Anchoring Transitions of Liquid Crystals for Optical Amplification of Phospholipid Oxidation Inhibition by Ascorbic Acid

Minmin ZHANG and Chang-Hyun JANG†

Department of Chemistry, Gachon University, Seongnam-Si, Gyeonggi-Do 461-701, Korea

There is considerable evidence that the antioxidant property of ascorbic acid (AH) is effective for reducing oxidative stress of phospholipids. Herein, a liquid crystals (LCs)-based method was developed for the optical amplification of resistance to phospholipid oxidation by AH. Phospholipid peroxidation initiated by free radicals was monitored from a homeotropic-to-planar anchoring transition of LCs via polarized optical microscopy. Alternatively, consistent homeotropic anchoring of LCs was observed when the oxidation caused by free radicals was blocked by AH.

Keywords Ascorbic acid, antioxidant, phospholipids, liquid crystals, anchoring transition

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4-cyanobiphenyl (5CB), was purchased from EM Industries (Hawthorne, NY). Copper specimen grids (50 meshes, 500 μm pitch, 420 μm hole, 80 μm bar, 25 ± 5 μm thickness) were obtained from GILDER GRIDS (Grantham, Lincs). Premium glass microscope slides were purchased from Fisher Scientific (Pittsburgh, PA). Ultrapure water, with a resistivity of 18.2 MΩ cm, was obtained from a Milli-Q system (Millipore, Bedford, MA).

Treatment of glass microscope slides with OTS
Glass slides were immersed in piranha solution [70% (v/v) sulfuric acid and 30% (v/v) H₂O₂] for 30 min at ~80°C. 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 and allowed to stay at room temperature for 30 min. The samples were then rinsed with methylene dichloride and dried under nitrogen.

Formation of AH-PAPC vesicles
AH–TBS solution was obtained by dissolving solid AH in TBS buffer (TBS consisting of 0.05 M Tris and adjusted to a pH of 5.8 prior to use). Phospholipids dissolved in chloroform (50 mg/mL) were dried with N₂ gas and desiccated under vacuum for at least 3 h. Dried phospholipids were resuspended with AH–TBS to a final concentration of 1 mM. The phospholipid suspension was then sonicated three times for 5 min each to clarify using a probe sonicator, filtered twice with a 0.22-μm filter, and typically used within 2 d after preparation.

Preparation of a glass slide-supported LC optical cell
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 ± 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 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 the aqueous solutions of interest. Each assay was independently performed at least six times.

Results and Discussion

LC-based technique for testing the antioxidation of AH
Prior to the identification of the antioxidation effects of AH on phospholipids, 400 μL of a solution containing AH-PAPC (in 0.05 M Tris, 0.138 M NaCl, and 0.0027 M KCl; the pH was adjusted prior to use) was reacted with Fenton reagent (5 μL FeSO₄ solution (50 mM) and 500 μL of hydrogen peroxide (H₂O₂) (25 mM)) for 2 h at room temperature with continuous shaking. Subsequently, 5CB (confined in copper grids) was immersed in these mixed solutions.

LC-based sensor for imaging oxidation effects of the Fenton reaction on bioactive phospholipids
LCs were employed for real-time and label-free characterization of phospholipid oxidation by the Fenton reaction. Figure 1D shows the representative chemical structure of LCs and oxidized phospholipids forming during oxidation of PAPC. 2-Arachidonoyl-1-palmitoyl-sn-glycero-3-phosphocholine (PAPC), owing to its amphiphilic properties, self-assembled at the aqueous–LC interface, causing the LC optical images to turn dark and indicating a homeotropic alignment of LC molecules at the interface (Fig. 1A). This result is consistent with previous research, which demonstrated that the orientational changes of LCs correspond to the formation of the phospholipid monolayer at aqueous-LC interfaces. In this study, phospholipid solution at a concentration of 0.5 mM was utilized as a detection substrate. After oxidation by Fenton reaction, oxidized PAPC which remain at the interface prevent self-assembling, inducing orientational changes of LCs from the homeotropic to planar transition. As a result, a dark-to-bright optical change was observed through polarized optical microscopy (Fig. 1B). Subsequently, the optical signals that represented the oxidation level of phospholipids at different concentrations of H₂O₂ were
observed in real time. The sensitivity of H$_2$O$_2$, a vital component of the Fenton reagent, was also determined to be 25 mM at room temperature.\textsuperscript{17} Compared with previous research by Brandi and Baatout,\textsuperscript{21,22} the detection limit is reasonable. Brandi et al. found that cell membrane permeability was markedly increased in cell membranes at H$_2$O$_2$ concentration >13.25 mM.\textsuperscript{21} We believe that the sensitivity of H$_2$O$_2$ can be still increased if we increase the temperature and prolong the incubation time.

