Therapeutic Uses of Antioxidant Liposomes

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1. Introduction

This chapter focuses on the use of antioxidant liposomes in the general area of free radical biology and medicine. The term antioxidant liposome is relatively new and refers to liposomes containing lipid-soluble chemical antioxidants, water-soluble chemical antioxidants, enzymatic antioxidants, or combinations of these various antioxidants. The role of antioxidants in health and disease has been extensively discussed, and many excellent reviews and books are available (1–3). Antioxidant liposomes hold great promise in the treatment of many diseases in which oxidative stress plays a prominent role. Oxidative stress is a physiological condition in which the production of damaging free radicals exceeds the in vivo capacity of antioxidant protection mechanisms to prevent pathophysiology. Free radicals are molecules with unpaired electrons, often highly reactive and damaging to biological systems. The biological membranes of subcellular organelles are a major site of free radical damage but proteins and DNA are also significant targets. Moreover, free radicals can alter cellular signal transduction pathways and stimulate the synthesis of inflammatory cytokines. Oxygen radicals and other reactive oxygen species (ROS) arise from the single electron reductions of oxygen.

\[ \text{O}_2 + \text{e}^- \rightarrow \text{O}_2^- \]  
(1)

\[ \text{O}_2^- + \text{e}^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 \]  
(2)

\[ \text{H}_2\text{O}_2 + \text{e}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{OH}^- \]  
(3)

\[ \text{OH}^- + \text{e}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} \]  
(4)

\[ \text{O}_2^- + \text{NO} \rightarrow \text{ONOO}^- \]  
(5)

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(4)

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(5)
In addition, the superoxide radical (\(O_2^•–\)) can react rapidly with nitric oxide to yield peroxynitrite (ONOO\(^–\)) as shown in Eq. 5. Peroxynitrite is a reactive nitrogen oxide species (RNOS) that can also cause damage to DNA, proteins, and membranes. Moreover, ONOO\(^–\) is likely to be generated during inflammation and the killing of bacteria. Free radicals are generated in both the aqueous and lipid compartments of cells and, to minimize their damaging effects, requires both lipid- and water-soluble antioxidants. Nevertheless, the potential clinical use of such bifunctional liposomes has been extremely limited (4).

A primary use of antioxidant liposomes has been to define the molecular mechanism of action for various antioxidants (5–13). Antioxidants such as butylated hydroxytoluene (BHT) and \(\alpha\)-tocopherol have also been used to prevent the oxidation of unsaturated fatty acid moieties in the phospholipids of liposomes during storage (14) or sonication (15). This chapter, however, focuses on the potential therapeutic uses of antioxidant liposomes. This is a rapidly evolving area of medical research not extensively reviewed. Most of the research to date has been accomplished using in vitro cell culture systems or animal models. Very few clinical trials have been attempted, yet obvious medical situations exist (as discussed later) in which antioxidant liposomes have enormous health-related significance. The preparation of antioxidant liposomes that can be targeted to specific sites in the body is also a promising area but awaits further research. Most chemical antioxidants are phytoceuticals whose properties have already been extensively studied and are generally regarded as nontoxic and safe for human consumption (1). In the following subheadings, we first review the varieties of antioxidants that have either been used in antioxidant liposomes or hold the promise of such utilization. We then focus on issues relating to the modes of administration and lastly describe the clinical uses of antioxidant liposomes for diseases in which oxidative stress plays a major role. Major emphasis is placed on the use of antioxidant liposomes for pulmonary diseases.

