Protection of DPPC phospholipid liposomal membrane against radiation oxidative damage by antioxidants

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ABSTRACT
Investigations in our laboratory on egg lecithin liposomes have recently showed a marked protection against damage by gamma radiation when cholesterol was present in the composition of vesicles suggesting a role of bilayer molecular architecture in the mechanism of free radical mediated lipid peroxidation (1). Present study was designed to determine the changes in bilayer permeability in DPPC unilamellar vesicles after exposure to gamma radiation by monitoring the leakage of pre-loaded carboxyfluorescein (CF), a marker loaded in aqueous interior of vesicle and fluidity alterations in the bilayer using fluorescence polarization of 1, 6 - diphenyl - 1, 3, 5 - hexatriene (DPH), a membrane bilayer probe. It was found that radiation doses of an order of magnitude higher were required to produce detectable changes in vesicles of DPPC than in the vesicles of egg lecithin suggesting a modulating role of chemical nature of composition in the membrane radiation sensitivity. It was significant to find that the leakage of CF from and incorporation of DPH into vesicle bilayer showed similar response pattern to radiation doses (0.1-6 kGy) which was also found to be dose rate dependent. Presence of antioxidants; alpha-tocopherol (0.15 mole %) in the bilayer membrane or ascorbic acid (0.1 mM) in the aqueous region significantly protected DPPC vesicles from radiation damage as determined from DPH uptake kinetics suggesting involvement of reactive free radicals of lipids as well as water radicals in the mechanism of membrane peroxidative damage. The magnitude of protection was found to increase with the increasing concentration of both these antioxidants but comparisons showed that α-tocopherol was far more effective in protecting the vesicles than ascorbic acid. These results contribute to our understanding of the mechanism of radiation oxidative damage and its modification by radical scavenging and/or organizational modulation which emphasize the importance of structure and composition of membrane in developing new strategies to reduce or eliminate the damaging effects of ionizing radiation on living cells.

INTRODUCTION
Ionizing radiation induced damage of cellular membrane is known to alter many structural and physiological processes leading to loss of normal cellular function including cell death (2). Oxidative damage of membrane is mediated by the free radicals generated in the cytosol and in the surrounding of the cell which are believed to be involved in radical chain reactions (3). Polyunsaturated fatty acyl chains in biological membrane are the critical targets which display susceptibility to attack by the radiation induced free radicals. Liposomes offer a suitable model membrane system to investigate the molecular mechanism of membrane damage induced by radiation since the composition of liposomes can be easily controlled and varied. We have employed a fluorescence probe, carboxyfluorescein (CF), to detect the changes in permeability of liposomal membrane (4-5). Fluorescence polarization measurement of diphenyl hexatriene (DPH) provides an important method to monitor the structural and fluidity changes in cellular and liposomal membrane (6-7) which has been employed to reveal structural alterations in irradiated unilamellar liposomes. Results from our laboratory have demonstrated that the presence of cholesterol in the composition of egg lecithin liposomal membrane produced a modulating effect on structural order of the membrane bilayer which reflected in significantly protecting liposomes against radiation damage (1). This report describes results of further investigations on radiation effects on unilamellar liposomes prepared from DPPC. It is shown that irradiation induced membrane damage exhibits a biphasic response to increasing doses of radiation. Results have also shown that incorporation of lipophilic antioxidant such as α-tocopherol or hydrophilic antioxidant e.g. ascorbic acid in the vesicles produced significant protective effect on the irradiated liposomes. Results have further shown that the tocopherol was more effective than ascorbic acid in protecting the damage to liposomal membrane against gamma radiation.

Keywords : Gamma radiation, liposome, Carboxyfluorescein, DPH fluorescence polarization, antioxidant

Abbreviations: DPPC, Dipalmitoylphosphatidylcholine; PBS, Phosphate buffer saline; CF, 6 - carboxyfluorescein ; DPH , 1,6-diphenyl - 1,3,5-hexatriene.
MATERIALS AND METHODS

Chemicals and reagents
DPPC, DPH and ascorbic acid were procured from Sigma. CF was obtained from Eastman Kodak Co. CF was used after purification to remove polar contaminants (8). Sephadex G-50 coarse was a product of Pharmacia Biotech. and α-tocopherol was supplied by Fluka. Aqueous solutions were prepared in distilled water and fluorescence measurements were made using PBS filtered through 0.22 micrometer membrane filter.

