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Photo-dynamic induction of oxidative stress within cholesterol-containing membranes: Shape transitions and permeabilization

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ABSTRACT

Photochemical internalization is a drug delivery technology employing a photo-destabilization of the endosomes and the photo-controlled release of endocyted macromolecules into the cytosol. This effect is based on the ability of some photosensitizers to interact with endosomal membranes and to photo-induce damages leading to its breakdown. The permeabilization efficiency is not quantitatively related to the importance of the damages, but to their asymmetric repartition within the leaflets. Using unilamellar vesicles and a chlorin, we studied the effect of the membrane's cholesterol content on its photo-permeabilization. First, the affinity of the chlorin for membranes was studied. Then, we asymmetrically oxidized the membranes. For DOPC/CHOL GUVs, we observed different shape transitions, in accordance with an increase followed by a decrease of the membrane effective curvature. These modifications are delayed by the cholesterol. Finally, the photo-permeabilization of GUVs occurs, corresponding to a pore formation due to the membrane tension, resulting from vesicles buddings. Cholesterol-rich GUVs permeabilization occurs after a lag, and is less important. These results are interpreted regarding both (i) the cholesterol-induced tightening of the lipids, its consequences on physical parameters of the membrane and on oxidation rate and (ii) the suggested ability of cholesterol to flip rapidly and then to relax the differential density-based stress accumulated during membrane bending.

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1. Introduction

The oxidation of unsaturated lipids plays a central role, both in biological functions and in pathogenesis of many diseases [1–4]. Oxidation alters the properties of biomembranes, including their fluidity and permeability [5,6]. These changes, in turn, affect the activity of membrane proteins, including membrane-bound enzymes, receptors and transport proteins [7–9].

The photochemical induction of oxidation is an effective and controlled way of inducing oxidation processes [10]. It is based on the ability of certain molecules, the photosensitizers, to generate Reactive Oxygen Species (ROS) upon light irradiation. The ROS react with surrounding biomolecules, generating photo-damages. This property, together with the preferential retention of certain photosensitizers by tumors as compared to normal surrounding tissues, has found an application in an anti-tumoral therapy, the Photodynamic Therapy (PDT) [11]. Indeed, a huge level of such light-induced damages leads to the death of the targeted cells. More recently, photosensitizer-induced lipid oxidation has been used to improve the delivery of macromolecular therapeutic agents to their intracellular targets by an approach called Photochemical Internalization (PCI) [12]. After the uptake by endocytosis, the degradation of the macromolecules in lysosomes is greatly reduced by the photodynamic destabilization of the endocytic vesicles membrane, increasing their biological activity. This approach demonstrated a huge and controlled increase of the drug efficacy, both *in vitro* and *in vivo* [13,14].

Because of their very short lifetime [15], photo-induced ROS have a limited diffusion length (10–20 nm), which imply a close association of photosensitizers with the targeted location [15–18]. For tetrapyrrole photosensitizers, the ability to cross membranes is governed by the charge of their lateral chains [19–23]. The chlorin e6 (Ce6), a second generation photosensitizer, is not able to cross the biological membranes [24,25]. Consequently, it interacts only with the monolayer in contact with the photosensitizer solution, i.e. labels the membrane asymmetrically. Recently, we thus labeled model membranes, Giant Unilamellar Vesicles (GUVs) composed of dioleoylphosphatidylcholine (DOPC), which is an unsaturated lipid. Under light-induced oxidation, we observed shape transitions and permeabilization of the GUVs. Our results demonstrated that, under light-induced asymmetric modifications, the curvature of the membrane is strongly modified. The budding induced by the curvature is responsible for a stretching of the membrane up to its lysis tension and its subsequent permeabilization. The key role of the asymmetry in this

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permeabilization of the membrane, related to its bending stress, was highlighted [26].

Biophysical studies of membranes, including experimental observations of vesicle shape transitions, led to the area difference elasticity (ADE) model [27]. This model successfully predicts the shape transformations of a vesicle, when its curvature or area to volume ratio is modified. The effective spontaneous curvature of the membrane used in this model comes from two distinct contributions: one due to the preferred packing of individual lipids and the other to the mismatch of the area of a monolayer compared to the other. When present in the outer leaflets of a membrane, lipids with cone shape will create a mismatch between the area of the hydrophobic and hydrophilic regions in the leaflet, inducing a positive spontaneous curvature. Likewise, an increase in the preferred area of lipids in the outer layer will induce an area difference between the two leaflets and increase the effective spontaneous curvature of the membrane. In pure phospholipidic membranes, the diffusion of lipids from one leaflet to the other (flip-flop) is very slow, and cannot equilibrate the area difference between leaflets.

