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The dynamics of giant unilamellar vesicle oxidation probed by morphological transitions

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ABSTRACT

We have studied the dynamics of Lissamine Rhodamine B dye sensitization-induced oxidation of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) giant unilamellar vesicles (GUVs), where the progression of the underlying chemical processes was followed via vesicle membrane area changes. The surface-area-to-volume ratio of our spherical GUVs increased after as little as ten seconds of irradiation. The membrane area expansion was coupled with high amplitude fluctuations not typical of GUVs in isoosmotic conditions. To accurately measure the area of deformed and fluctuating membranes, we utilized a dual-beam optical trap (DBOT) to stretch GUV membranes into a geometrically regular shape. Further oxidation led to vesicle contraction, and the GUVs became tense, with micron-scale pores forming in the bilayer. We analyzed the GUV morphological behaviors as two consecutive rate-limiting steps. We also considered the effects of altering DOPC and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (RhDPPE) concentrations. The resulting kinetic model allows us to measure how lipid molecular area changes during oxidation, as well as to determine the rate constants controlling how quickly oxidation products are formed. Controlled membrane oxidation leading to permeabilization is also a potential tool for drug delivery based on engineered photosensitizer-containing lipid vesicles.

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1. Introduction

Lipid bilayer membranes are the structural barriers that set boundaries of the cell and its compartments. They serve as the platforms on which membrane proteins are localized and play a central role in a host of physiological processes. The ability of lipid bilayers to perform their physiological role depends on the integrity of the membrane structure [1]. Lipid oxidation causes modification and/or loss of essential membrane functions [2] and has been identified in pathological conditions such as cancer [3,4] and aging-associated conditions such as atherosclerosis [5] and Alzheimer's disease [6]. Despite the strong connection of membrane oxidation to human health, the specific molecular mechanisms connecting lipid oxidation to the membrane's roles in disease etiology and pathogenesis are not well understood. Here, we use a light-induced model of lipid oxidation to probe the kinetics of lipid oxidation by observing oxidation-linked changes to the morphology of giant unilamellar lipid vesicles (GUVs).

The key oxidation process that occurs within the lipid bilayer involves oxidative species such as reactive oxygen species (ROS) attacking unsaturated lipids. The products of the oxidation reactions depend on the type of lipids involved (including mono- and polyunsaturated) and the particular oxidative species mediating the attack [7]. However, the most commonly observed fundamental process can be viewed as a series of key chemical events. First, the oxidation of an unsaturated lipid molecule is initiated by the abstraction of the allylic hydrogen adjacent to the double bond and the reaction with molecular oxygen to form a carbon-centered peroxyl radical. This in turns initiates the oxidation of neighboring lipid molecules, and the lipid tails are left modified with a hydroperoxy group [8,9], and further oxidation leads to lipid tail scission into a truncated lipid molecule and tail fragment [10].

Singlet oxygen ($^1O_2$) is an ROS frequently encountered during the visualization of the lipid membrane by fluorescence microscopy. When irradiated, photosensitive molecules such as porphyrin derivatives and fluorescent rhodamine dyes can transfer their energy to molecular oxygen (O_2) to generate the more reactive $^1O_2$ [11,12]. Biomedically, single oxygen production by photosensitization is utilized by photodynamic therapy (PDT) for targeted destruction of malignant tissues [13]. Singlet oxygen is also naturally-occurring, resulting when UVA rays photosensitize endogenous porphyrins [14].

Singlet oxygen-induced effects have been noted to alter membrane phase behavior [15,16]. Other reports have noted more drastic changes to the lipid membrane morphology induced by singlet oxygen-related

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reactions. For the general case of phospholipid membrane oxidation, molecular dynamics simulations of a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) membrane reveal membrane bending and pore formation within nanoseconds of oxidation, especially with scission of both acyl tails [17]. Investigations adding photosensitizers to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and DOPC GUVs reported changes to the membrane bending modulus [18] and area expansion modulus [19] with lipid oxidation. Others observed increases in the membrane surface area accompanied by vesicle shape fluctuations when irradiating porphyrin-labeled pure POPC or POPC-containing GUVs [16,20]. In addition, a study using DOPC GUVs in different concentrations of methylene blue solution also observed post-expansion membrane area contraction accompanied by the loss of optical contrast across the membrane; this was interpreted as the result of pore formation [21]. Similar effects have also been reported in polymersomes composed of polyethyleneoxide-*b*-polybutadiene (PEO-*b*-PBD) diblock copolymer with the chromophore chlorin e6, where the vesicles grew larger over three minutes and also shrunk afterwards [22]. In this study, the morphological changes previously observed by others are utilized to constructing a kinetic model of oxidation progress. This modeling approach provides a generalizable framework for quantifying and predicting the results of photosensitization-induced oxidation processes. Additionally, this work provides the first visual confirmation of micron-sized pore formation accompanying vesicle shrinkage in the latter stage of oxidation. We show that careful observation of pore dynamics can be used to estimate changes in the line tension of the membrane.

