Astaxanthin (Asx), 3,3′-dihydroxy-β,β-carotene-4,4′-dione; Fig. 1 chemical structure) is a common pigment found in algae, fish, and birds. Astaxanthin has been reported to be more effective than a representative carotenoid, β-carotene, for prevention of singlet oxygen, as well as lipid peroxidation in biological membranes. The direct radical scavenging capability of Asx has been confirmed using the synthetic radical 1,1-diphenyl-2-picrylhydrazyl. Previously, we suggested that the potent antioxidation activity of astaxanthin in biomembranes could be attributable to the conjugated polyene and terminal ring moieties of Asx that trap radicals on the membrane surface, they would be available to react with exogenous free radicals in the aqueous phase. Therefore, liposome encapsulated Asx (Asx-lipo) was expected to be an ideal formulation for measurement of Asx hydroxyl radical scavenging ability in aqueous solution.

In this study, Asx-lipo was prepared, and its hydroxyl radical scavenging activity in aqueous solution was examined and compared with the activity of the well known antioxidants β-carotene and α-tocopherol also in liposome formulations. Moreover, the protective effect of Asx-lipo on hydroxyl radical-induced cytotoxicity was examined. This report constitutes the first study of the hydroxyl radical scavenging capability of Asx in aqueous solution.
purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, U.S.A.). Egg phosphatidylcholine (EPC) and dimyristoyl phosphatidylcholine (DMPC) were obtained from NOF Corporation (Tokyo, Japan). Luminol reagent was purchased from Nacalai Tesque (Kyoto, Japan). NIH3T3 cells were obtained from RIKEN BRC CellBank (Wako, Japan). All other reagents were of the highest grade commercially available.

**Preparation of Liposomal Formulations of the Antioxidants Astaxanthin, β-Carotene, and α-Tocopherol**

EPC liposome and DMPC liposome encapsulated antioxidant formulations were prepared using a lipid hydration method. Chloroform solutions containing 1µmol of EPC or DMPC, and various amounts of the antioxidants Asx, β-carotene, or α-tocopherol were dried to a thin film under vacuum using a rotary evaporator (N-1000, EYELA, Tokyo, Japan). The dried lipid film was hydrated with 0.1 mL of 10 mM Tris buffer (pH 7.4) at room temperature to obtain a liposomal suspension, and the particles were diminished in size by sonication using a bath-type sonicator (AU-25C, Aiwa, Tokyo, Japan). The diameter of liposome encapsulated antioxidant was measured by the dynamic light scattering method using a Zetasizer nano (Malvern Instruments Ltd., U.K.).

**Measurement of Chemiluminescence Intensity Dependent on Hydroxyl Radical Production by Fenton Reaction**

Hydroxy radical generation was evaluated by chemiluminescence intensity according to the previously reported method. To generate hydroxyl radicals, 0.1 mL of 0.25 M H2O2 (final concentration: 385 µM) and 0.2 mL of 1 mM FeSO4 solution (final concentration: 48 µM) were mixed in a test tube. Hydroxyl radicals are known to be generated by the Fenton Reaction (final lipid concentration: 0.96 mM of EPC or DMPC, and 0.2 mM of 1 mM FeSO4 solution (final concentration: 330 µM), and 0.2 mL of 1 mM FeSO4 solution (final concentration: 385 µM) were mixed in a test tube. Hydroxyl radicals are known to be generated by the Fenton reaction as follows; Fe2++H2O2→Fe3++OH−+·OH.

Therefore, the generated amount of hydroxyl radicals might be 385µM in theory. Then, 5s after mixing, chemiluminescence intensity was measured for 2 min by a Luminescence-PSN (ATTO, Tokyo, Japan).

**Measurement of Absorption Spectra of Astaxanthin in Liposomes before and after Incubation with Fenton’s Reagent**

Absorption spectra of Asx-lipo suspension before and after incubation with FeSO4 and H2O2 were measured by microplate-reader infiniteM200 (Tecan Group Ltd., Swiss). The concentrations of liposomal suspension, FeSO4 solution, and H2O2 were the same as described above. Chemiluminescence intensity was measured 5 s after mixing for 2 min by a Luminescence-PSN. The absorption spectrum of EPC liposomes without astaxanthin was subtracted from each absorption spectrum of Asx-lipo to cancel liposomal turbidity. Therefore, the absorption spectra presented in Fig. 4 are differential spectra between liposomes with and without astaxanthin.