Preparation of liposomes
Liposomes were prepared by the film hydration method (9). 20 mM of DPPC was dissolved in chloroform in a round bottom flask. The organic solvent was evaporated under vacuum leaving an uniform thin-film on the inner walls of the flask which was further dried for 3 h. The film was hydrated with 1 ml of 10 mM PBS (pH 7.4) using few glass beads. The milky suspension of multilamellar vesicles (MLV) was sonicated (20 kHz, 200 W, model w-220 F, Heat Systems Ultrasonics, New York) for 45 min with a titanium probe (Dia. = 0.19 cm). During hydration and sonication the temperature of DPPC liposome suspension was maintained at 45°C. The sonicated liposomal suspension was centrifuged (1000 g, 10 min.) to remove titanium particles and the supernatant was used for experiments after appropriate dilutions in PBS. The average diameter of sonicated vesicle suspension was predominantly about 100 nm.

α-Tocopherol was incorporated in liposomal membrane during the thin film preparation. DPPC was mixed with the antioxidant in chloroform to obtain a final concentration of 0.15 mol %. Ascorbic acid was encapsulated in DPPC liposomes by hydration of the dried lipid film with 0.1 mM ascorbic acid solution in PBS (pH 7.4).

To encapsulate CF in DPPC vesicles, the lipid film was hydrated with a 100 mM CF solution prepared in PBS followed by usual procedure of sonication. The unilamellar vesicles obtained after sonication were filtered through Sephadex G-50 gel minicolumn to separate the non-encapsulated probe material (5) and subsequently the suspension was dialyzed overnight against PBS at 25°C.

Irradiation of liposomes
The unilamellar liposomes prepared from DPPC (6 kGy ;100 Gy / min. or upto 700 Gy ;11 Gy / min.) were irradiated at 25°C by 60 Co γ-rays. Liposomal suspension was diluted 200 times with PBS before irradiation. The pH of PBS and fluorescence of CF (100 mM in PBS) was not changed after irradiation at 25°C upto 6 kGy.

Determination of vesicle permeability
Membrane permeability in irradiated DPPC vesicles was monitored by leakage of CF from liposomes after exposure to a dose of 0.7 and 6 kGy followed by incubation at room temperature upto 7 h. Self quenched concentration of CF (> 50 mM) in liposomes developed an intense fluorescence when it was diluted after leakage in outer aqueous environment. The fluorescence intensity of CF was measured at 25°C (λex = 490 nm, and λem = 520 nm, Ex / Em band width = 5 / 10 nm) by spectrofluorimeter (LS 50 B Model, Perkin Elmer). Permeability of liposomes was expressed in terms of relative fluorescence intensity which was the ratio between intensity of a particular liposome sample and the total fluorescence obtained by disruption of liposomes by Triton X-100 (0.01 %), and multiplied by 100.

Labeling with DPH and polarization measurement in liposomes
Radiation induced changes in membrane fluidity of liposomes were measured by fluorescence polarization of DPH (1). Irradiated DPPC liposomes were labeled with 10 μM DPH in PBS at 25°C (L / P molar ratio 100). The fluorescence intensity and depolarization of DPH in labeled liposomes were measured in PBS at 25°C at excitation wavelength, 357 nm (band width = 5 nm) and emission wavelength, 425 nm (band width = 5 nm) by spectrofluorimeter (LS50 B, Perkin -Elmer) equipped with in-built system for polarization of excitation and emission light, and correction of the grating factor (G). The fluorescence from liposomes without the probe was always less than 1 % as compared to the labeled liposomes.
RESULTS

CF fluorescence in irradiated vesicles

DPPC liposomes encapsulated with CF were γ-irradiated for increasing doses (0.3-6 kGy) followed by incubation at 25 °C. The fluorescence intensity of liposomes measured after different time of incubation showed that the degree of CF leakage from the DPPC liposomes was dependent on the applied radiation dose (Table 1). The percent release of CF from vesicles irradiated for 0.7 kGy dose was three times higher compared to control which was found to decrease at higher radiation doses and the leakage of CF from the liposomes was insignificantly small following radiation dose of 6 kGy up to 7 h incubation.

Table 1. CF leakage from irradiated liposomes. DPPC liposomes encapsulating CF (100 mM) and suspended in PBS were γ-irradiated for increasing doses (Dose rate: 100 Gy/min.) followed by incubation at 25 °C. The leakage of liposomes was monitored after 0 and 7 h of incubation as mentioned in Materials and Methods.