**Role of AH in phospholipid oxidation**

It is well-known that AH works as a donor antioxidant, it could be considered a valid alternative to protect lipids from oxidation. As we mentioned above, when 5CB films were exposed to phospholipids oxidized by the Fenton reaction, the optical images of 5CB changed from dark to bright colors (Figs. 1A and 1B). However, after AH was added into the mixed aqueous solution of PAPC and Fenton reagent and incubated for 2 h at room temperature with continuous shaking, 5CB films immersed under 400 μL of this solution remained dark, indicating a homeotropic state in LC orientation (Fig. 1C). After 1 h passed, the uniformly dark appearance of the LCs was still sustained. We observed the optical response for more than 4 h, the image remained black during contact with the solution, maintaining the homeotropic alignment. These results indicate that the presence of antioxidants in the phospholipid oxidation process is an effective way to protect the lipid from oxidative damage.

We confirmed antioxidation of AH by field emission scanning electron microscopy (FE-SEM). Figure 2 shows SEM images of the PAPC membrane with and without the addition of AH prior to the initiation of Fenton oxidation. Phospholipid membrane specimens that were pre-incubated with different solutions were transferred onto a silicon wafer. After the membrane was dried overnight at room temperature, SEM images were obtained using an FE-SEM (JEOL Corp., JSM 6700F) at 15.00 kV. The SEM image shows that the phospholipid membrane has a porous morphology after oxidation by the Fenton reaction (Fig. 2A). Instead, after AH was dispersed as an aqueous solution directly in Fenton reagent, the oxidation effect of free radicals on phospholipids was substantially reduced, as shown in Fig. 2B. It is clear that the antioxidant activity derived from AH protected the phospholipid from being destroyed. We also used UV/Vis spectroscopy to confirm the antioxidation of AH on phospholipid oxidation (Fig. 3). The absorption of a conjugated diene at 235 nm was used to determine the concentration of hydroperoxides.\textsuperscript{23} To calculate absorbance at a given wavelength, the computer in the spectrophotometer simply takes the intensity of light at that wavelength before it passes through the sample ($I_0$), divides this value by the intensity of the same wavelength after it passes through the sample ($I$), then takes the log 10 of that number:

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A = \log\frac{I_0}{I}
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The illustration in Fig. 3 reveals the peroxide value as a function of oxidation time. It is apparent that the antioxidation of AH inhibited phospholipid oxidation.

After we confirmed the antioxidant activity of AH, we monitored the antioxidation of AH in more detail. We next studied in vitro phospholipid oxidation in the presence of different concentrations of AH. We found that the antioxidant activity was concentration-dependent. A series of mixed solutions of PAPC pre-incubated with Fenton reagent and AH at different concentrations was introduced into the LC optical chamber. When 400 μL of 500 μM PAPC solution pre-incubated with Fenton reagent and 500 μM AH was introduced into the 5CB film chamber, a dark appearance (Fig. 4B) was displayed as an optical response, indicating a homeotropic orientation of LCs at the aqueous/LC interface. This result was comparable to
the optical response of LC contact with pure PAPC solution (Fig. 4A). Next, we employed AH solution at a concentration of 230 μM; the same dark image of LC was obtained, suggesting a homeotropic alignment of LCs at the aqueous/LC interface (Fig. 4C). Then, we carried out the same detection using AH at a concentration of 170 μM. Contrary to the above results, the LCs adopted a partially bright appearance when they were immersed under PAPC solution that had been pre-incubated with the mixed solution of Fenton reagent and 170 μM AH, corresponding to a tilted alignment of LCs at the aqueous/LC interface (Fig. 4D). When we lowered AH concentration to 85 μM, the LCs immediately became entirely bright in optical appearance and remained bright for 1 h, indicating that the orientation of LC transitioned to planar anchoring (Fig. 4E). This appearance was similar to the one we obtained from PAPC solution that was solely pre-incubated with Fenton reagent (Fig. 4F), where LCs produced a bright response and indicated a planar alignment at the aqueous-LC interface. These findings strengthened the notion that there is an increase in phospholipid oxidation, which is reflected by increased luminosity in LC images. In an effort to quantify the luminosity of the LC image, we used the ImageJ software to convert images to grey scale, and the average pixel brightness of each analyzed region was calculated. The luminosity of the optical images of 5CB was evaluated after contact with PAPC solution pre-incubated with Fenton reagent and AH solutions at different concentrations. The dynamic response of LC to AH at different concentrations, included an inverse S-curve typical for LC-based imaging response, showing the threshold of antioxidation (Fig. 4G). From the results above, it is clear that antioxidant activity has a strongly positive correlation with AH concentration.

Conclusions

In summary, we demonstrated an LC-based detection technique for label-free characterization of phospholipid oxidation by the Fenton reaction. Phospholipid oxidation disrupted the integrity and organization of the lipid membrane and induced a bright appearance in the aqueous-LC interface, and AH was used as an effective antioxidant to protect the lipid from oxidative damage. After the addition of AH solution, the optical response reverted back to a dark appearance, indicating that the phospholipid monolayer formed at the aqueous-LC interface, prior to the oxidation. Moreover, the results suggest that AH showed concentration-dependent activity.

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