1994

Delivery Of Superoxide Dismutase To Fetal Rat Lung Epithelial Cells Byph-Sensitive Liposomes.

Page T. Briscoe
University of Alabama at Birmingham

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road. Ann Arbor. MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9512250

Delivery of superoxide dismutase to fetal rat lung epithelial cells by pH-sensitive liposomes

Briscoe, Page T., Ph.D.

University of Alabama at Birmingham, 1994

U·M·I

300 N. Zeeb Rd.
Ann Arbor, MI 48106

DELIVERY OF SUPEROXIDE DISMUTASE TO FETAL RAT LUNG EPITHELIAL CELLS BY PH-SENSITIVE LIPOSOMES

by

PAGE BRISCOE

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry and Molecular Genetics in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1994

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Major Subject Biochemistry

Name of Candidate Page T. Briscoe

Title Delivery of Superoxide Dismutase to Fetal Rat Lung Epithelial Cells by pH-Sensitive Liposomes

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of the prematurely born infant. A major contributor to the progression of this disease is therapeutic hyperoxia to ensure oxygenation of tissues. Hyperoxia increases tissue oxygen radical production with this overproduction exceeding the antioxidative capacity of the infant lung, causing injury and impairing repair mechanisms. Based upon these observations, this research is directed towards increasing the lung cell content of an antioxidant enzyme, CuZn superoxide dismutase (CuZn SOD). Since the native form of CuZn SOD is membrane-impermeable, the vector for intracellular delivery of CuZn SOD chosen was liposome membranes. The specific liposomes developed herein become destabilized at a low pH and thus are termed pH-sensitive liposomes. To test the efficacy of enzyme delivery by this modality, an in vitro model was used, primary cultures of fetal rat lung distal epithelial cells (FRLE cells). Enhancement of liposome delivery occurred when a targeting molecule was incorporated. To this end, surfactant protein A (SP-A) was used to target liposomes to type II cells. In this study, it was determined that pH-sensitive liposomes incorporate 10 ± 4% of SOD activity added originally, are unilamellar and homogenous in size

(200 ± 100 nm diameter), and stable for one week. The cultured FRLE cells appeared to exhibit a receptor for SP-A, with K_d = 4 ± 0.2 μg/ml and capacity = 130 ± 3 ng/10^6 cells. Liposomes prepared with incorporated SP-A delivered 2-fold more SOD to FRLE cells than non-targeted liposomes. Other studies also suggested that the delivery of SOD to FRLE cells by pH-sensitive liposomes is cytoprotective against oxidant stress produced by paraquat or xanthine oxidase plus xanthine. These pH-sensitive liposomes have promising therapeutic potential, especially in the treatment of BPD and other oxidant-mediated lung and vascular pathologies.

Abstract Approved by: Committee Chairman B A Freeman

Program Director Jeffrey A. Engler

Date 9/28/94 Dean of Graduate School James L. Gordon

DEDICATION

Mark J. Winn

Carol N. Winn

Devon Winn

I miss you and your encouragement and laughter.

ACKNOWLEDGEMENTS

I am indebted to my graduate committee members, Jeff Esko, Ph.D., Leo Hall, Ph.D., Tony Thompson, Ph.D., Dale Parks, Ph.D., and my mentor Bruce Freeman, Ph.D. for their invaluable guidance and encouragement throughout this work. I am also grateful to Dr. Freeman for the many opportunities he has given me to help advance me in my career in science.

I owe thanks to many others for their help and personal encouragement. I thank Mark Winn for showing me the true meaning of scientific endeavor, and for spreading to me his enthusiasm for this field.

I give special thanks to Ben Gilliam for his encouragement, and also for his technical assistance with word processing and the illustrative material included in this work.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

CHAPTER

LIST OF TABLES

LIST OF FIGURES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

BPD	bronchopulmonary dysplasia
RDS	respiratory distress syndrome
ROS	reactive oxygen species
SP-A	surfactant protein A
NADH	β-nicotinamide adenine dinucleotide
$O_2^{\bullet -}$	superoxide
H_2O_2	hydrogen peroxide
OH•	hydroxyl radical
SOD	superoxide dismutase
OH^-	hydroxyl anion
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
PAN	peroxyacylnitrate
GPX	glutathione peroxidase
GSH	glutathione
HDL	high density lipoprotein
Chol	cholesterol
SA	stearylamine

DPPC	dipalmitoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DOSG	dioleoylglycerosuccinate

CHAPTER 1

INTRODUCTION

Reactive oxygen species are produced as byproducts of the metabolic processes of all aerobic organisms. These species are partial reduction products of oxygen and can exert cytoproliferative, cytostatic, and cytocidal effects if there is disruption of the tenuous balance between tissue antioxidant defenses and net rate of oxidant production. The progressive pulmonary injury which prematurely born infants suffer from is one of the most straightforward clinical manifestations of oxygen radical-induced tissue injury. Thus, the principal objective of this dissertation research was to develop a more effective means for modulating lung tissue antioxidant defenses by using fetal lung cells as an *in vitro* model.

Biochemistry of Reduced O_2 Species

The cells of an aerobic organism use O_2 as an electron acceptor during oxidative phosphorylation, which provides the cell with high energy phosphate compounds. The advantage of O_2 as an oxidant is that the redox potential for reducing O_2 to water (H_2O) is very high (0.816 V), meaning that it has a high affinity for accepting additional electrons and is readily reduced. During the process of reoxidation of NADH (reduced β-nicotinamide adenine dinucleotide) for energy formation in the mitochondria, cytochrome oxidase tetravalently reduces O_2 to H_2O without the release of reactive oxygen species (ROS) (Fridovich 1986).

Ideally, O_2 is tetravalently reduced without forming reactive intermediates, but even under normal conditions the cells produce ROS by univalent and divalent stepwise reduction of O_2. About 1-5% of the O_2 consumed during energy production within the mitochondria normally escapes from this cytochrome oxidase-catalyzed pathway to undergo univalent reduction (Reilly, Schiller, and Bulkley 1991). The univalent reduction of O_2 forms superoxide ($O_2^{\cdot -}$), termed a free radical because of an unpaired electron in the outer orbital. Superoxide is notable in that it can act either as a mild oxidant or as a reductant (Forman and Boveris 1984). Other ROS derive from sequential univalent reductions of O_2, including hydrogen peroxide (H_2O_2), a 2 e^- reduction, and hydroxyl radical (OH·), a 3 e^- reduction product. The four electron reduction of O_2 yields water, as catalyzed by the cytochrome c oxidase complex.

Dismutation of $O_2^{\cdot -}$ yields H_2O_2 (Equation 1), occurring either spontaneously or when catalyzed by superoxide dismutase (SOD). The rate of spontaneous dismutation of $O_2^{\cdot -}$ at pH 7.4 is $2 \times 10^5\ M^{-1} \cdot s^{-1}$ and is $2 \times 10^9\ M^{-1} \cdot s^{-1}$ when catalyzed by CuZn SOD.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2\ H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{(Equation 1)}$$

Without trace metals, the resulting H_2O_2 is a relatively stable compound with oxidative capacity, though the oxidation reactions of H_2O_2 are fairly slow (Grisham, McCord, and Sylvester 1986). In the presence of iron and $O_2^{\cdot -}$, H_2O_2 can produce OH· (Equations 2 and 3) via the iron-catalyzed Fenton reaction or Haber-Weiss reaction (Cohen 1985). In this reaction, $O_2^{\cdot -}$ reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), which then reacts with H_2O_2 to yield OH· and hydroxyl anion (OH^-) (Grisham, McCord, and Sylvester 1986). The hydroxyl radical is one of the most reactive

molecules in biological systems, with a rate constant for reaction with organic molecules of up to 10^{10} $M^{-1}\cdot s^{-1}$ (Grisham, McCord, and Sylvester 1986).

$$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \qquad \text{(Equation 2)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\cdot \qquad \text{(Equation 3)}$$

In physiologic milieu, lactoferrin, transferrin (Thomas, Morehouse, and Aust 1985), ADP- Fe^{3+} (Grisham, McCord, and Sylvester 1986), hemoglobin, and ferritin (Grisham, McCord, and Sylvester 1986) can provide the iron needed for these reactions. These reactions in concert with a stimulator of production of ROS can cause further damage to cellular components.

Cellular Production and Reactions of ROS

There are numerous intracellular sources of ROS with sources ranging from molecules to organelles. Also, certain tissue cell types are often major sources of O_2 radicals (Table 1). Many low molecular weight molecules and proteins produce ROS by autoxidation, photooxidation, and during catalytic cycling. There are several disease states in which the oxygen free radicals are implicated in playing a key role in causing and exacerbating biochemical damage (Table 2, adapted from Menzel 1984).

Reactive O_2 species react with and can damage many cellular components. Proteins, lipids, and nucleic acids are readily oxidized by ROS. A variety of proteins are susceptible to scission and inactivation by ROS, with all of these targets susceptible to the indiscriminate reactivity of OH· (Table 3). Also, the lipids of cell

Table 1. Sources of reactive O_2 species

Source of Oxidant Stress	Example	Reference
Cells	neutrophils	Fridovich 1984a
	monocytes	Fridovich 1984a
	macrophages	Fridovich 1984a
Intracellular		
Small molecules	catecholamines	Fridovich 1984a
	reduced ferredoxins	Fridovich 1984a
	hydroquinones	Fridovich 1984a
	Fe(II)	Freeman and Crapo 1982
	pyrogallols	DiGuiseppi and Fridovich 1984
	thiols	Fridovich 1984a
	homocysteine	Olszewski and McCully 1993
	flavin	Muller 1987
Proteins, Enzymes	reduced cytochrome c	Fridovich 1984a
	myoglobin	Fridovich 1984a
	hemoglobin	Fridovich 1984a, Fridovich 1983
	xanthine oxidase	Fridovich 1983
	flavin dehydrogenases	Fridovich 1983
	NADH dehydrogenase	DiGuiseppi and Fridovich 1984
	cytochrome P_{450} reductase	DiGuiseppi and Fridovich 1984
	cyclooxygenase	Reilly, Schiller, and Bulkley 1991
	lipoxygenase	Reilly, Schiller, and Bulkley 1991
	cytochrome b_5	Fridovich 1983
Extracellular		
Air pollutants		
	ozone (O_3)	Freeman and Crapo 1982

Table 1. Continued.