2. Lipid-Soluble Antioxidants

The lipid-soluble antioxidants that can be incorporated into liposomes include vitamin E (tocopherols and tocotrienols) (16), ubiquinones (17), retinoids (18–20), carotenoids (21,22), lipid-soluble flavonoids (e.g., quercetin, hesperetin, naringenin) (23), tamoxifen (24,25), as well as synthetic lipid-soluble antioxidants such as BHT, tertiary butylhydroquinone (TBHQ), and probucol. Tocopherols can readily be incorporated into both monolayers of unilamellar liposomes in a monomeric form (16). Furthermore, tocopherol in liposomes can undergo spontaneous intermembrane transfer to an acceptor membrane without the fusion of the tocopherol liposome (16). This intermembrane transfer is more pronounced when the tocopherol liposome contains
polyunsaturated fatty acids (16). R,R,R-α-tocopherol and R,R,R-α-tocotrienol are forms of vitamin E that have the same aromatic chromanol head group but differ in the structure of their hydrocarbon tails. R,R,R-α-tocotrienol is, however, a better peroxyl radical scavenger than R,R,R-α-tocopherol in phosphatidylcholine liposomes (26).

β-Carotene (a carotinoid) can be incorporated into liposomes to a maximum of about 0.5 mol% (based on phospholipid) whereas tocopherol can be incorporated at levels as high as 30 mol%. The ability of β-carotene in liposomes to inhibit free radical mediated lipid peroxidation appears, however, to be much lower than that of α-tocopherol (27). β-Carotene at 0.45 mol% (of phospholipid) is, however, a more powerful inhibitor of singlet oxygen mediated lipid peroxidation than α-tocopherol at 0.45 mol% (28). α-Tocopherol at 4.5 mol% is, however, also effective at inhibiting both free radical lipid peroxidation as well as singlet oxygen mediated lipid peroxidation (28). Singlet oxygen can be generated by photosensitization and this reactive oxygen species may contribute to light-induced skin toxicity as well as the aging of skin.

The lipids used in the preparation of antioxidant liposomes also provide an opportunity to introduce antioxidant capacity into liposomes. For example, plasmalogens (1-alkenyl, 2-acyl-) phospholipids are thought to have antioxidant properties (29,30). Liposomes constructed with ethanolamine plasmalogen inhibit both iron- and copper-dependent peroxidation in the presence of preformed lipid hydroperoxides (31). Koga et al. have synthesized a novel phospholipid containing a chromanol structure as its polar head group (12,32). This phosphatidyl derivative of vitamin E is almost as effective an antioxidant as α-tocopherol in unilamellar liposomes subjected to free radicals generated in the lipid phase. The potential therapeutic value of liposomes with antioxidant phospholipids has not been explored but this is an obvious area for future research.

A major advantage of antioxidant liposomes is their ability to simultaneously contain (and deliver) both water- and lipid-soluble antioxidants. This is particularly important in the case of liposomes with both tocopherol (TOH) and ascorbate (Asc), as it has been demonstrated that ascorbate can regenerate tocopherol from the tocopheroxyl radical (TO•) (33).

\[
TO' + Asc \rightarrow TOH + Asc^* (6)
\]

### 3. Water-Soluble Antioxidants

The water-soluble antioxidants that can be used in antioxidant liposomes include ascorbate (vitamin C), urate, glutathione, N-acetylcysteine (NAC), lipoic acid (or dihydrolipoic acid which is its reduced form), pro-cysteine, and water-soluble flavonoids (as in pycnogenol). Dihydrolipoic acid is somewhat
unique because it can quench peroxyl radicals generated both in the aqueous phase and in membranes (34). Chemical antioxidants generally act by donating an electron to a free radical (thereby quenching the free radical) or by serving as a substrate for an antioxidant enzyme. Glutathione, for example, is itself an antioxidant (6) and can also function as a substrate for glutathione peroxidase, a key (selenium containing) antioxidant enzyme that converts lipid hydroperoxides (LOOH) or H₂O₂ into the corresponding lipid alcohols (LOHs) or H₂O. Chemical antioxidants can also be chelators of transition metal ions that catalyze lipid peroxidation reactions. Urate, which is present at very high concentrations in human plasma, is an excellent antioxidant that can both chelate transition metal ions and also quench aqueous free radicals (35). Recently, we have observed 50–60% protection by N-acetylcysteine in the generation of free radicals in lungs by mustard gas induced lung injury in guinea pigs (Das and coworkers unpublished observations).