**Estimation of Cytotoxicity by Hydroxyl Radical Produced by Fenton Reaction**

NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C and in a 5% CO2 atmosphere for 24h. The medium was removed, and then DMEM containing 1 mM of liposomes was added to the cells. The cells were incubated with the medium containing liposomes for 30 min at 37°C. After incubation, the medium was removed, and the cells were incubated with DMEM containing 10 mM FeSO4 and 10 mM H2O2 at 37°C for 30 min. After incubation, DMEM was removed, and the cells were collected by treatment with trypsin. The collected cells were stained with 0.4% trypan blue, and the numbers of total cells and stained cells were counted.

**Statistical Analysis**

Statistical significance was determined using Student’s t-test. p values <0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

**Preparation of liposomes containing astaxanthin, β-carotene, and α-tocopherol**

In this study, we chose EPC, which is used widely for liposome formulation, as a base lipid of liposomes to encapsulate Asx. Liposomes were prepared by the conventional lipid film hydration method. The average diameter of EPC liposomes without Asx (EPC-lipo) was approximately 150 nm, although the values widely varied (Table 1). EPC-lipo encapsulating Asx (Asx-EPC-lipo) were prepared by the same method as for EPC-liposomes. The average diameter of EPC-lipo containing various amounts of Asx showed a tendency to increase with increasing amounts of Asx (Table 1). For comparison with Asx-EPC-lipo, EPC-lipo containing β-carotene (β-caro-EPC-lipo) and α-tocopherol (α-toco-EPC-lipo) were prepared. The average size of β-caro-EPC-lipo also increased with an increase in β-carotene content; however, the diameter of α-toco-EPC-lipo was not dependent on the amount of α-tocopherol (Table 1). These results suggest that the increase in diameter from EPC-lipo was due to Asx and β-carotene intercalation into the lipid membrane via the polyene portion of the structures. The effect of incorporating α-tocopherol on liposomal size differed from the carotenoids presumably because its smaller size and amphiphilic structure would more readily maintain the liposome size (Fig. 1), although the hydroxyl group of the chroman ring has also been suggested to locate around the hydrophobic/hydrophilic interface of lipid membranes similar to astaxanthin.

**Effect of Astaxanthin Liposomes on Chemiluminescence Intensity Depending on Generation of Hydroxyl Radical by the Fenton Reaction**

In order to evaluate the scavenging ability of Asx for hydroxyl radicals, we examined the effect

<table>
<thead>
<tr>
<th>Amount of antioxidant (mol%)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx-EPC-lipo</td>
<td>151.4±125.0</td>
<td>170.1±119.4</td>
<td>168.1±133.1</td>
<td>198.6±140.7</td>
<td>333.6±80.2</td>
</tr>
<tr>
<td>β-Car-EPC-lipo</td>
<td>151.4±125.0</td>
<td>166.3±80.2</td>
<td>203.8±56.7</td>
<td>213.8±99.2</td>
<td>216.3±92.4</td>
</tr>
<tr>
<td>α-Toco-EPC-lipo</td>
<td>151.4±125.0</td>
<td>114.1±51.0</td>
<td>95.3±28.5</td>
<td>108.9±31.1</td>
<td>188.6±232.4</td>
</tr>
<tr>
<td>Asx-DMPC-lipo</td>
<td>112.5±51.2</td>
<td>—</td>
<td>—</td>
<td>232.9±268.9</td>
<td>171.8±50.7</td>
</tr>
</tbody>
</table>

