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Imaging the oxidation effects of the Fenton reaction on phospholipids at the interface between aqueous phase and thermotropic liquid crystals

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The lipid peroxidation process has attracted much attention because of the growing evidence of its involvement in the pathogenesis of age-related diseases. Herein, we report a simple, label-free method to study the oxidation of phospholipids by the Fenton reaction at the interface between an aqueous phase and immiscible liquid crystals (LCs). The different images produced by the orientation of 4-cyano-4′-pentylbiphenyl (5CB) corresponded to the presence or absence of oxidized 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG). The oxidation effects of the Fenton reaction on DOPG were evaluated by monitoring the orientational response of liquid crystals upon contact with the oxidized DOPG solutions. DOPG was oxidized into chain-changed products containing hydroxy, carbonyl, or aldehyde groups, resulting in the rearrangement of the phospholipid layer. This induced the orientational transition of LCs from homeotropic to planar states; therefore, a dark to bright optical shift was observed. This shift was due to the Fenton reaction preventing DOPG to induce the orientational alignment of LCs at the aqueous/LC interface. We also used an ultraviolet spectrophotometer to confirm the effects of oxidation on phospholipids by the Fenton reaction. Using this simple method, a new approach for investigating phospholipid oxidation was established with high resolution and easy accessibility.

[**Key words:** Oxidation; Phospholipids; Liquid crystals; Orientational transition; Fenton reaction]

Phospholipids are the most abundant lipids in cell membranes. Because of their amphiphilic nature, they form spherical lipid bilayers known as liposomes or vesicles (1,2). They are important in mammalian cell biology because they act as lipid mediators by providing a permeable barrier as well as substrates for synthesis (3,4). The role of lipid oxidation in disease is still a subject of considerable research, and within this area, there is growing interest in the roles of oxidized phospholipids, which have been detected in pathological conditions, often at raised levels compared to those from subjects with normal physiology (5). There is substantial evidence that the oxidized phospholipids of oxidized low-density lipoprotein accumulate *in vivo* and play an important role in cardiovascular disease (6–8).

The Fenton reaction is defined as the oxidation of organic substrates by a mixture of hydrogen peroxide and ferrous iron (9). The oxidizing intermediates involved in Fenton reactions are thought to cause damage to phospholipids and play a significant role in the aging process and a variety of diseases, such as cancer (10). The Fenton reaction is a key reaction in the oxidation of phospholipids. Notably, this reaction is thought to occur in heart diseases, such as ischemia and reperfusion (11). Many researchers doubt that the significance and existence of the Fenton reaction in biological systems is due to the low concentrations of H_2O_2 and free iron. They also suggest that the high and undiscriminating reactivity of hydroxyl radicals produce great limitations on diffusion and cause more wide-ranging damage to biomolecules (10).

Assays for identifying and characterizing the oxidation of phospholipids have been the subject of many studies. Development of these assays include: (i) separation of phospholipid oxidation products by mass spectrometry (12), (ii) biological functions of oxidized phospholipids (13), (iii) identification of new families of bioactive phospholipids generated by immune cells (3), and (iv) measurement of the oxidation of phospholipids by physicochemical tests (14). Reis et al. (12) used mass spectrometry coupled with high performance liquid chromatography (HPLC) separation techniques (HPLC-MS) to study phosphatidylcholine peroxidation products. They developed a reverse-phase liquid chromatography method coupled to electrospray mass spectrometry for the separation of glycero-phosphatidylcholine peroxidation products formed by the Fenton reaction. In addition, Domingues et al. (15) used tandem mass spectrometry with a method of soft ionization (electrospray and matrix-assisted laser desorption ionization) to characterize changes in fatty acyl chain. Qian and Buettner (16) also used electron paramagnetic resonance spin trapping to examine iron-induced free radical oxidations. All of these large facility-based methods are time consuming and require sophisticated instrumentations.

In the past decade, long-range orientational anchoring of liquid crystals has become one of the most considerable tools in the field of biological assays, particularly based on the effects of interfacial

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interactions of biomolecules. The property of birefringence of LCs, due to their anisotropic nature, allows the orientational change of LCs to be observed under a polarized optical microscopy, which is visible with naked eye. We recently have shown that phospholipid monolayer spontaneously assembled at the aqueous/liquid crystal interface is correlated to the orientation of LCs (17–19). The biological enzyme activity involved in combining phospholipids is demonstrated to trigger orientational transition of LCs that can be readily visualized under crossed polarizers. This correlation provides principles to transduce and amplify biological enzymatic events that occur at these interfaces.