Source of Oxidant Stress	Example	Reference
Air pollutants		
	nitrogen oxides	Freeman and Crapo 1982
	sulfur dioxide	Elstner and Oswald 1991
	peroxyacyl nitrates	Halliwell, Gutteridge, and Cross 1992

Table 2. Diseases and metabolic processes that involve ROS overproduction

System	Process
Cardiovascular	Reperfusion arrhythmias
	Myocardial stunning
	Atherosclerosis
	Ischemia/Reperfusion
Pulmonary	Respiratory distress syndrome
	Ischemia/Reperfusion
	Bronchopulmonary dysplasia
	Hyperoxia
	Emphysema
Central nervous system	Stroke
	Hyperbaric oxygen
	Ischemia/Reperfusion
Gastrointestinal tract	Ischemia/Reperfusion
	Neonatal necrotizing enterocolitis
	Ulcer-producing stress
Other processes	Aging
	Muscular dystrophy
	Rheumatoid arthritis
	Cataractogenesis
	Chronic alcohol toxicity

Table 3. Targets of reactive O_2 species

Target	ROS Involved	Reference
Proteins		
Thiols of proteins	O_3, nitrogen oxides	Kono and Fridovich 1982 Elstner and Oswald 1991
Protein tyrosines	O_3, nitrogen oxides, PAN	Kono and Fridovich 1982 Elstner and Oswald 1991 Halliwell, Gutteridge, and Cross 1992
Primary amines	PAN	Halliwell, Gutteridge, and Cross 1992
Hemoglobin	$O_2^{\cdot-}$, H_2O_2	DiGuiseppi and Fridovich 1986
Catalase	$O_2^{\cdot-}$	Kono and Fridovich 1982
Myeloperoxidase	$O_2^{\cdot-}$	DiGuiseppi and Fridovich 1986
Glyceraldehyde-3-phosphate dehydrogenase	$O_2^{\cdot-}$	Halliwell, Gutteridge, and Cross 1992
Creatine phosphokinase	$O_2^{\cdot-}$	DiGuiseppi and Fridovich 1986
SOD	H_2O_2	Bray et al. 1974
Non-proteins		
Fatty acid olefins	O_3, nitrogen oxides	Menzel 1984
Indole acetic acid	PAN	Mudd 1984

Table 3. Continued.

Target	ROS Involved	Reference
NADPH, NADH	PAN, O_3	Halliwell, Gutteridge, and Cross 1992
Nucleic acids	PAN, H_2O_2	Halliwell, Gutteridge, and Cross 1992 Imlay and Stuart 1988
Olefins	PAN, $O_2^{\cdot -}$	Halliwell, Gutteridge, and Cross 1992
α-Tocopherol	$O_2^{\cdot -}$	DiGuiseppi and Fridovich 1986
Cholesterol	$O_2^{\cdot -}$	DiGuiseppi and Fridovich 1986
Lipid peroxides	$O_2^{\cdot -}$	DiGuiseppi and Fridovich 1986

membranes can become peroxidized during overproduction of ROS. Lipid peroxidation occurs generally in three steps (equations 4-9, X· is a free radical): initiation, chain propagation, and chain termination (Stark 1991). Polyunsaturated lipids, lipid carbon-centered radicals, lipid alkoxyl radicals, lipid peroxyl radicals, and lipid hydroperoxides are represented as LH, L· , LO·, LOO· , and LOOH, respectively (Cohen 1985).

Initiation:	$LH + X\cdot$	$\longrightarrow$	$L\cdot + XH$	(Equation 4)
Propagation:	$L\cdot + O_2$	$\longrightarrow$	$LOO\cdot$	(Equation 5)
	$LOO\cdot + LH$	$\longrightarrow$	$LOOH + L\cdot$	(Equation 6)
	$LOOH + Fe^{+2}$	$\longrightarrow$	$LO\cdot + OH^-$	(Equation 7)
	$LO\cdot + LH$	$\longrightarrow$	$LOH + L\cdot$	(Equation 8)
Termination:	$LOO\cdot + LOO\cdot$	$\longrightarrow$	nonradical species	(Equation 9)
	$LOO\cdot + L\cdot$	$\longrightarrow$	nonradical species	

Exposure of cells, hence their DNA, to ozone (O_3), hyperbaric hyperoxia, H_2O_2 , and $O_2^{\cdot-}$ generating drugs can result in mutagenic effects and cause chromosome deletions, sister-chromatid exchanges, and single-strand breaks (Imlay and Stuart 1988). Oxygen radicals may attack DNA at either deoxyribose or nucleotide bases, creating hydroxylated or dimerized products. Cellular defenses against DNA damage mediated by oxygen toxicity include DNA excision-repair, antioxidant enzymes, and a deliberate depletion of NADH, which could otherwise drive an ongoing Fenton reaction (Imlay and Stuart 1988).

Antioxidant Defenses

Cellular defenses against the overproduction of free radicals are composed of various antioxidants which lower the steady state concentration of free radicals, thereby lowering damage inflicted by radicals. There are two major types of antioxidants: enzymatic and low molecular-weight expendable scavengers (Table 4).

Superoxide dismutase catalyzes the dismutation of $O_2^{\cdot -}$ to H_2O_2 (as shown in Equation 1). Superoxide dismutase was first described in 1969 (McCord and Fridovich 1969), and was shown to be identical to previously described proteins to which no catalytic function had been ascribed, i.e. cerebrocuprein, hemocuprein, and erythrocuprein. All SODs are metalloenzymes containing a redox-active transition metal ion at the active site (Omar, Flores, and McCord 1992).

There are three types of cytosolic superoxide dismutase: manganese-containing SOD (Mn SOD) of prokaryotes and the matrix of mitochondria, iron-containing SOD (Fe SOD) found mostly in prokaryotes and some plants, and copper-zinc SOD (CuZn SOD), mostly cytosolically located in eukaryotic cells, chloroplasts, and some bacteria. From amino acid sequence analysis, the Mn SOD and Fe SOD are very similar, while the CuZn SOD is not highly homologous with other SODs (Fridovich 1989). All the SODs have remarkably similar specific activities, despite significant differences in structure and sequence (Fridovich 1984b).

Table 4. Antioxidants

Type	ROS Specificity	Reference
Enzymatic		
CuZn SOD	$O_2^{\cdot -}$	Fridovich 1984b
Mn SOD	$O_2^{\cdot -}$	Omar, Flores, and McCord 1992
Fe SOD	$O_2^{\cdot -}$	Omar, Flores, and McCord 1992
EC-SOD	$O_2^{\cdot -}$	Fridovich 1984b
Catalase	H_2O_2	Bray et al. 1974
Glutathione Peroxidase	H_2O_2, alkyl hydroperoxides	Thomas, Geiger, and Girotti 1993 Krinsky 1992 Flohe 1984
Low molecular weight		
Glutathione (GSH)	Substrate for GPX tocopherol radical	Menzel 1984 Krinsky 1992
Thiols	General ROS	Menzel 1984
Metallothionein	H_2O_2	Cubatsu and Meneghini 1993
High density lipoprotein	General ROS	Fahey and Sundquist 1991
α-Tocopherol	Lipid radicals	Menzel 1984 Flohe 1984
Ascorbate	General ROS	Menzel 1984

There is also another type of CuZn SOD: extracellular SOD (EC SOD). This heparin-binding glycosylated SOD is termed extracellular SOD because it was first found in plasma. Extracellular SOD (EC SOD) is a secretory glycoprotein with most enzyme distribution being membrane-associated. The cDNA of EC SOD contains a homologous central region to the cytosolic CuZn SOD cDNA, but with additional sequences at both the amino and carboxy termini. The carboxy terminus has a highly positively charged 26 residue amino acid sequence, facilitating high affinity binding for sulfated polysaccharides and adherence to cell surfaces (Bray et al. 1974).

Catalase catalyzes the reaction shown in Equation 10. It is present in most tissues in peroxisomes and mitochondria and is specific for H_2O_2 detoxification (Radi et al. 1991). High concentrations of H_2O_2 will result in maximum enzyme catalytic activity.

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2 \qquad \text{(Equation 10)}$$

Selenium-containing glutathione peroxidase (GPX) also catalyzes reduction of H_2O_2 as well as organic hydroperoxides such as LOOH (equation 11). Glutathione peroxidase plays an important role in cellular antioxidant defense by scavenging low concentrations of H_2O_2, due to its low K_m compared with catalase and by preventing decomposition of LOOH to damaging free radical intermediates (Thomas, Geiger, and Girotti 1993). This occurs in conjunction with phospholipase A_2, forming lysophospholipid as an intermediate (Krinsky 1992).

$$ROOH + 2\ GSH \longrightarrow GSSG + ROH + H_2O_2 \qquad \text{(Equation 11)}$$

Selenium-containing GPX (Flohe 1984) contains selenol (a selenocysteine) at the catalytic site (Chiu, Lubin, and Shohet 1984). Glutathione peroxidase reduces diverse hydroperoxide species, including hydrogen peroxide, ethyl hydroperoxide, tert-butyl hydroperoxide, linoleic acid hydroperoxide, prostaglandin G_2, progesterone 17 α-hydroperoxide, cholesterol 7 β-hydroperoxide, and peroxidized DNA (Flohe 1984). There is also a form of GPX that directly reduces LOOH using glutathione (GSH) as the reductant, termed phospholipid hydroperoxide GPX, as well as a non-selenium containing GPX that acts only on H_2O_2 (Fahey and Sundquist 1991).