The average diameter of liposomes was measured by dynamic light scattering. Data shown are the means with S.D. from multiple measurements (n=5).
of EPC-lipo encapsulation of various amounts of Asx on the chemiluminescence intensity that depended on hydroxyl radical generation by the Fenton reaction in aqueous solution. As shown in Fig. 2, the chemiluminescence intensity decreased in a dose-dependent manner with respect to Asx in EPC-lipo. In particular, relatively low concentrations (<20 µM) of Asx significantly reduced the chemiluminescence intensity. This result indicates for the first time that Asx encapsulated in EPC-lipo membranes can scavenge hydroxyl radicals in aqueous solution. As suggested previously, the terminal rings of Asx would be located at the membrane surface, and the polyene moiety would be embedded in the hydrophobic region of the lipid membrane6); therefore, the terminal rings of Asx on the liposome surface would be available to scavenge hydroxyl radicals.

β-Caro-EPC-lipo and α-toco-EPC-lipo also reduced the hydroxyl radical dependent chemiluminescence intensity. It has been reported that α-tocopherol in liposomal membranes and micelle structures can scavenge hydroxyl radicals generated in aqueous solution.19) Recently, direct observation of β-carotene reaction with hydroxyl radical was confirmed using a photo-Fenton reagent to generate hydroxyl radicals in acetonitrile/tetrahydrofuran solution,19) although similar measurements have not been performed in aqueous solution. Therefore, this is the first report regarding the hydroxyl radical scavenging reactions of carotenoids including β-carotene and Asx in aqueous solution. The concentrations of antioxidants encapsulated in EPC-lipo producing 50% inhibition of chemiluminescence intensity (IC 50) are summarized in Table 2. The IC 50s of β-caro-EPC-lipo and α-toco-EPC-lipo were 20 and 40 µM, respectively. The IC 50 value of Asx-EPC-lipo was lower than that of α-toco-EPC-lipo, which indicates Asx encapsulated in EPC-lipo is a more potent scavenger of hydroxyl radicals generated in aqueous solution than the liposome formulation of α-tocopherol. The potent hydroxyl radical scavenging activity of Asx may be due to dual radical trapping by the terminal ring as well as the conjugated polyene moieties of Asx on the membrane surface and within the membrane.6) On the other hand, the radical trapping site of α-tocopherol is limited to a phenolic group, although α-tocopherol diffusing freely in the lipid bilayer would effectively react with hydroxyl radicals generated on the outer layer surface of liposomes because of the location of α-tocopherol is not fixed in the lipid membranes.20) In this experimental condition, luminol molecules would localize at the membrane interface due to its hydrophobicity. Therefore, Asx might competitively scavenge hydroxyl radicals with luminol at the interface of lipid membranes.

**Effect of Unsaturated Acyl Chains in EPC on Hydroxyl Radical Scavenging of Asx-Lipo** Since EPC consists of unsaturated fatty acids, which can react with ROS, we were interested in ascertaining whether the unsaturated acyl chains of EPC were responsible for the preventative effect of Asx-EPC-lipo on the hydroxyl radical dependent chemiluminescence intensity. In order to confirm the participation of unsaturated acyl chains in hydroxyl radical scavenging by Asx-EPC-lipo, DMPC, which consists of saturated fatty acids, was used as a control base lipid for liposome encapsulated Asx. The size of DMPC liposomes without Asx (DMPC-lipo) also increased slightly with an increase in the amount of Asx, although the

![Graph showing effect of Asx-EPC-Lipo on Chemiluminescence Intensity](image)

**Table 2. IC 50 Values of Hydroxyl Radical Scavenging by EPC-Lipo Encapsulated Asx, β-Caro, and α-Toco**

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>IC 50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxanthin</td>
<td>18</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>20</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>40</td>
</tr>
</tbody>
</table>

IC 50 values were obtained from the dose-dependent decrease by EPC-lipo encapsulated Asx, β-caro, and α-toco on chemiluminescence intensity induced by hydroxyl radical production.