In the present study, we developed an LC-based approach for the label-free characterization of oxidized phospholipids by the Fenton reaction occurring at the interface between aqueous phase and immiscible liquid crystals. We attempted to explore the activities of iron(II) and hydrogen peroxide towards 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG) membrane at the aqueous/LC interface and their correlation to the orientational transitions of LCs. The impacts of different catalysts and pH on the Fenton oxidation were also explored. To the best of our knowledge, an LC has never been utilized to report the effects of oxidation of phospholipids by the Fenton reaction before. This study suggests the applicability and superiority of a label-less and low-cost LC-based approach for identifying phospholipids oxidized by the Fenton reaction and for screening their potential uses.

MATERIALS AND METHODS

Materials Tris buffered saline (TBS; 0.05 M Tris, 0.138 M NaCl, and 0.0027 M KCl, pH 8.0), sulfuric acid, hydrogen peroxide (30% w/v), octyltrichlorosilane (OTS), DOPG, sodium chloride, hydrochloric acid, sodium hydroxide, chloroform, ethanol, methanol, methylene dichloride, and capillary tubes were purchased from Sigma–Aldrich (USA). Liquid crystal, 4′-pentyl-4-cyanobiphenyl (5CB), was purchased from EM industries (Hawthorne, NY, USA). Copper specimen grids (50 meshes, 500 μm pitch, 420 μm hole, 80 μm bar, 25 μm ± 5 μm thickness) were obtained from Gilder Grids (Grantham, UK). Premium glass microscope slides were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water, with a resistivity of 18.2 MΩ cm, was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Treatment of glass microscope slides with OTS Glass microscope slides were cleaned in accordance with a previously described procedure (17). Briefly, the slides were immersed in the piranha solution [70% (v/v) sulfuric acid and 30% (v/v) hydrogen peroxide] for 30 min at ~80 °C (warning: piranha solution reacts strongly with organic compounds and should be handled with extreme caution; do not store the solution in closed containers). The slides were then rinsed with water, ethanol, and methanol and dried under a stream of gaseous nitrogen, after which they were heated at 120 °C overnight prior to OTS deposition. A 0.5 mM OTS solution was prepared and the piranha-cleaned slides were immersed in 0.5 mM OTS in a heptane solution at room temperature for 30 min. The samples were then rinsed with methylene dichloride and dried under nitrogen. Next, films of nematic LC [4-cyano-4′-pentylbiphenyl (5CB)] were deposited into the pores of gold grids on octadecyltrichlorosilane (OTS)–coated glass substrates, the OTS-coated glass caused the LC to assume a perpendicular (homeotropic) orientation at the glass surface. Any sample that did not exhibit homeotropic anchoring of 5CB was rejected.

Preparation of a glass slide-supported LC optical cell A glass slide-supported LC optical cell was prepared as previously reported (19). Simply put, OTS-treated glass slides were fixed to the bottom of an eight-well chamber slide with silicone mounting medium. Subsequently, transmission electron microscopy grids (50 meshes and 25 μm ± 5 μm thickness) were placed onto the slide. The copper grid was then impregnated with 2 μL of 5CB using a Hamilton syringe. By heating to its isotropic phase (>35 °C), excess 5CB was removed from the LCs with a 20-μL capillary tube. The grids containing LCs were then immersed into aqueous solutions of interest. Each assay was performed at least six times independently.

Preparation of phospholipid vesicles LCs, decorated with phospholipid membrane, were prepared from DOPG phospholipid solution following previously reported procedures (20). Simply put, phospholipids dissolved in chloroform (50 mg/mL) were dried under a stream of nitrogen and desiccated under vacuum for at least 3 h. Dried phospholipids were resuspended with Tris-buffered saline (TBS, consisting of 0.05 M Tris and adjusted to a pH of 5.8 prior to use). The final concentration of DOPG phospholipid solution was 1 mM. The turbidity of the resulting solution represented the presence of large multilamellar vesicles. The phospholipid suspension was then sonicated three times each for 5 min to clarify using a probe sonicator. Then, the solution was filtered twice with a 0.22-μm filter and typically used within 2 d after the preparation. Prior to the formation of the DOPG membrane, 400 μL of DOPG solution was added to one of the wells supported on the glass slide. The DOPG membrane was then formed by touching a copper grid impregnated with 5CB to the DOPG solution in the well.