Low molecular weight antioxidants include glutathione (GSH), vitamin E, vitamin A, ascorbic acid, and high density lipoproteins (HDL). GSH protects against microsomal lipid peroxidation by maintaining protein thiols and vitamin E (α-tocopherol) in the reduced state (Palamada and Kehrer 1993). Vitamin E is involved in Equations 12 and 13 and reduces peroxy radicals to yield the hydroperoxide, which can be reduced to non-toxic hydroxy components by glutathione peroxidase (Thomas, Geiger, and Girotti 1993), thereby inhibiting lipid radical propagation reactions. High density lipoproteins also take part in the protection of plasma lipids from peroxidation (Klimov et al. 1993).

$$ROO\cdot + \alpha TH \longrightarrow ROOH + \alpha T\cdot \qquad \text{(Equation 12)}$$

$$ROO\cdot + \alpha T\cdot \longrightarrow ROOH + \alpha TH \qquad \text{(Equation 13)}$$

Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic pulmonary syndrome occurring in prematurely born infants with respiratory distress syndrome (RDS) treated with positive pressure ventilation and oxygen (O_2) supplementation for respiratory insufficiency (Northway 1992a). Because of this treatment and their impaired or immature antioxidant defense mechanisms, infants born between 24 and 32 weeks gestation frequently suffer oxidant-mediated lung injury (O'Brodovich and Mellins 1985, Morley 1991). Surfactant delivery before first breath (prophylactic treatment) and after O_2 supplementation (rescue treatment) lowers the O_2 requirement of the infant and the severity of RDS (Kwong et al. 1985). Because of successful surfactant therapy and O_2 supplementation, these infants survive RDS, but about 20% of the survivors manifest BPD (Northway 1992a; Northway 1992b; Merritt et al. 1986). This indicates the need for additional therapy targeted for BPD in conjunction with surfactant therapy used for RDS.

The mechanisms of pulmonary damage following hyperoxia and the specific sequelae of BPD development are controversial. There are various theories as to what causes this disease, including lung immaturity, barotrauma from positive airway pressure ventilation, O_2 toxicity, respiratory infections after airway intubation, and increased lining fluid (Southal and Samuels 1990). The theory that O_2 toxicity is a major contributor to the onset and continuation of BPD development is worth study,

because the endogenous rate of generation of reactive O_2 species (ROS) increases in the lung with hyperoxia (Freeman and Crapo 1981, Freeman, Topolosky, and Crapo 1982). Premature infants are also at additional risk for O_2-mediated damage because of low activities of pulmonary antioxidant defenses, which increase only during late gestation (Tanswell and Freeman 1984b; Yam, Frank, and Roberts 1978).

One of the earliest manifestations of O_2 toxicity in the premature infant lung is alveolar epithelial and endothelial damage (Matalon and Egan 1981), resulting in increase in alveolar epithelial permeability (Clement et al. 1992), lowered type II cell proliferation, and inhibition of lung injury repair (Burri 1991). The basement membrane as well as pulmonary cells are at risk for oxygen-related damage. The basement membrane is necessary for the development of terminal alveolar sacs (Burri 1991), which begins shortly after birth. The lowered antioxidant enzyme activity level and the increased ROS steady production in the premature infant lung cause an antioxidant enzyme to ROS imbalance. It is likely that the antioxidant enzyme to ROS imbalance damages both pulmonary cells and the basement membrane, and, in addition, activates inflammatory responses, thereby altering the normal maturation, development, and repair of the lung. These observations indicate that enhancing the level of antioxidant enzymes in the premature infant lung may alleviate the processes that lead to the development of BPD. Since the type II cells of the lung produce surfactant and differentiate into type I cells when there is injury and loss of oxidant-

sensitive type I cells, it would be beneficial to specifically deliver antioxidants to pulmonary type II cells and vicinal cells, promoting lowered ROS induced injury, normal surfactant production and function, and repair of the lung tissue.

Surfactant Production and Type II Cells

The type II cell is the major source of pulmonary tubular myelin and surfactant. Type II cells are of epithelial origin and their function is not only to produce surfactant but also to differentiate to type I cells when there is injury to the lung that destroys type I cells. To protect these cells is to protect the lung's ability to repair itself since type I cells cover 90% of the alveolar surface and are critical for maintaining the air blood barrier. Type II cells synthesize and store surfactant in specialized organelles called lamellar bodies. Type II cells both secrete lamellar bodies and ingest surfactant by endocytosis from their environment. Pathways for surfactant clearance include: uptake by type II cells, alveolar macrophages, type I cells, tracheal epithelial cells, and microciliary cells; and movement up the airways and into the esophagus, degradation in alveoli, and movement through the epithelium and endothelium into blood or lymph (Doleman and Bast 1990). Type II cells can internalize surfactant components to then be recycled or degraded. Surfactant protein A, the major protein component of surfactant, is localized to coated vesicles and endosomes (Doleman and Bast 1990). Adult type II cells express a high-affinity receptor for SP-A (Wright, Borchelt, and Hawgood 1989, Kuroki, Mason, and Voelker

1988) suggesting that SP-A can be used as a ligand for the targeted delivery of liposomes.

Liposomes as Drug Delivery Vehicles

To help alleviate injury associated with oxidant-associated diseases, several methods have been utilized to increase antioxidant levels in cells and tissues, including the modification of antioxidant proteins with polyethylene glycol (PEG), formation of chimeric antioxidant enzyme genes, and the use of liposomes as vectors to deliver antioxidant enzymes (Table 5). There has been little clinical success to date in using native SOD as a therapeutic agent. The native CuZn-SOD is cell-impermeable, has a very short circulating half-life, and has a bell-shaped dose-response curve, wherein SOD becomes toxic at high doses (Omar et al. 1990). Superoxide dismutase must be delivered efficiently in order to lower its systemic toxicity and increase the efficiency of its delivery to specific tissues. The research described herein shows that entrapment of drugs, including antioxidant enzymes, into liposomes is a promising method for highly efficient and specific delivery to tissues.

Liposomes are spherical lipid bilayers that enclose an aqueous interior and are produced by several different techniques. They form spontaneously when lipids are dispersed in aqueous media, but vary greatly in size and organization. In an excess of water, phospholipids and other polar amphiphiles form closed concentric bilayer membranes, entrapping water and dissolved solutes (e.g., drugs) in the process

Table 5. SOD modifications for enhancing pharmacologic efficacy

Modification	Effects and advantages	Disadvantages
Native SOD	No modification necessary	Inefficient intracellular delivery
(Omar et al. 1990)		Expensive
		No targeting
Polyethylene glycol attachment	Allows endocytosis of active enzyme	No targeting
(Walther et al. 1993)	Increased circulating $t_{½}$	
(Zhu et al. 1993)	Lowered immunogenicity	
Fusion genes with heparin binding sequences	Promotes binding to proteoglycans	Partial targeting
(Mao et al. 1993)		
Liposome entrapment	Efficient delivery	Limited shelf-life
(Zhu et al. 1993) (Tanswell, Olson, and Freeman 1990)	Can be targeted Increased circulating $t_{½}$	
(Tanswell and Freeman 1987)	Lowered immunogenicity	
	Low systemic toxicity	
	Useful for many compounds	

(Gregoriadis and Florence 1993). Sizes can range from tens to hundreds of nanometers in micellar, bilayer, inverted micellar (hexagonal H_{II} phase), unilamellar, or multilamellar liposome form, depending upon lipid content and composition. Average liposome size can be adjusted by sonication, detergent dialysis, microfluidization, homogenization, and other techniques.

Liposomes (phospholipid-based vesicles) have been investigated since 1970 as a vector for the delivery or targeting of drugs to specific sites in the body. Many hundreds of drugs, including antitumor and antimicrobial agents, peptide hormones, enzymes, other proteins, vaccines and genetic material have been incorporated into the aqueous or lipid phase of liposomes of various sizes and compositions by an ever increasing number of technologies (Gregoriadis and Florence 1993). Liposomes have the potential to be tailored in a variety of ways to ensure the production of formulations that are optimal for clinical use (Torchilin et al. 1992). They are versatile in terms of size, composition, surface charge, and bilayer fluidity; are able to incorporate almost any drug regardless of solubility; and are able to carry on their surface cell-specific ligands (Gregoriadis and Florence 1993). Thus ligands, including antibodies, bind to their receptors on the surface of cells in a highly specific fashion, while the attached liposomes can introduce their drug contents into the interior of the cell through endocytosis or other pathways. They can also be manipulated to deliver to specific target cells, have increased half life *in vivo* by co-coating the liposomes

with polyethylene glycol (PEG) (Torchilin et al. 1992), and deliver drugs efficiently to target cells.