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Radiation induced membrane fluidity changes in liposomes

Fig. 1. shows the dose rate dependent changes in fluorescence polarization of DPH in γ-irradiated DPPC liposomes labeled with DPH at 25 °C for 17 h in PBS. Decrease in DPH polarization was found in vesicles irradiated up to 1 kGy which, however, showed an increase at higher doses of irradiation (up to 6 kGy). For a constant radiation dose, the observed polarization changes in liposomes were more prominent when irradiation was carried out at lower dose rate. The maximum fluidization of DPPC liposomal membrane was observed at 500 and 300 Gy at the dose rate of 100 and 11 Gy/min., respectively.

![Fig. 1. Fluorescence polarization of DPH in liposomes irradiated by 60 Co γ-rays at different dose rates 100 Gy/min. (□) and 11 Gy/min. (■). Subsequently, liposomes were labeled with DPH for 17 h at 25 °C and polarization of liposome samples was measured in PBS.](image-url)
Modification of DPH uptake by antioxidants

DPPC liposomes prepared with or without antioxidants were γ-irradiated followed by incubation with DPH at 25 °C. The fluorescence intensity of liposomes was measured at different time of incubations in PBS which was found to increase as a function of incubation period and a saturation in DPH uptake was observed after 17 h of incubation. However, a significant decrease in rate of DPH uptake was observed in vesicles irradiated for 6 kGy. Compared to unirradiated controls, 27% decrease in DPH uptake was observed in irradiated liposomes (6 kGy). However, no change in the rate of DPH entry was found between unirradiated liposomes and the liposomes irradiated but containing α-tocopherol (0.15 %) in their composition. On the other hand, 10 % reduction in DPH uptake was observed in liposomes containing 0.1 mM ascorbic acid (Fig. 2) and further increase in the concentration of the antioxidant produced insignificant increase in the radioprotective effectiveness.

![Graph showing DPH uptake kinetics in irradiated liposomes.](image)

DISCUSSION

Oxidative damage of cellular membrane by ionizing radiation has been known to cause impairment of the functional behavior of membrane and also enzymes and receptors (2). Modification of damage at membrane level offers an important strategy for the protection of normal cells against unavoidable therapeutic / accidental radiation exposure. This work was aimed to investigate the mechanism of gamma radiation induced damage in model membrane employing fluorescence technique and modification of liposomal damage by the presence of aqueous and membrane specific antioxidants. Alterations in membrane fluidity in irradiated model (1) as well as cellular membrane (10) have been reported employing DPH fluorescence probe. Moderate doses of gamma radiation are known to significantly increase membrane permeability of HeLa cells (11). The increase in leakage of CF fluorescence up to a dose of 0.7 kGy and reduced leakage of CF at 6 kGy from irradiated liposomes (Table 1) suggests a dose dependent bi-phasic change in membrane permeability after irradiation of liposomes. The observed increase in membrane permeability for moderate doses of radiation shows radiation induced increase in bilayer permeability. On the other hand, for irradiations at higher doses the induced oxidative damage
seems to stabilize the liposomal membrane. This inference was further supported by observed similar bi-phasic pattern in terms of fluorescence polarization of DPH in liposomes as a function of radiation dose. It was interesting to find that liposomes irradiated at high dose rate showed an increased membrane fluidity (upto 500 Gy) as inferred from the observed decrease in DPH polarization and on irradiation for further higher doses the membrane was found to show increased rigidity properties. The maximum fluidization of liposomal bilayer was for a dose exposure of 300 and 500 Gy for dose rate of 11 and 100 Gy/min., respectively. These results suggest that for a defined radiation dose, greater membrane damage occurs at lower radiation dose rate (Fig. 1). As can be seen from fig. 2 the reduced uptake of DPH occurred in liposomes without antioxidants irradiated for 6 kGy. However, liposomes composed of either α-tocopherol in the membrane or ascorbic acid in aqueous interior showed an enhanced entry of DPH suggesting recovery from radiation damage in presence of antioxidants. It was also shown that increase in DPH uptake was higher in presence of α-tocopherol than ascorbic acid suggesting that antioxidants specifically located in bilayer membrane displayed greater radioprotective ability than water soluble antioxidants against the radiation induced damage to vesicular membrane.

REFERENCES