A baseline understanding of the dynamics of lipid oxidation is of broad utility. Rhodamine-based dyes are broadly used in imaging studies; understanding the precise conditions under which they can be expected to lead to oxidative damage–and potential artifactual results–is therefore important. Rhodamine dyes have also been considered as potential photosensitizers in photodynamic therapy (PDT) [11,23]. Understanding the kinetics of oxidation produced by the photosensitizer is the key to designing such therapies, and the framework of our kinetic model is extensible beyond rhodamine fluorophores to other photosensitizers, such as porphyrin chromophores [24]. Despite many observations of light-induced oxidation of the lipid membrane, the dynamics of the underlying chemical events is largely unexplored, so the main goal of this study is to provide a kinetic model applicable to the complex process of oxidation while retaining simplicity by constraining to rate-limiting processes. Based on the morphological changes observed by others [16,20–22] and in this study, the process is apparently a two-step process involving membrane area expansion followed by contraction. Bateman and Gee [25] described the rate of light-induced oxidation of non-conjugated olefins to be first-order in which the effective rate constant varies proportionally with light absorption, which directed the focus of our study toward the dependence of oxidation dynamics on the intensity of the excitation light.

2. Materials and methods

2.1. Materials

The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (RhDPPE) were purchased from Avanti Polar Lipids. Sucrose, glycerol, 6-hydroxy-2,5,7,8-tetrametylchromane-2-carboxylic acid (Trolox), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich. Glucose and Rhodamine B were purchased from Alfa Aesar, chloroform was from Macron Fine Chemicals, and sodium azide was from BDH Chemicals. Deuterated chloroform was from Cambridge Isotope Laboratories, Inc.

2.2. GUV preparations

Our standard GUV composition consisted of 9:1 molar ratios of DOPC to RhDPPE. In reducing DOPC concentrations to 85 and 75%, the unsaturated lipid was substituted with DPhPC such that RhDPPE was maintained at 10% of the total composition; DPhPC was selected as a substitute that would have a fluidity and phase behavior similar to that of an unsaturated lipid without susceptibility to oxidation. To investigate rhodamine content dependence, RhDPPE was substituted with unlabeled DPPE.

The GUVs were formed using the electroformation method pioneered by Angelova and coworkers [26]. A lipid solution (in chloroform) was deposited on an indium-tin oxide (ITO)-coated side of a glass slide (Delta Technologies), inside the perimeter enclosed by a silicone o-ring (13/16″ ID, 1″ OD, Sterling Seal & Supply) attached to the slide by silicone vacuum grease (Dow Corning). After drying under vacuum overnight, the lipid film was hydrated with a 200 mM sucrose solution in 10 mM HEPES buffer at pH 7.40 such that the final lipid concentration was 30–40 μg/mL. The electroformation apparatus was completed by attaching another ITO-coated slide to the o-ring with vacuum grease, with the conductive side facing toward the lipid film. Electrodes connecting each of the ITO-coated slides to a function generator (Hewlett–Packard/Agilent Technologies) allowed for the application of an AC field at 10 Hz and 1.3 V for 1 h at room temperature. Vesicles were used within a day of electroformation. Lipid mixtures containing rhodamine dye were always shielded from ambient light to avoid photobleaching and other unintended oxidation effects.

For dual-beam optical trap (DBOT) experiments, which require a higher density of GUVs in solution, GUVs were formed by hydrating the lipid film dried on a layer of agarose hydrogel [27–29]. The GUVs were formed in 500 mM sucrose solution in 20 mM HEPES buffer at pH 7.00, a condition used in the previous work with a DBOT [30,31].

2.3. Basic photooxidation experiments

Electroformed RhDPPE-labeled GUVs were observed with epifluorescence microscopy on a Nikon TI-E inverted microscope, using illumination filtered through a green excitation filter (528–553 nm bandpass, 540 nm cut-on wavelength). Excitation light was provided by a 130 W mercury lamp (Intensilight, Nikon). The maximum irradiation intensity through the objective (Apo TIRF 60× Oil/NA 1.49, Nikon) was measured by a laser power meter (Thorlabs, Inc.) to be 5.93 mW at 561 nm. Intensity was reduced in binary ratios using neutral density filters. We utilized asymmetry in aqueous solutions to facilitate microscopy observations: the GUVs formed in sucrose were transferred to an isoosmotic glucose solution (200 mM in 10 mM HEPES at pH 7.40). Since sucrose solutions are denser than glucose solutions at the same concentration, the GUVs sedimented to rest on the glass coverslip, minimizing their distances to the objective and their mobility. Figure S1 illustrates the described arrangement.

To estimate line tension in the pore-forming regime of DOPC GUV oxidation, pore closure was delayed by performing the experiments in ten-fold elevated aqueous viscosity. Both 200 mM glucose and sucrose solutions were formed in a 1:1 v/v glycerol–water mixture. The viscosity of the resulting sucrose solution was measured with an Ubbelohde viscometer (Cannon Instrument Company) to be 10.3 mPa·s.

2.4. Chemical analysis of oxidized lipids

In preparation for nuclear magnetic resonance (NMR) spectroscopy, lipid samples (in chloroform) were dried onto the bottom of a glass vial under an argon stream and placed under vacuum for at least 1 h. The samples were irradiated with the green excitation light for 1 h. Samples were redissolved in ~800 μL of deuterated chloroform and scanned on a Varian VNMRS-500 2-channel NMR spectrometer at 25 °C. Samples

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