One type of liposome that can be developed to have desirable pharmacological properties is the pH-sensitive liposome. pH-sensitive liposomes are made with lipids that destabilize the liposomal structure at low pH conditions (pH 4). Liposomes prepared with dioleoylphosphatidylethanolamine (DOPE) and diacylsuccinylglycerols are sensitive to lowered pH conditions and are stable in plasma (Collins, Litzinger, and Huang 1990). The succinyl group of the amphiphiles is protonated under acidic conditions. This leads to reduced interbilayer repulsion and a loss of head group hydration, which promotes destabilization of the membrane into an inverted hexagonal phase formation. Since the endosomal compartment of the cell is maintained at pH 5-6, the sensitivity of the liposomes to lowered pH allows destabilization and fusion of the liposomes in the endosome and subsequent cytosolic delivery of intra-liposomal contents (Illustration 1).

Intravenous injection of liposomes is normally followed by their interaction with at least two distinct groups of plasma proteins: opsonins and high density lipoproteins (HDL). Opsonins mediate liposome endocytosis by the fixed macrophages of the reticuloendothelial system (RES) and circulating monocytes by absorbing onto the surface of vesicles. High density lipoproteins remove phospholipid molecules from the vesicle causing their disintegration and release of encapsulated solutes at rates

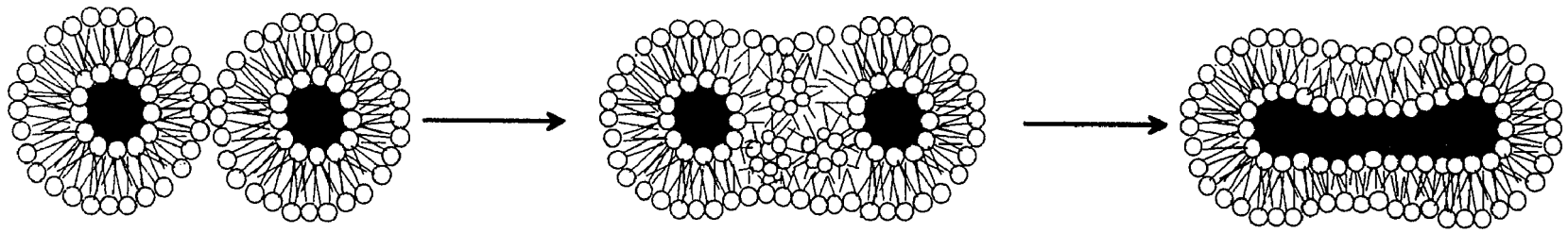


Illustration 1. Fusion of pH-sensitive liposomes

dependent on the extent of bilayer damage (Gregoriadis and Florence 1993). Rates of uptake by the RES are influenced by a number of parameters including vesicle size, surface charge and bilayer fluidity (Torchilin et al. 1992). Recent work has shown that a hydrophilic vesicle surface achieved by the use of polyethyleneglycol and other hydrophilic molecules substantially prolongs the half-life of liposomes having an average diameter of up to 100 nm (Collins, Litzinger, and Huang 1990). Some of the realistic prospects of liposome development for clinical use include cancer chemotherapy, antimicrobial therapy, immunological adjuvants in vaccines, diagnostic imaging, ocular delivery of drugs (Gregoriadis and Florence 1993) and intratracheal instillation to the lung for treating airway and pulmonary diseases.

In Vitro Model and Hypothesis

Because there are many uncontrollable variables associated with whole animal studies, it is desirable to develop an *in vitro* cell model of fetal lung injury, which will also help reveal the efficacy of liposome-mediated antioxidant enzyme delivery for attenuating oxidant lung injury and consequent BPD. To study the delivery of antioxidants to fetal lung, an *in vitro* model using fetal rat lung distal epithelial cells (FRLE cells) was used. Fetal lung distal epithelial cells readily divide in culture media containing 10% fetal calf serum (Jassal et al. 1991), making them amenable for study in large numbers. The isolation of these cells produces a primary culture of very high purity, as determined by staining for a epithelial cell-specific intermediate

filament, cytokeratin. Also, these monolayers show characteristic dome formations when plated at high density.

The hypothesis guiding this dissertation research is based on the observation that BPD development is strongly correlated with hyperoxic treatment and increased ROS production. Also, alveolar macrophages and neutrophils are often activated during pulmonary disease processes, which increases net lung ROS production. Thus, oxygen radicals are being overproduced both intracellularly and extracellularly (Illustration 2). Supplementation of the premature infant lung with antioxidants may then prevent many problems associated with BPD. The use of pH-sensitive liposome to deliver SOD has potential to reduce the production of ROS intracellularly, allowing type II cells to remain viable until maturation of the lung occurs (Illustration 3).

The hypothesis guiding this dissertation research is that entrapment of SOD into pH-sensitive liposomes formed with SP-A will efficiently deliver SOD to cultured FRLE cells, thereby attenuating oxidant stress damage. This method of antioxidant delivery can then be further studied in animal models, prior to clinical application as a treatment for BPD and other oxidant-related lung pathologies.