4. Entrapped Antioxidant Enzymes

The application of antioxidant liposomes to problems of medical interest has primarily been with liposomes containing entrapped antioxidant enzymes. Recombinant biotechnology has provided the means to obtain large (i.e., commercial) quantities of human antioxidant enzyme but these enzymes do not normally penetrate the plasma membrane of cells and have a short half-life when introduced into the body by intravenous injection. Turrens has reviewed the potential of antioxidant enzymes as in vivo pharmacological agents (36). The attachment of polyethylene glycol (PEG) to antioxidant enzymes increases their in vivo half-lives and their effectiveness in preventing pulmonary oxygen toxicity in rats (37). The various procedures for preparing liposomes with entrapped antioxidant enzymes have been evaluated by Aoki et al. (38). This group and others (39) have found that positively charged liposomes have a superior trapping efficiency for superoxide dismutase (which has a negative charge).

Early work by Freeman et al. (40) has shown that porcine aortic endothelial cells treated with liposomes with entrapped superoxide dismutase (SOD liposomes) can dramatically increase their cellular SOD levels and thereby protect the cells from oxygen-induced injury. In a key paper, Beckman et al. (41) found that endothelial cells treated with liposomes containing entrapped superoxide dismutase and catalase (SOD+CAT liposomes) can increase the cellular specific activity of these enzymes by at least 40-fold within 2 h. These results are particularly important because endothelial cells are a major site for oxidative damage. Moreover, intravenous antioxidant liposomes would certainly make contact with vascular endothelial cells under in vivo conditions.
5. Modes of Administration

Antioxidant liposomes can be administered topically, intratracheally, intravenously, by inhalation in an aerosol form, or by intramuscular injection. Topical administration can certainly be long term and is of considerable interest to the cosmetic industry in treating specific skin disorders such as psoriasis. α-Tocopheryl acetate in liposomes has been found to have a better dermal absorption than free α-tocopheryl acetate (42). Topical administration of antioxidant liposomes could also be useful in situations where individuals were exposed to toxic substances (e.g., chemical warfare agents) causing skin damage by free radical mechanisms. Inhalation and intratracheal administration can be useful for those situations in which pulmonary tissues are subjected to oxidative stress such as with influenza infection or inhalation of toxic substances such as paraquat (4).

Intravenous administration would primarily be limited to situations in which oxidative stress is a component of an acute trauma or disease. The intravenous use of antioxidant liposomes has the potential for rapidly increasing the plasma and tissue concentration of antioxidants far beyond what oral administration could achieve. Moreover, the proteolytic and bioselective processes of the gastrointestinal tract do not limit the types of antioxidants that can be administered via intravenous antioxidant liposomes. For example, it is known that plasma levels of α-tocopherol are about 10 times higher than the levels of γ-tocopherol despite the fact that dietary levels of γ-tocopherol are at least two times that of α-tocopherol. Nevertheless, γ-tocopherol has a unique chemical ability to detoxify peroxynitrite that is not shared with α-tocopherol (43). Peroxynitrite is a powerful oxidant formed by the reaction of nitric oxide with superoxide radicals (see Eq. 5) and may be an important mediator of acute oxidant tissue damage. It is reasonable to suspect, therefore, that medical situations could arise in which it would be desirable to rapidly increase plasma (and tissue) levels of γ-tocopherol. The poor bioavailability of orally administered γ-tocopherol makes this very difficult to accomplish. This limitation could, however, be overcome by the intravenous administration of liposomes containing γ-tocopherol.

Vitamin E used in oral supplements is often in the form of a tocopheryl ester such as tocopheryl acetate or tocopheryl succinate. Tocopheryl esters are not, however, absorbed and must first be acted upon by intestinal esterases to liberate the unesterified tocopherol. It is interesting, therefore, that α-tocopheryl succinate but not α-tocopherol has been found to inhibit the activation of nuclear factor κB (NFκB) in cultured macrophages (44). NFκB is a key transcription factor that regulates the expression of many inflammatory cytokines. α-Tocopheryl succinate can be incorporated into liposomes, and intravenous injection would deliver this form of vitamin E to phagocytic cells.
Oral administration of tocopheryl succinate would not, however, be expected to deliver this form of vitamin E to cells.