![Graph showing effect of Asx Encapsulated in EPC-Lipo Consisting of Unsaturated Acyl Chain and DMPC-Lipo Consisting of Saturated Acyl Chains on Hydroxyl Radical Dependent Chemiluminescence Intensity](image)
average diameter of DMPC-lipo was less than that of EPC-lipo (Table 1). As shown in Fig. 3, DMPC liposomes encapsulating Asx (Asx-DMPC-lipo) also decreased the hydroxyl radical dependent chemiluminescence intensity in a dose-dependent manner. The Asx-DMPC-lipo had almost the same scavenging effect on hydroxyl radicals as Asx-EPC-lipo. In addition, chemiluminescence intensity of DMPC-lipo without Asx was also the same as that of EPC-lipo without Asx (Supplemental Fig. 1). These results indicate that the unsaturated acyl chains of EPC did not participate in the scavenging capability of Asx-EPC-lipo and that Asx itself in liposomal membranes is responsible for hydroxyl radical scavenging.

Evaluation of Direct Reaction of Asx in Liposomes with Hydroxyl Radical Recently, the direct reaction of β-carotene with hydroxyl radicals was evaluated from the decrease of characteristic peaks in visible and near-infrared absorption spectra. To obtain evidence regarding direct scavenging of hydroxyl radical by Asx, absorption spectra of Asx-lipo before and after the addition of Fenton’s reagent were measured (Fig. 4). Asx-lipo gave an absorption peak at 470 nm, which was almost the same wave length as reported previously for Asx in acetonitrile/tetrahydrofuran solution, which was dependent on the amount of Asx encapsulated within the liposomes. Upon addition of Fenton’s reagents into the suspension, peak absorbance values for Asx at both 40 μM and 80 μM concentrations decreased as shown in Fig. 4. These results indicate that the conjugated polyene chain moiety was disrupted by hydroxyl radicals and suggests that Asx in liposomes scavenged hydroxyl radicals by direct reaction with its polyene moiety. Asx might react with hydroxyl radicals at the interface of lipid membranes, since hydroxyl radicals would be generated by Fe²⁺/H₂O₂ accumulated on the surface of liposomal membranes. The decrease in the absorbance of 80 μM Asx was almost double that of the 40 μM concentration, although the difference in chemiluminescence intensity at 40 μM and 80 μM of Asx was not significant, i.e., the absorbance change of Asx did not correspond to hydroxyl radical scavenging shown in Figs. 2 and 3. Therefore, 40 μM Asx would be enough to scavenge hydroxyl radicals in this condition. Higher decrease in the absorbance of 80 μM Asx than that of 40 μM Asx might be due to reaction of surplus Asx with Asx radicals generated by hydroxyl radical scavenging.

Effect of Asx-EPC-Lipo on Cytotoxicity Induced by Hydroxyl Radical Since Asx-EPC-lipo showed a significant scavenging effect on hydroxyl radicals in aqueous solution, Asx-EPC-lipo was also expected to protect cells from hydroxyl radical induced cytotoxicity. Previously, we reported that topical administration of Asx-EPC-lipo protected skin from UV-induced damages. Thus, we examined the protective effect of Asx-EPC-lipo on hydroxyl radical-induced cytotoxicity in the cultured mouse skin fibroblast cell line, NIH3T3 cells. As shown in Fig. 5, Asx-EPC-lipo prevented hydroxyl radical-induced cytotoxicity in a dose-dependent manner. Since Asx-lipo would be internalized into cells via endocytosis after a 30 min incubation and removal of non-internalized free liposomes, the preventative effect was probably due to radical scavenging on the cell surface by Asx distributed in the cell plasma membranes.

CONCLUSION

In this study, Asx-EPC-lipo was prepared, and its capability to scavenge hydroxyl radical in aqueous solution was examined. Asx-EPC-lipo demonstrated significant hydroxyl radical scavenging activity in aqueous solution, which was more potent than either EPC-lipo encapsulated β-carotene or α-tocopherol. The absorption spectrum of Asx in liposomes was reduced in the presence of hydroxyl radical, which indicates that Asx reacted directly with hydroxyl radical. Moreover, Asx-EPC-lipo prevented cell cytotoxicity induced by hydroxyl radical generated in an aqueous phase. This is the first report regarding the hydroxyl radical scavenging capability of Asx in aqueous solution.
REFERENCES