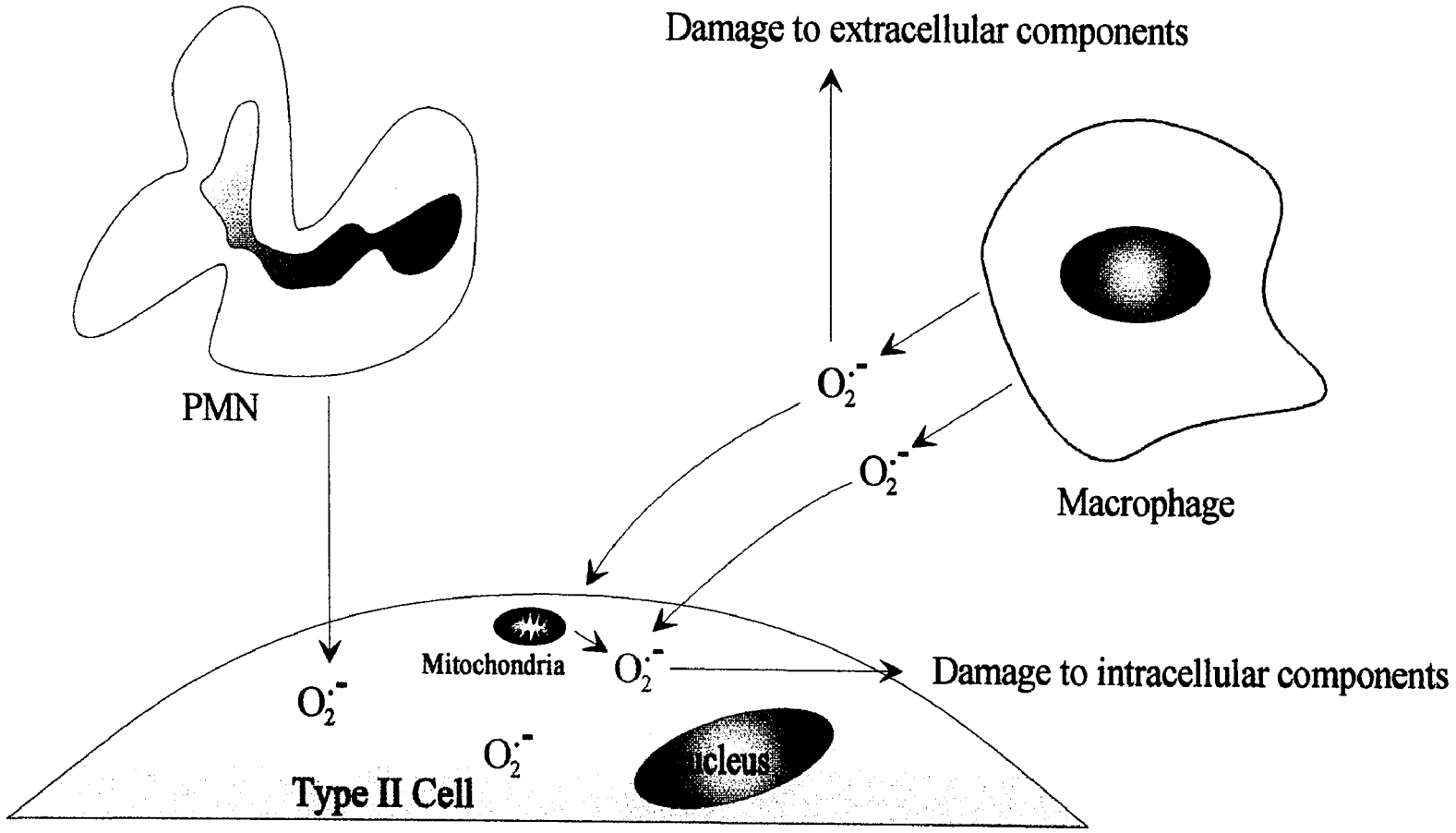


Illustration 2. Superoxide radical sources in the lung

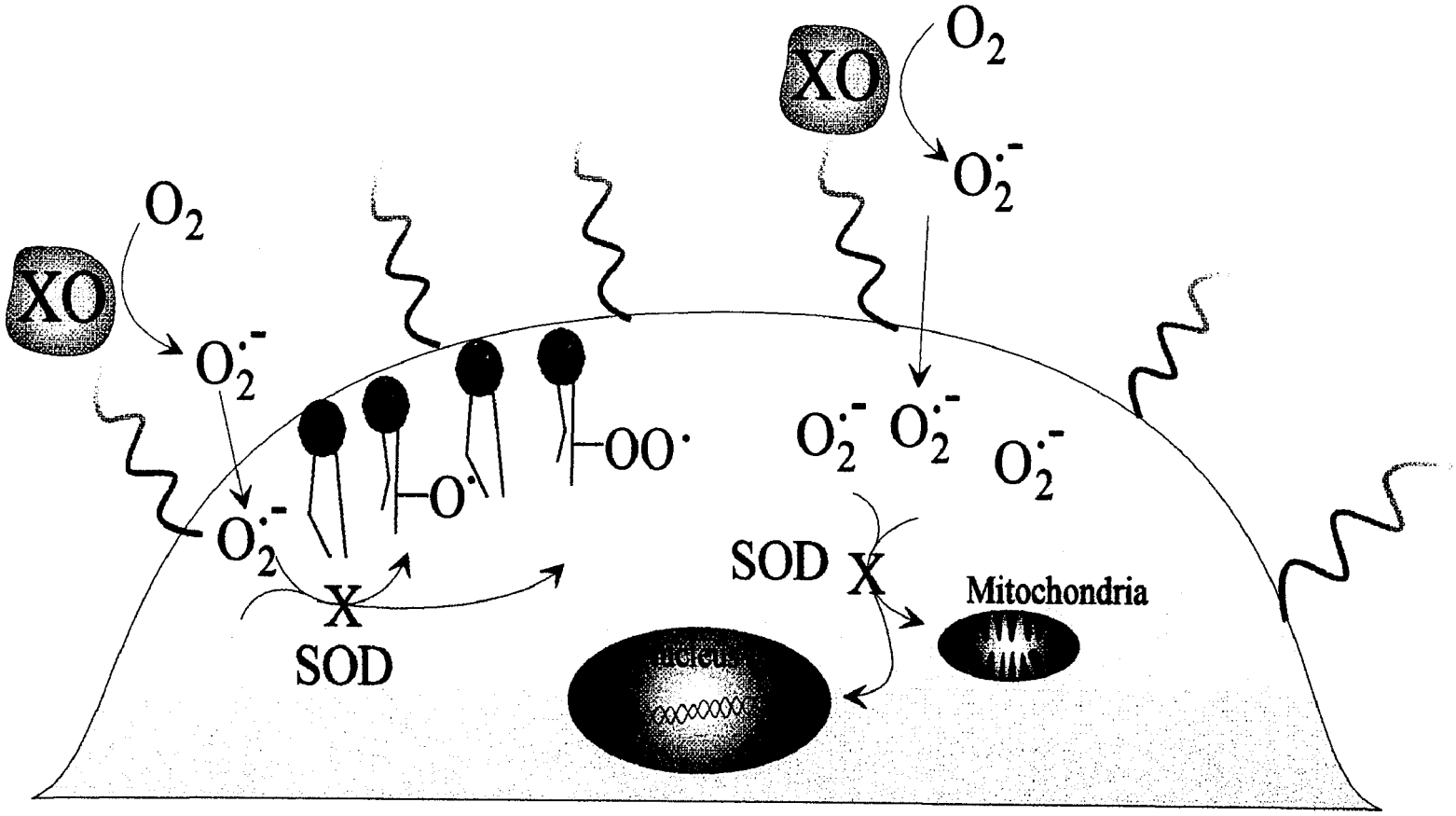


Illustration 3. SOD protection of type II cells from $O_2^{\cdot-}$

CHAPTER 2

MATERIALS AND METHODS

Materials

Reagents and Supplies

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3- succinate (DOSG), and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Superoxide dismutase was obtained from Diagnostic Data, Inc. (Mountain View, CA), catalase from Worthington Biomedical Corporation (Freehold, NJ), BCA protein assay reagents and Iodo-beads from Pierce Biochemicals (Rockford, IL). BioGel A-1.5 m agarose was obtained from Bio-Rad (Richmond, CA) and filter holders from Fisher (Pittsburgh, PA). Polycarbonate membranes were obtained from Costar Nucleopore (Cambridge, MA). Trypsin (0.05% trypsin, 0.53 mM EDTA), antibiotic/antimycotic mixture , HBSS (Ca^{2+}, Mg^{2+} free), and DMEM (Dulbecco's modified essential medium) were obtained from Gibco Laboratories (Grand Island, NY). Deoxyribonuclease I (DNase) was obtained from Worthington Biochemical corporation (Freehold, NJ). Adenine hydrochloride [8-^{14}C-], $Na^{125}I$ (~17 Ci/mg, pH 8-11), and tritiated inulin were obtained

from DuPont NEN Research Products (Boston, MA). All other materials and collagen (type IV) were obtained from Sigma (St. Louis, MO).

Equipment

The Polaron E7500 freeze-fracture device used in freeze-fracture of liposomes was obtained from VG Microtech (Uckfield, East Sussex, England), and the resulting freeze-fracture grids were examined and photographed using a JEOL 100 CX-II electron microscope. The liposome extruder was obtained from Lipex, Inc. (Vancouver, Canada). The Nicomp Submicron Particle Sizer was obtained from Nicomp (Santa Barbara, CA).

Methods

Cell Culture

Fetal rat lung distal epithelial cell cultures (FRLE) were prepared as previously described (Jassal et al. 1991). The lungs of day 19 gestation fetal rats were dissected out and major airways and vessels were removed. The lungs were rinsed in Hank's balanced salt solution (HBSS) without calcium or magnesium ions (-Ca^{2+}, -Mg^{2+}) four times. The rinsed lungs were minced and vortexed gently to remove red blood cells. This rinsing and vortexing step was repeated several times until a clear supernatant was obtained. The minced lung tissue was then digested in a trypsinizing flask for 20 min at 37° C with 0.125% w:v trypsin and 0.4 mg/ml DNase. The trypsinization was stopped by adding Eagle's minimal essential medium (MEM) containing 10% (v:v)

heat-inactivated fetal calf serum (FCS-HI). Fetal calf serum is heat-inactivated by incubation at 56° C for 30 minutes. The resulting cell suspension from the trypsinization is eluted through a filter holder containing 100 mm mesh nylon cloth. The filtered mixed cell suspension was pelleted by centrifugation at 420 x g for 5 min for a total of three times, resuspended to 15 ml in MEM containing 0.1% (w:v) collagenase, and incubated at 37° C for 15 min. Collagenase activity was neutralized by adding an equal amount (15 ml) of MEM with 10% FCS-HI (v:v). Epithelial cells and fibroblasts were sedimented at 420 x g for 5 min, resuspended in MEM with 10% FCS-HI (v:v), and cultured at 37° C in 75 cm^2 tissue culture flasks twice for 1 hr, to permit the differential adherence of fibroblasts. The non-adherent cells were sedimented at 240 x g for 3 min and resuspended in fresh MEM with 10% FCS-HI, plated in 75 cm^2 culture flasks or 12-well plates, and incubated overnight at 37° C, during which time the epithelial cells attached. Non-adherent cells were removed from all cultures after a 24-hour incubation and the adherent cells were used for experiments. This isolation protocol gives a population of epithelial cells of high purity as determined by staining for the epithelial cell-specific intermediate filament cytokeratin (Jassal et al. 1991).

Iodination of Proteins

Iodination of superoxide dismutase and SP-A was conducted using Iodo-beads (Pierce Biochemicals, Rockford, IL). For this, SOD (1 mg) or SP-A (0.26 mg) was

incubated with 5 mCi $Na^{125}I$ in 0.5 ml PBS (0.01 M KH_2PO_4, 2.7 mM KCl, 137 mM NaCl pH 7.4) with 3 beads for 10 min. The ^{125}I-SOD solution was removed from the beads and unincorporated $Na^{125}I$ was removed by gel exclusion chromatography using Sephadex G-25. The resulting void volume collected was tested for the proportion of radioactivity that was bound to protein. This was done by using a 1 ml aliquot and precipitating the protein with an equal volume of 10 % trichloroacetic acid (TCA). The sample was centrifuged and the resultant supernatant and pellet were analyzed in a gamma counter. The radioactivity was 85% precipitable.

Cytotoxicity Assays

Cytotoxicity was determined by two methods. The first was by determining the percent release of a ^{14}C-adenine prelabel. Within two hours of incubation of cells with ^{14}C-adenine, the radioactive label becomes incorporated into ATP (65%-75%), ADP (15%-20%), and 5'AMP (10%-15%) (Shirhatti and Krishna 1985). Upon damage of cells in culture, the radioactive macromolecules are released, indicative of cell damage. ^{14}C-adenine (4 μCi) was added to 20 ml MEM, and 0.5 ml added to each 4 cm^2 well and incubated for 2 hr. The cells were rinsed 3 times with HBSS ($-Ca^{+2}$, $-Mg^{+2}$) before liposomes were added. At the end of the experiment, the amount of radioactivity in the media was counted and compared to the total amount incorporated into cells (100% lysis). One-hundred percent lysis was determined by adding 1% CHAPS (3-[(3-cholamidopropyl) -dimethylamino] -1-propanesulfonate)) in PBS to a 6

separate wells of a 12-well tissue culture dish before the start of the experiments to indicate 100% lysis. The second method used to determine cell damage was the determination of the number of cells that detach during incubation. To determine the protective effect of liposomes containing antioxidants against X/XO-derived oxidant stress, the number of detached cells after treatment with xanthine and xanthine oxidase was counted. The media from each well was collected, and the number of cells in the media was determined using a cell counter (Coulter Electronics, Inc., Hialeah, FL). This type of approach has been described before as an assay for damaged cells (Panus et al. 1992). Detached cells are cells that are damaged enough to lift away from the plastic culture dish, but are not necessarily dead. This damage could be due to both intracellular and extracellular damage. The extracellular damage could mimic basement membrane damage, which would correlate with damage in the *in vivo* setting.