It is very significant that Cu,ZnSOD liposomes administered by intravenous injection can penetrate the blood–brain barrier and significantly elevate brain levels of SOD activity within 24 h (46,47). Moreover, the intravenous administration of Cu,ZnSOD liposomes to rats can reduce cerebral infarction caused by ischemia (47) and also inhibit learning dysfunction caused by a low dose of total body irradiation (48). Surprisingly, intraperitoneal injection of SOD liposomes has also been found to increase the brain levels of SOD in gerbils and to inhibit ischemia/reperfusion oxidative stress (49).

A major problem with conventional liposomes is that they are recognized by the immune system as foreign substances and are rapidly removed from circulation by the phagocytic cells of the reticuloendothelial system. The Kupffer cells of the liver are the most abundant population of phagocytic cells in the body. In some circumstances, however, the uptake of conventional liposomes by hepatic Kupffer cells can actually be an advantage. Carbon tetrachloride (CCl4), for example, is known to induce hepatotoxicity by a free radical mediated mechanism. Yao et al. (45) found that intravenous administration of liposomes containing vitamin E (TOH liposomes) was very effective in decreasing mortality in mice given a lethal dose of CCl4. The TOH liposomes were found to primarily accumulate in the Kupffer cells of the liver.

In recent years considerable advances have been made in the design of stealth liposomes that are not well recognized by the immune system and therefore have a much longer half-life in circulation than conventional liposomes. Stealth technology employs liposomes with a polymer coating of polyethylene glycol–phosphatidylethanolamine (PEG–PE). Recently, the preparation of pH-sensitive stealth liposomes has been described (50). These liposomes have a prolonged circulation in vivo and destabilize at mildly acidic pH, thereby being particularly efficient at delivering a water-soluble compound into a cell’s cytoplasm. The use of stealth antioxidant liposomes is very new with an increasing commercial interest in their potential therapeutic applications.

6. Antioxidant Liposomes and Oxidative Stress

Increasing evidence suggests that oxidative stress is an important factor in the aging process and in the etiology of many chronic diseases such as atherosclerosis, ischemic heart disease (51), and cancer (52,53). Schwartz et al. (54) at the Harvard School of Dental Medicine have used the hamster cheek pouch tumor model to explore the potential anticancer use of various antioxidants. This group found that β-carotene liposomes injected into the oral squamous cell carcinoma of the hamster caused a lysis of the tumor cells but not of normal cells (54). Retinoids have also been shown to be clinically
effective in treating diverse premalignant and malignant conditions such as cutaneous T-cell lymphomas, leukoplasia, squamous cell carcinomas of the skin, and basal cell carcinomas (55,56). Several investigators have documented dramatic improvement in patients with acute promyelocytic leukemia after treatment with all-trans-retinoic acid (57–59). However, the side effects of oral all-trans-retinoic acid therapy are similar to effects seen with vitamin A: headaches, other central nervous system problems, and dryness of mucosal tissues, erythema, and desquamation of skin. When incorporated in liposomes, all-trans-retinoic acid-associated toxicity is markedly reduced whereas antitumor properties, that is, growth inhibition and differentiation induction of all-trans-retinoic acid are maintained or even enhanced (60,61). Phase I and phase II clinical studies found that plasma levels of all-trans-retinoic acid were maintained at high concentrations even after prolonged treatment of patients with all-trans-retinoic acid-liposomes (62). In general, the use of retinoids is safe and induces complete remission in 80–90% of acute promyelocytic leukemia patients. However, chronic oral administration results in reduced plasma levels associated with disease relapse in the majority of patients; this can be circumvented by using all-trans-retinoic acid liposomes.

Oxidative stress also contributes to the pathology observed in acute medical problems such as heart attack (51,63–66), respiratory distress syndrome (67), trauma (3), irradiation (48), cold injury (68), and certain types of infectious diseases such as influenza and human immunodeficiency virus (HIV) infection. Evidence suggests that trauma to the brain results in the overproduction of superoxide radicals that may contribute to edema (69,70). Antioxidant liposomes containing SOD have been used effectively to treat posttraumatic brain edema (69,70) and neurological dysfunctions in rats (71).