Preparation of oxidized phospholipid vesicles The phospholipid vesicles prepared as described above were diluted to a final concentration of 0.5 mM and the mixture was vortexed. Oxidative treatments using Fe(II) and H_2O_2 were performed as per the procedure detailed in a previous publication (21). 5 μL $FeSO_4$ solution (50 mM) and 500 μL of hydrogen peroxide (H_2O_2) at a certain concentration are added to 500 μL of the phospholipid vesicles. This mixture was then left to react at room temperature for 2 h under continuous shaking.

LC-based technique for testing the oxidation of phospholipid Prior to the identification of the oxidation effects of hydrogen peroxide on phospholipids, 400 μL of a solution containing oxidized phospholipids (in 0.05 M Tris, 0.138 M NaCl, and 0.0027 M KCl; the pH was adjusted prior to use) was added to individual wells. 5CB (confined in copper grids) was then immersed in these oxidized phospholipid solutions.

Optical examination of LC texture The optical texture of the LCs was examined using a Nikon eclipse LV100 POL microscope equipped with crossed polarizers in transmission mode. The sample was placed on a rotating stage between the crossed polarizers. All optical images were captured using a digital camera (DS-2Mv, Nikon, Tokyo, Japan) mounted on the microscope with a resolution of 1600 × 1200 pixels, a gain of 1.00×, and a shutter speed of 1/10 s.

Determination of phospholipid oxidation by UV/Vis spectroscopy The percent oxidation of the phospholipid was determined using Beer's Law by calculating the peroxidized phospholipid concentration from the absorbance at 235 nm. An aliquot (50 μL) of the reaction mixture was taken out and diluted with ethanol to 500 μL. The absorbance at 235 nm was measured with a Varian 50 Bio UV/Vis spectrophotometer.

RESULTS AND DISCUSSION

LC-based method for imaging oxidation effects of the Fenton reaction on bioactive phospholipids The oxidation of lipids has been a topic of interest in biochemical and food sciences for a long time. The basic principles of non-enzymatic free radical effects on phospholipids are well established, although questions about detailed mechanisms remain unanswered. In this LC-based technique, the phospholipid monolayer at the aqueous/LC interface was used as the oxidation substrate. Phospholipids, because of their amphiphilic nature, form spherical lipid bilayers known as liposomes and vesicles. When phospholipid vesicles are exposed to a hydrophobic surface, they fuse with the surface and form a planar membrane, leading to the formation of a lipid monolayer. Previous studies have demonstrated that the presence and organization of amphiphiles at the interface between liquid crystals (LCs) and an immiscible aqueous phase is coupled to the orientational ordering of the LCs. Brake et al. (30) reported that contact the interface of a thermotropic LC with an aqueous solution of phospholipid resulted in the spontaneous assembly of a monolayer of phospholipid at the aqueous/LC interface, corresponding to a dark image in the optical response. Fig. 1 shows the schematic illustration of the experimental system. A phospholipid monolayer was prepared by exposing the 5CB-filled copper grid to an aqueous solution containing 500 μM of DOPG. Following the exposure, the initially bright optical images of 5CB became uniformly dark (Fig. 1A), indicating the self-assembly of DOPG at the aqueous/LC interface. Films of 5CB laden with DOPG were then washed with TBS buffer five times and were stable for more than a week. However, when 5CB films were exposed to phospholipids oxidized by the Fenton reaction, the optical images of 5CB evolved from dark to bright colors (Fig. 1B). This observation is in accordance with previous studies demonstrating that the orientational changes of LCs correspond to the formation of the phospholipid monolayer at aqueous/LC interfaces (22). We also examined the optical response of LCs after introducing the Tris buffer solution (TBS) into the chamber containing LCs and observed a bright appearance (Fig. 2A), which was attributed to the planar orientation of LC molecules at the aqueous/LC interface.