Non-pH-sensitive Liposome Preparation and Analysis

Stock lipid solubilized in chloroform was dried in a 15 ml thick wall conical scintered-glass tube using a stream of argon gas and was further evaporated in vacuo using a rotoevaporator for a minimum of 1 hr at 50° C. Multilamellar vesicles (MLVs) were produced by adding enough aqueous buffer solution to make the final lipid solution 100 mg phospholipid/ml. To produce VET_{200}s (vesicles of extrusion technique, size 200 nm), liposome solutions were frozen in liquid nitrogen and thawed

at 40° C a total of 5 times. The resulting MLVs were extruded 10 times through 2 polycarbonate membranes of 400 nm pore size (Illustration 4). Extruded liposomes were then centrifuged at 100,000 - 150,000 xg for 1 hour. Supernatant was removed and the pellet was resuspended in the appropriate aqueous buffer.

pH-sensitive Liposome Preparation and Analysis

DOPE (35 mmol) and DOSG (35 mmol) dissolved in 3 ml chloroform were evaporated under a stream of nitrogen gas and the resulting lipid film was rehydrated by adding 1 ml 7 mM HEPES, 0.21 M sucrose, pH 8.0 (HEPES/Sucrose buffer). The lipid was hydrated for 48-72 hours at 4° C while carefully maintaining pH at range 8.0-9.0 by periodically adding tetraethyl ammonium hydroxide (TEA-OH). Superoxide dismutase (10 mg/ml final concentration), and in some cases SP-A (50 mg/ml final concentration), was dissolved in HEPES/sucrose buffer. The dissolved proteins were then added to lipid emulsions, which were then extruded 10 times through 2 stacked polycarbonate membranes of 400 nm pore size. For experiments using SP-A, the SP-A was allowed to associate with the rehydrated lipids for 15 minutes before SOD was added. Unincorporated protein was washed from liposomes by centrifugation 2 times at 150,000 x g for 1 hour. Liposome size was determined by quasielastic light scattering using a Nicomp Submicron Particle Sizer. Liposome destabilization in an acidic environment was detected by incubating liposomes that contained ^{3}H-inulin or ^{125}I-SOD at varying pH conditions. The resultant preparations

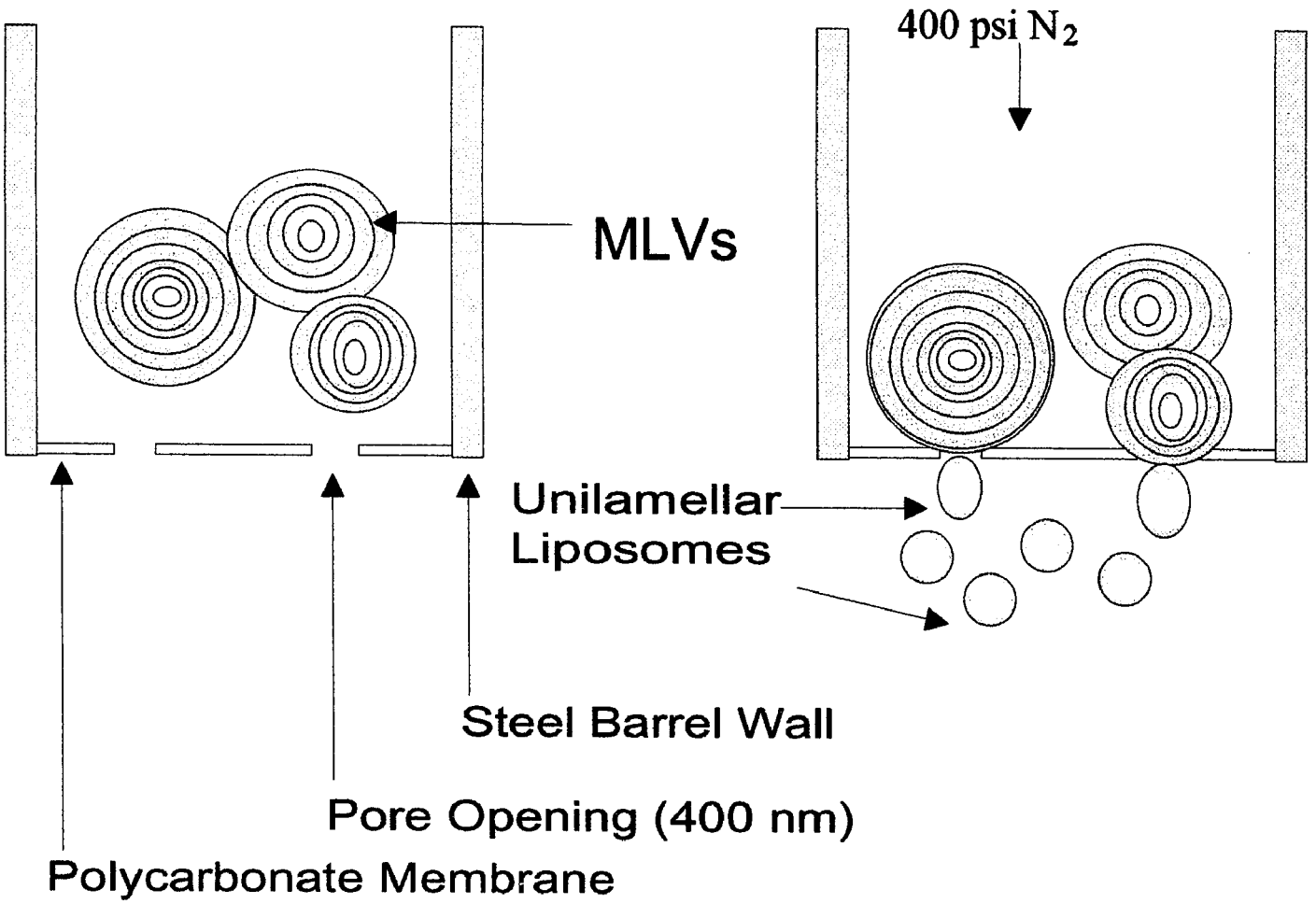


Illustration 4. Extrusion of multilamellar liposomes to unilamellar liposomes

were centrifugally eluted through 3 ml bed volume columns of Bio-Gel A-1.5 m agarose beads. The void volume, containing intact liposomes, was counted in a scintillation or gamma counter (Illustration 5). To quantitate the extent of lipid incorporation into liposomes, ^{14}C-cholesteryl oleate (0.001 mCi) was added to lipids before liposome preparation. For stability assessment, liposomes were prepared containing 100 mM entrapped calcein. The release of liposomal contents over time was detected by the fluorescence yield of calcein (λ_{ex}=490 nm and λ_{em}=520 nm) from a 100 µl liposome aliquot diluted to 1 ml in HEPES/Sucrose buffer before (F_i) and after (F_f) addition of 10 ml of Triton X-100 for a final concentration of 1%. Liposome content release was calculated by:

$$1- \frac{F_f - F_i}{F_f} x\ 100 - \%\ leakage$$

Freeze-fracture electron microscopy

Samples (10-20 µl) of liposomes sedimented at 150,000 x g for 1 hour were loaded into copper holders and rapidly frozen with a liquid nitrogen jet freezer. Samples were then freeze-fractured and evaporation-coated with carbon and platinum using Polaron E7500 freeze-fracture devices and photographed using a JEOL 100 CX-II electron microscope.