Retinopathy of prematurity is a leading cause of blindness in premature and low birth weight infants who are often treated with high levels of oxygen due to surfactant deficiency. Considerable evidence (72) indicates that oxidative stress is a major contributor to this disease. In an animal model, Niesman et al. (73) found that intraperitoneal administration of SOD encapsulated in PEG-modified liposomes resulted in a significant increase in retinal SOD activity and an improved tolerance to high oxygen levels. Despite the enormous health-related significance, there are no clinical trials testing the efficacy of antioxidant liposomes to treat retinopathy of prematurity.

7. Pulmonary Applications of Antioxidant Liposomes

7.1. Potential Clinical Applications

Premature children often suffer from respiratory distress syndrome because they lack the capacity to synthesize pulmonary surfactant (74). Surfactant is
necessary to maintain proper expansion of the small air sacs in the lung. If surfactant levels are low, the small air sacs in the lungs collapse resulting in poor oxygen delivery (hypoxia) to tissues. Infants deficient in surfactant therefore require treatment with high levels of oxygen to prevent damage to their vital organs. Unfortunately, premature infants are often deficient in antioxidants that are necessary to protect organs from injury caused by high concentrations of oxygen. The combination of surfactant deficiency and the presence of oxygen free radicals promote the development of chronic lung disease (bronchopulmonary dysplasia or BPD). BPD is a major cause of morbidity and mortality in premature infants. An estimated 50% of all neonatal deaths result from BPD or its complications. In the adult form of respiratory distress syndrome (ARDS), antioxidants such as N-acetylcysteine are recognized for their role in reducing the duration of acute lung injury \((75,76)\). The rationale for using antioxidant liposomes to treat respiratory distress in premature infants or adults is certainly compelling and supported by the animal models detailed below. However, almost no clinical trials have been initiated.

7.2 Animal Models

Shek et al. \((77)\) have discussed the general application of liposomes for improved drug delivery to pulmonary tissues. These authors point out that the delivery of drugs to the lung via liposomes is particularly useful because it can minimize extrapulmonary side effects and potentially result in increased drug retention time. In addition (as discussed previously), liposomes for delivery by inhalation or instillation can encapsulate enzyme and/or chemical substances that cannot be delivered by an oral route. Smith and Anderson \((78)\) demonstrated that intratracheally administered liposomes (with phosphatidylcholine, cholesterol, and stearylamine) have a long retention time (\(> 5\) d) in the mouse lung. Liposomes with entrapped Cu,Zn superoxide dismutase and catalase (Cu,ZnSOD+CAT liposomes) were intratracheally instilled in rabbits and the alveolar distribution of the antioxidants measured after 4 and 24 hours \((79)\). The results indicate that Cu,ZnSOD+CAT liposomes could increase both SOD and CAT activities in distal lung cells, including alveolar type I, alveolar type II cells, and macrophages. More recent studies by Walther et al. \((80)\) have shown that intratracheal administration of CuZn-CAT liposomes to premature rabbits can increase the lung SOD activity and protect against hyperoxic lung injury. Moreover, intratracheal delivery of SOD liposomes or CAT liposomes does not down-regulate mRNA synthesis of these enzymes in the premature rabbit lung \((81)\).

Archer et al. \((82)\) have made effective use of the isolated perfused rat lung to study the role of oxygen radicals in modulating pulmonary vascular tone. This group showed that the generation of oxygen radicals (from xanthine–xanthine
oxidase) decreased pulmonary vascular presser response to alveolar hypoxia. Either pretreatment of the lung with desferrioxamine or a mixture of superoxide and catalase liposomes inhibited decreases in pulmonary vascular reactivity. Superoxide dismutase administered free in solution or combined with catalase in liposomes, increased the normoxic pulmonary arterial pressure, and enhanced vascular reactivity to angiotensin II and hypoxia (82).

