16 carbon atoms in the chain are largely present in the natural composition of membranes [15]. Moreover, homogeneous DPPC bilayers can be obtained in well-known and reproducible ways. Detailed structural

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studies on DPPC membranes are widely present in the literature [16,17].

Two preparative approaches were used in order to introduce resveratrol into the supported model membranes. In the first approach, bilayers were pre-formed on the solid substrate. Resveratrol was then inserted in an aqueous subphase in contact with the bilayer. In the second approach, resveratrol was co-mixed with the DPPC molecules before the composite bilayers were deposited onto the solid substrate. Depending on the preparation, apparently opposed structural effects of resveratrol on the lipid bilayer were found. The effect of resveratrol in the lipid bilayer in the presence of cholesterol was also studied.

2. Materials and methods

2.1. Materials

Resveratrol (3,4′,5-trihydroxystilbene, ABCR GmbH&Co, Germany) was used from powder form as delivered. Sodium chloride salt and anhydrous dimethyl-sulfoxide (DMSO) (Sigma–Aldrich Co. LLC., USA) were of purity >99.9%. Head- and chain-deuterated lipids (d13-DPPC and d62-DPPC) and d7-cholesterol (Avanti Polar Lipids Inc., USA) were dissolved in chloroform for further use. Silicon wafers ($80 \times 50 \times 10\,mm^3$, (1 1 1) surface, roughness <3 Å; Synchrotronix, France) were used as substrates for the lipid bilayer deposition. The silicon substrates were cleaned by sonication in chloroform, acetone and ethanol sequentially, and the surfaces were made hydrophilic by a UV–ozone treatment (30 min) immediately before being used for bilayer deposition. H_2O was taken from a purification system (resistivity > 18 MΩ cm; MilliPore Inc., USA) and D_2O (>99.9% purity) was provided by the Institut Laue-Langevin (ILL, Grenoble, France).

2.2. Sample preparation

The samples studied with neutron reflectometry consisted of solid supported lipid bilayers immersed in an aqueous subphase, all being sealed in the ILL reflectometry solid/liquid cells [18]. Bilayers were prepared by using the Langmuir–Blodgett/Langmuir–Schaefer (LB/LS) techniques [19,20] or by the vesicle fusion technique [16,21,22]. The procedure for the LB/LS techniques is described in previous work [19,20]. The bilayer deposition was performed at room temperature. Thereafter all samples were equilibrated at 62 °C in the ILL reflectometry chamber before measurements. Resveratrol was introduced into the host bilayer by contacting the membrane with an aqueous subphase of water:DMSO (3:1, v/v, resveratrol content 2.5 mg/ml) at 62 °C for 1 h. The solvent mixture of water/DMSO was used because of the very low solubility (~0.03 mg/ml) of resveratrol in pure water [23]. After the incubation, the subphase was thoroughly exchanged by flushing with pure water. The vesicle fusion procedure started with the preparation of chloroform solutions of DPPC with and without resveratrol. The chloroform was evaporated from the solution until a thin film was formed. This lipid film was then dispersed into an aqueous sodium chloride solution (100 mM) by vortexing. Pulsed probe sonication (Bandelin Electronic GmbH, Berlin, Germany) was applied for 30 min at room temperature in order to form small unilamellar vesicles of 20–50 nm in diameter [24]. The final average lipid concentration in the vesicle suspension was ~0.5 mg/ml. The phase transition temperature of the deuterated DPPC may be lower by ~5 °C [25,26] as compared to the hydrogenated DPPC bilayer (42 °C). The vesicle suspension was injected into the water subphase of the sample cell at a temperature (62 °C) safely above the main phase transition of the pure lipid bilayer. The system was incubated for ~30 min at this temperature, such that the vesicles spontaneously fused onto the support and formed a planar bilayer [16,21,22]. Finally, the system was thoroughly flushed with pure water to remove the remaining lipid vesicles from the bulk phase. The temperature of the reflectometry chamber was maintained at 62 °C.

For the experiment with cholesterol, a d13-DPPC bilayer containing d7-cholesterol in the proportion 6:1 (DPPC:cholesterol, mol/mol) was prepared by means of the LB/LS technique. Resveratrol was introduced into the bilayer with the incubation approach from the subphase.

2.3. Neutron reflectometry

Neutron reflectometry [14] bases on the reflection of a neutron beam at interfaces as described by the Fresnel equations. The reflectivity, $R(q_z)$, is defined as the ratio between the measured intensities of the reflected and incident neutron beams at a given scattering vector, q_z, which is related to the de Broglie wavelength, λ, of the neutron and the grazing angle of incidence, θ, by $q_z = (4\pi/\lambda) \cdot \sin\theta$. The analysis of the measured reflectivity curve leads to the determination of the scattering length density (SLD) profile $\rho(z)$ along the surface normal [14]. The SLD depends on the composition along the z-direction according to the relation $\rho(z) = \sum_i \nu_i(z) \cdot b_i$, where $\nu_i(z)$ is the number density of the ith nucleus around the depth z and b_i is its atomic scattering length for neutrons.

All measurements were performed using the reflectometers D17 [27] and FIGARO [28] at the ILL, in time of flight mode, with an incident beam of wavelength distribution from 2 Å to 20 Å. Two grazing angles were chosen: 0.8° and 3.2° on D17, and 0.62° and 3.0° on FIGARO. This geometry allowed covering a q_z – range from $0.009\,Å^{-1}$ to $0.25\,Å^{-1}$ (the highest value of q_z being limited by the background). The SiO_2 layer on a bare silicon substrate was initially characterized by neutron reflectivity measurements in the contrast liquids H_2O and D_2O. The temperature of the sample was controlled by a water bath during the measurement. It was set to 62 ± 2 °C at which the supported lipid bilayer is in the L_α – fluid phase [29]. For the binary system, the membrane is also in its L_α – state [30]. The measured neutron reflectivity data were fitted by use of a slab model [31]: the composite films at the surface of the silicon wafer were divided into n sublayers with thickness t_n, SLD of the dehydrated material ρ_n, water volume fraction φ_n and interfacial roughness $\sigma_{n,n+1}$. The SLD of a sublayer thus comprises contributions of ρ_n and the SLD of the water contained in the layer. In the case of a supported bilayer, the different slabs correspond to: (1) the SiO_2 layer, (2) a thin water layer, (3) the inner headgroup region, (4) the hydrophobic tail region and (5) the outer headgroup region pointing toward the bulk water subphase. The hydrophobic core comprised both the inner and the outer chain layers. In order to increase the confidence in the model that best fitted the data, the reflectivity from the bilayer was measured in different contrasts. Then the data set from the different contrasts were fitted simultaneously under the constraint of obtaining identical structural parameters. The contrasts consisted of a subphase containing pure D_2O, pure H_2O or a mixture of H_2O and D_2O with SLD matching $3.5 \times 10^{-6}\,Å^{-2}$ (called 35MW) and of silicon ($2.07 \times 10^{-6}\,Å^{-2}$, called SMW) or of air (called 0 MW). The SLD values of the dry materials, used for the fitting procedure as start parameters, are listed in Table ST1 (supporting information). All data treatment was performed with the Motofit program [32].

2.4. Surface pressure–area isotherm measurement

Surface pressure–area isotherms were measured on a teflon trough (Langmuir trough 1212D, NIMA, Sweden) and at room temperature. The trough was filled with degassed water. The pressure

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