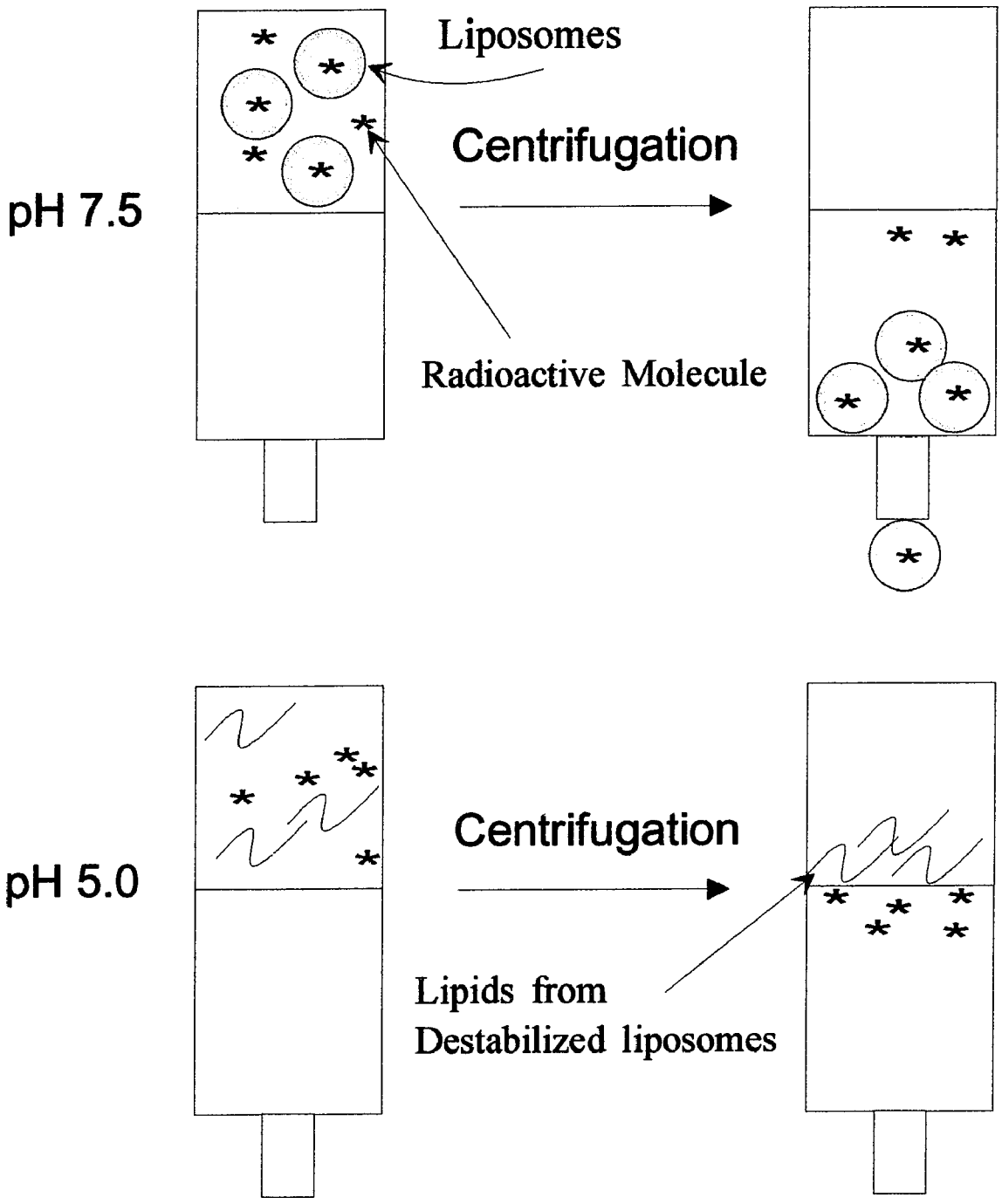


Illustration 5. Centrifugation of intact liposomes in void volume

^{125}I-SP-A binding to cells

Surfactant protein A was isolated from human lung lavage as described previously (Wright et al. 1987). Fetal rat lung distal epithelial cells were isolated, plated into 12-well plates, and used within 72 hours of isolation. Cells were removed from the incubator, placed on ice, and rinsed 3 times with ice cold binding medium (MEM, 1% w:v bovine serum albumin, and 50 mM HEPES pH 7.4). The cells were then incubated with radioactively labeled SP-A at the indicated concentration. After a 60-minute incubation on ice, the cells were rinsed three times with ice cold binding medium and solubilized with 0.1 N NaOH. The solubilized cells were assayed for radioactivity in a gamma counter.

Biochemical analysis

Protein content of cells was measured using bicinchoninic acid (Smith et al. 1985). Protein assays were performed using a 96-well plate and a microplate reader. The sample (30 µl) was put into a well, and 200 µl of the reagent was added. The samples were incubated for 30 minutes at 37° C. The absorbance was read at 560 nm and compared to a BSA standard to determine the protein content of each sample. The activity of CuZn superoxide dismutase (EC 1.15.1.1.) was assayed by inhibition of xanthine oxidase-dependent ferricytochrome c reduction (McCord and Fridovich 1969). Reduced ferricytochrome c absorbs light at 550 nm. During the assay, the oxidized form of ferricytochrome c is used to monitor the production of superoxide produced

from xanthine oxidase with xanthine as substrate. The $O_2^{\cdot -}$ reduces the oxidized cytochrome c, thereby increasing the absorbance at 550 nm. If this rate is inhibited by the addition of sample, then the sample contains superoxide dismutase. By definition, one unit of SOD activity is the amount that inhibits the rate of ferricytochrome c reduction by 50%. The following calculation was used to determine the activity of SOD present:

$$\frac{XO\ Rate}{SOD\ Rate} - 1 = U\ SOD\ activity$$

where XO rate is the rate of reduction of ferricytochrome c with xanthine and xanthine oxidase, and the SOD rate is the rate obtained when the sample is also added (Kobayashi et al. 1978). DNA content was estimated fluorometrically (Fiszar-Szafarz, Szafarz, and de Murillo 1981), employing 3,5-diaminobenzoic acid (DABA) as the reagent. This compound forms a fluorescent compound with DNA that can be observed at excitation λ = 408 nm and emission λ = 508 nm. The sample (brought to 200 µl with 1N NaOH) and 200 µl of DABA were added together and heated for 30 minutes in a 60° C water bath. The samples were compared to a standard to determine the DNA content of each sample.

CHAPTER 3

RESULTS

Cell Culture and SP-A Receptor Studies

The FRLE monolayers had a characteristic growth pattern and dome formation as described previously (Jassal et al. 1991). When incubated overnight in MEM without serum before experiments, cell monolayers retained these characteristics (Fig. 1, Fig. 2).

When FRLE cells were incubated with 1 mg/ml SP-A, time-dependent SP-A binding to cell monolayers was observed (Fig. 3). SP-A binding occurred rapidly over a 10 min period, reaching near maximal binding at 20 min. There was no additional binding beyond 20 min (Fig. 3). From this, all subsequent SP-A and SP-A-liposome binding experiments were performed for 60 min to assure complete binding. Specific high-affinity cell-binding SP-A was calculated by subtracting non-specific binding from total binding (Fig. 4). Total binding was indicated by binding of ^{125}I-SP-A alone (no excess non-radioactive SP-A), while non-specific binding was the ^{125}I-SP-A binding in the presence of excess non-radioactive SP-A. SP-A binding saturation

Figure 1. Photomicrograph of FRLE cell dome formation in culture. This demonstrates epithelial cell dome formations in culture, indicative of healthy growth on confluent monolayers with intact tight junctions. Cells on the plastic surface are in focus, with the top of the dome out focus.

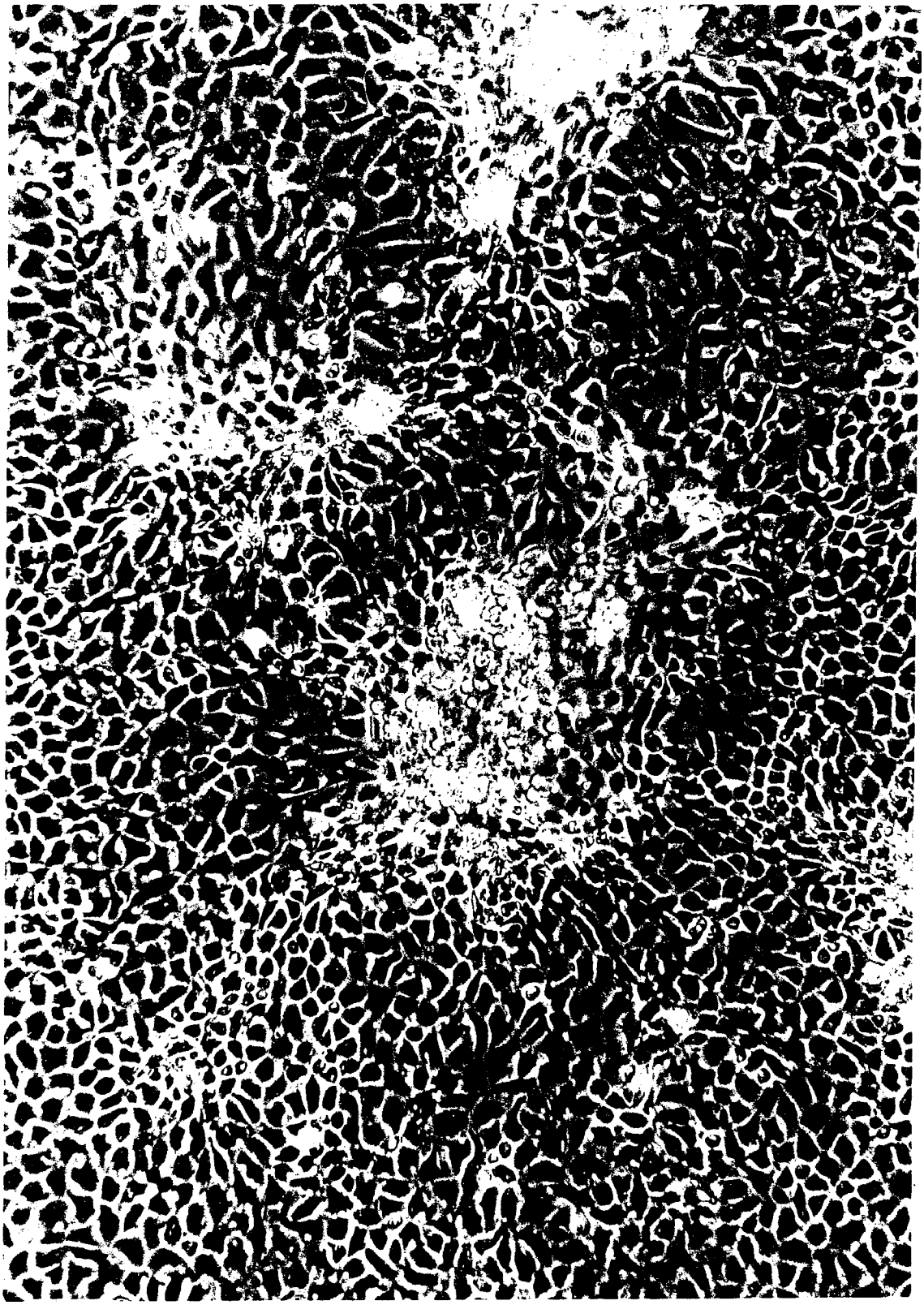
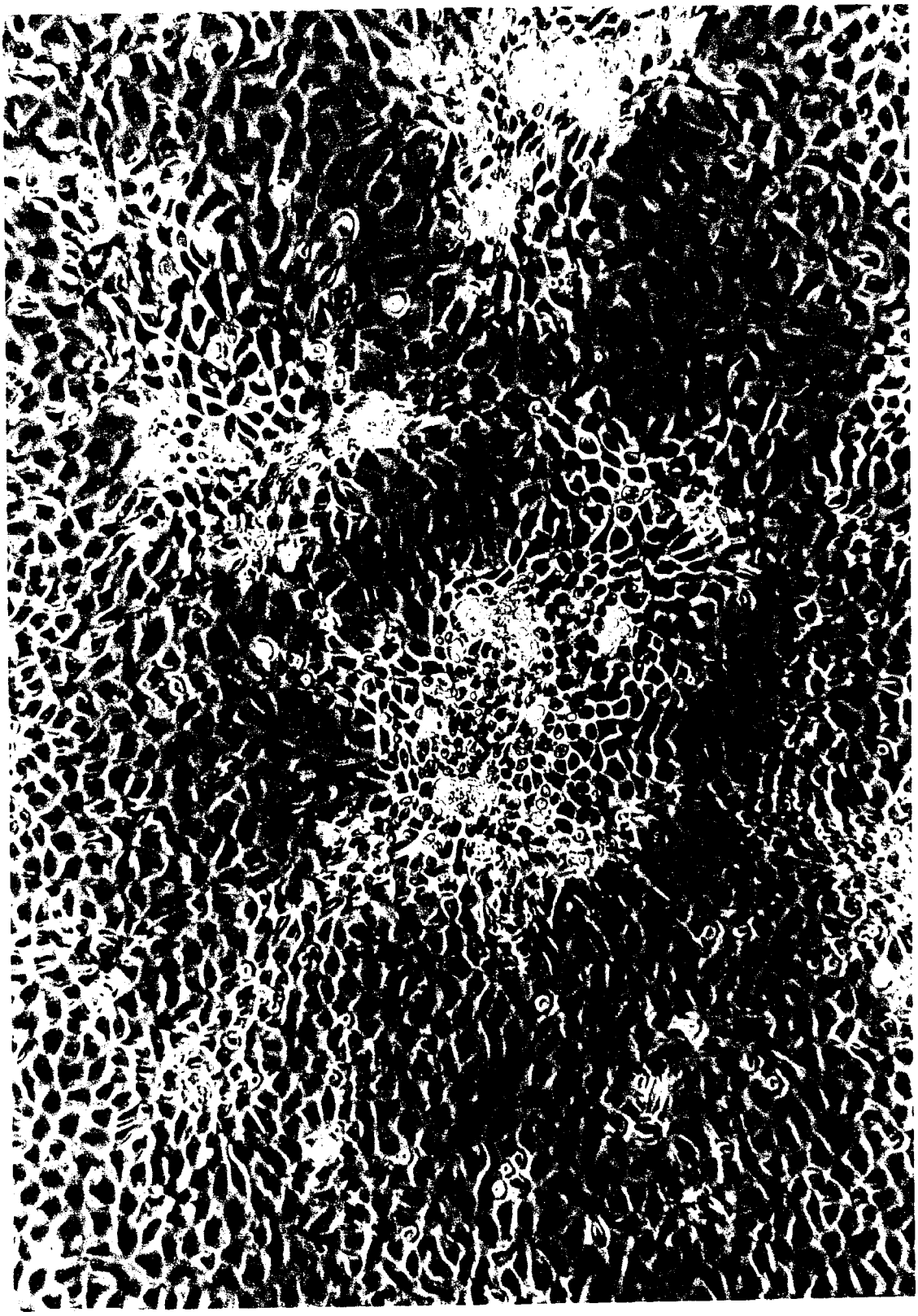