In a rat model, Freeman et al. (83) have shown that intravenous injection of SOD liposomes or CAT liposomes can increase (two- to fourfold) the lung-associated specific activity of these antioxidant enzymes and also provide resistance to oxygen injury. Intravenous injection of non-entrapped (i.e., free) SOD or CAT (in the absence or presence of control liposomes) neither increased the specific lung activities of these enzymes nor provided resistance to oxygen toxicity. Similarly, intratracheal administration of SOD liposomes or CAT liposomes (negatively charged and multilamellar) to rats resulted in a significant elevation of lung SOD or CAT activity as well as resistance to pulmonary oxygen toxicity (84).

Barnard et al. (85) have demonstrated that instillation of cationic SOD+CAT liposomes in a rabbit model was effective in preventing the increase in pulmonary filtration coefficient (a sensitive index of microvascular permeability) owing to free radical initiated lung injury. Repair of lung injury was inhibited by inhalation of elevated oxygen concentrations. This is of particular importance to the preterm human infant who may be exposed to elevated oxygen concentrations for weeks or months that could result in the chronic pneumopathy known as bronchopulmonary dysplasia. Treatment with liposome-encapsulated SOD and catalase conferred protection against the cytotoxic effects of 50% and 95% oxygen (86,87) and also protect against cell death (88).

Briscoe et al. (89) have evaluated the delivery of SOD to cultured fetal rat pulmonary epithelial cells via pH-sensitive liposomes. A fivefold increase in cellular SOD activity was observed after the culture cells were incubated with the pH-sensitive SOD liposomes (89). Fetal pulmonary epithelial cells express a high affinity receptor for surfactant protein A (SP-A). This receptor can be used to target liposome delivery to these cells by incorporating SP-A during the preparation of the SOD liposomes (89,90). The presence of SP-A in the SOD liposomes facilitates their uptake by pulmonary epithelial cells (89,90).

Considerable evidence suggests that oxidative injury to lung tissues can be mediated by neutrophils (91). Phorbol myristate acetate (PMA) has often been used to induce neutrophil-mediated lung injury in animal models. It is significant, therefore, that liposomes (dipalmitoylphosphatidylcholine) with \( \alpha \)-tocopherol are able to counteract some PMA-induced lung injury in a rat model (91). In contrast, rats pretreated with blank liposomes (no \( \alpha \)-tocopherol) showed no protection from PMA-induced lung injury (91).
Paraquat has also been used to induce oxidative lung injuries in animal models (4). Suntres and Shek (4) have compared the ability of α-tocopherol liposomes (TOH liposome) or liposomes with both α-tocopherol and glutathione (TOH+GSH liposome) to inhibit paraquat-induced lung damage in a rat model. Lung damage was assessed by increases in lung weight (caused by edema) and decreases in lung activities of angiotensin converting enzyme (ACE) that reflects damage to endothelial and alveolar type II epithelial cells. These investigators found that both TOH liposomes and TOH+GSH liposomes were equally effective in preventing loss of lung ACE activity but that TOH+GSH liposomes were more effective in preventing injury to alveolar type II epithelial cells (4). Interestingly, neither antioxidant liposome was effective in preventing lung edema (4).

Liposomes encapsulated with catalase (CAT liposomes) have also been found to be efficacious in preventing chronic pulmonary oxygen toxicity in young rats (92). In this work, rats were treated with 100% oxygen for 8 d and also given daily intratracheal injections of the CAT liposomes (with 160 U of CAT) that prevented chronic lung toxicity. Liposomes encapsulated with superoxide dismutase (SOD liposomes) or with lower levels of CAT (50 U or 70 U) did not prevent the chronic lung changes. SOD+CAT liposomes are also effective in protecting lung tissues from bleomycin-induced injury as evidenced by decreased levels of lipid peroxidation products (93).

Acknowledgments

This work was supported in part by grants from the Department of Defense (DOD; DAMD17-99-9550 to W. L. S., M. S., and S. K. D.), USDA National Research Initiative Competitive Grants Program (Proposal no. 9600976 to W. L. S.), Natural Source Vitamin E Association (to W. L. S.), and NIH grant 2S06GM0837 (to S. K. D.)

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