This is however not the case for a biological membrane with a more complex composition. In particular, a variety of amphiphilic molecules, such as cholesterol (0.1 to 0.4 of molar fraction [28,29]), are able to flip rapidly from a leaflet to the other [30–32]. This rapid flip-flop has been suggested to be an efficient pathway for the relaxation of the stress due to the leaflet area difference accumulated during membrane bending [33]. Moreover, the cholesterol is known to be an agent promoting membrane order. Most of the physical and functional membrane properties depend on cholesterol and its interaction with the other lipid species [28,29,34,35]. For dioleoylphosphatidylcholine (DOPC), this interaction can be described by the "umbrella model" and increases the membrane cohesion and mechanical stiffness [36]. It rigidifies the bilayers, reducing the free volume available for diffusion and decreasing the rate of lateral diffusion. The solubility limit of cholesterol in DOPC bilayers has been measured to be 0.67, corresponding to a regular distribution of the cholesterol molecules in the lipid bilayer up to solubility limit [37–39]. The cholesterol-induced tightening of the packing of lipids in the bilayers through its effect on the fluidity, viscosity and lateral diffusion, consequently decrease the membrane permeability [40], and also affects the rate of peroxidation of liposomal lipids. It is however impossible to predict the effect of membrane fluidity on the rate of its peroxidation [41]. Recently, GUV composed of DOPC/CHOL in 1:1 ratio have been reported to fluctuate following oxidative stress [42].

Here, we photochemicaly induce lipid oxidation in DOPC/CHOL-GUVs to address the effect of cholesterol content in the dynamics of a photo-oxidative membrane and on its photo-permeabilization. The photosensitizer used, the Ce6, allows an asymmetric targeting of the external bilayer of the membrane (called "targeted leaflet") and a fine control of the location of the oxidation [26]. Our results are discussed in relation with the technology of photochemical internalization, PCI.

2. Materials and Methods

2.1. Chemicals

All chemicals were purchased from Sigma (USA), except dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphocholine (DPPC) and cholesterol (CHOL) from Avanti Polar Lipids (USA), and chlorin e6 from Frontier Scientific (USA). Chlorin stock solution (5 mM) was prepared in ethanol and kept at −18 °C. The experimental Ce6 aqueous solutions were prepared, used without delay and handled in the dark. The osmolarity of the solutions was checked with an osmometer (Löser Messtechnik, Germany).

2.2. Vesicles formation

Lipids mixtures with various contents (DOPC/CHOL 10:0, 9:1, 8:2, 7:3 and 5:5 mol/mol or DPPC/CHOL 10:0, 9:1, 8:2) were made in chloroform. Large Unilamellar Vesicles (LUVs) were prepared by extrusion method. After evaporation of chloroform, lipids were dispersed in phosphate buffer pH 7.4 by vortexing. The liposome suspension was extruded 8–10 times through a stack of two polycarbonate membrane filters (Poretics, Livermore, CA) with pores of 50, 100 or 200 nm using an extruder device (Avanti Polar Lipids, USA).

Giant Unilamellar Vesicles (GUVs) with an average diameter of 10–20 μm were formed by the electroformation method [43] as reported previously [26]. Lipids mixtures in chloroform were deposited on ITO-covered glass plates. A chamber was made from two such glass plates and a Teflon spacer of 4 mm and the solvent was dried in vacuum. The chamber was then filled with a solution of 300 mM sucrose and an AC field was applied between the plates for 4 h.

For experiments, the GUVs were mixed with a 300 mM glucose solution. The density difference between sucrose and glucose caused the GUVs to sediment to the bottom of the chamber. The difference in optical index between sucrose inside and glucose outside the vesicle allowed phase contrast microscopy observation.

For DPPC containing GUVs, the preparation and experiments were performed at 60 °C, up to the transition temperature of the lipid mixture.

2.3. Steady-state interaction

For the steady-state study of the interaction of the Ce6 with LUV, fluorescence spectra were measured with an Aminco Bowman Series 2 spectrofluorimeter (Edison, NJ, USA). Recording was generally started 2 min after the preparation of the solutions under study. Liposome solutions were prepared at different concentrations of lipids. 10 μl of 10 μM Ce6 solution were added to 2 ml of vesicle preparation and the fluorescence spectra were recorded. In order to correct the spectra for small differences in Ce6 concentration arising from experimental inaccuracy, 20 μl of Triton-X100 were added after measurement leading to disruption of vesicles and solubilization of all Ce6 in the Triton micelles. The spectra were normalized accordingly.

Data thus obtained were treated as described elsewhere [24,44,45]. The global binding constant, K_B, was derived from changes in the fluorescence signal at a wavelength corresponding to the maximum of fluorescence emission of Ce6 incorporated into the membrane. We used the previously derived relationship:

$$F = F_0 + \frac{(F_\infty - F_0) \cdot K_B \cdot [lip]}{1 + K_B \cdot [lip]} \quad (1)$$

where F_0, F_∞ and F are respectively the fluorescence intensities corresponding to zero, total and intermediate incorporation of Ce6 into vesicles. Lipids being in large excess, the saturation of the bilayer is far to be reached and it can be assumed that Ce6 binding does not affect the properties of the model membrane. Then, regardless of the number of Ce6 molecules incorporated into a vesicle, [*lip*] was assumed to be equivalent to the total lipid concentration added, i.e. the sum of the cholesterol and DOPC concentrations.

2.4. Asymmetric labeling of GUVs

Giant Vesicles were asymmetrically labeled with Ce6 into the outer leaflet as reported in previous work [26]. The Ce6 concentration used was 25 μM.

2.5. Observation and illumination

GUVs were observed under an inverted microscope. The instrumental set-up was based on a Nikon Eclipse TE 300 DV inverted microscope equipped with a high numerical aperture phase oil

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