Figure 2. Photomicrograph of FRLE cell dome formation in culture. The tops of the domes are shown in the focal plane, and the monolayer is out of the focal plane.



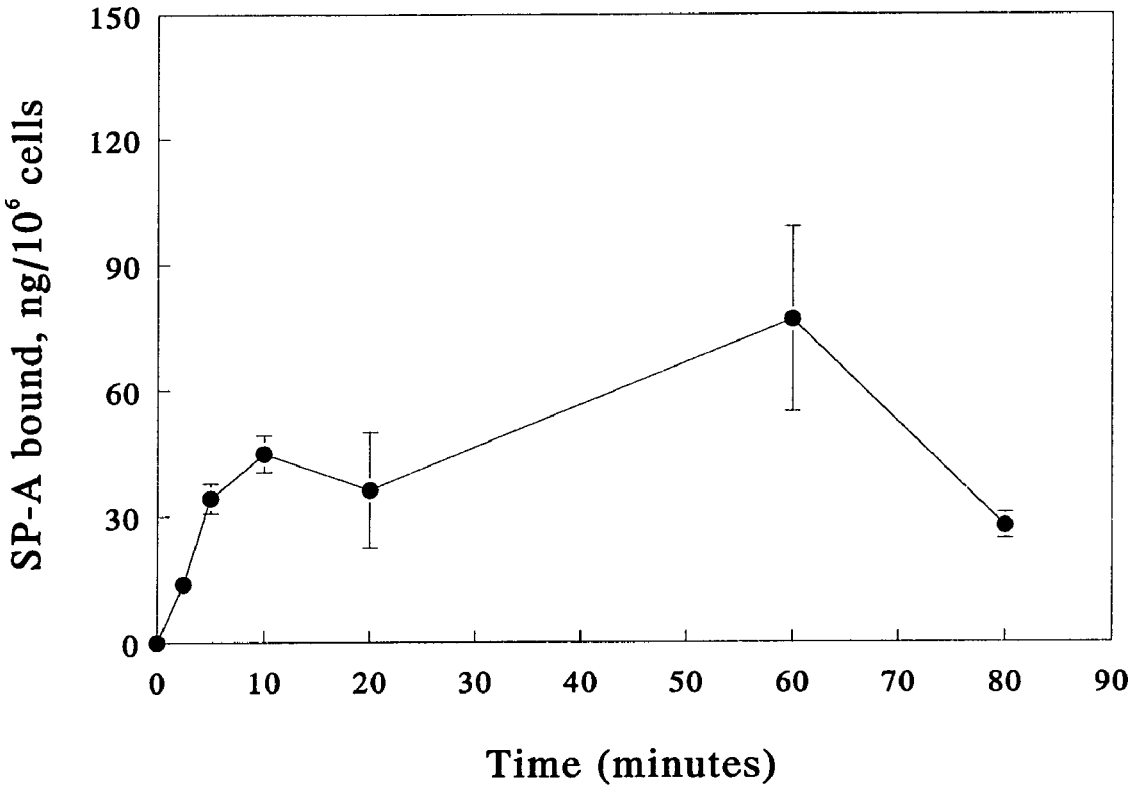


Figure 3. Time-dependent binding of SP-A to cultured FRLE cells. Cells were incubated for one hour at 4°C with 0.5 ml of 1 mg/ml ^{125}I-SP-A. Cells were rinsed with HBSS and solubilized with 0.1 N NaOH. Cell-associated radioactivity was determined at the indicated time points. Data represent mean ± SD, n=6.

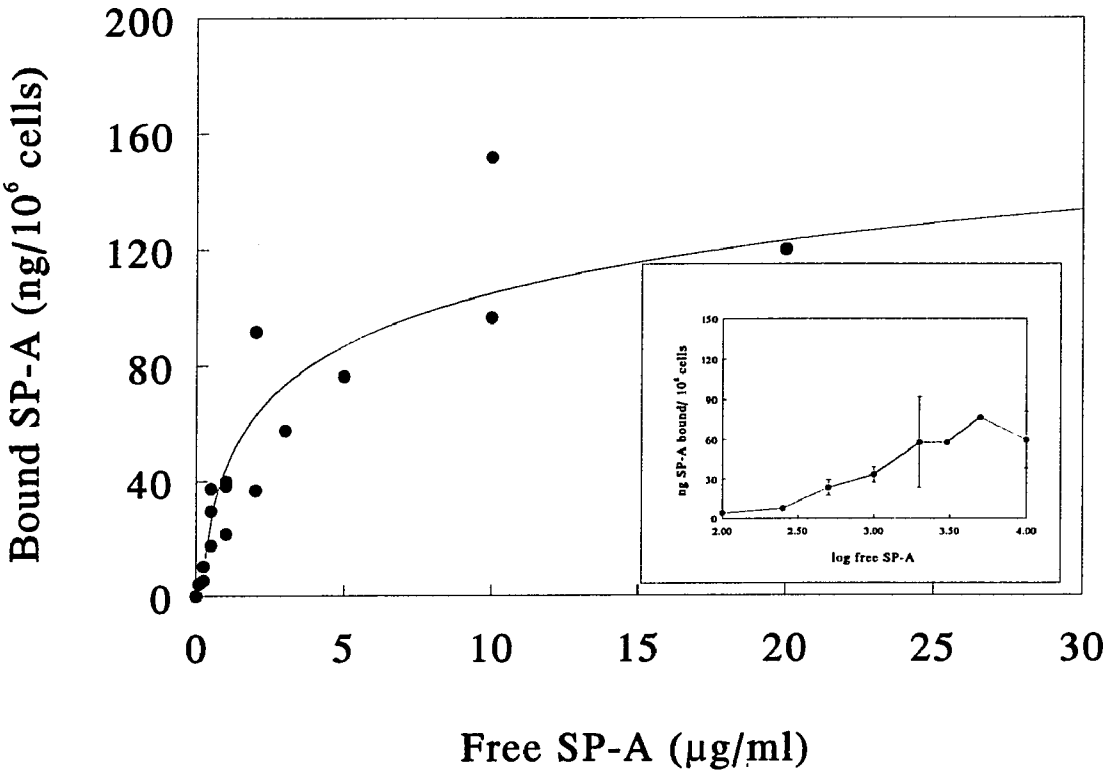


Figure 4. Specific binding of SP-A to cultured FRLE cells. The data were fitted to a ligand binding curve with one receptor and the K_d and binding capacity then extrapolated. These data represent triplicate determinations from four separate experiments and represent mean ± SEM. Inset. Klotz plot of binding data. The log concentration multiplied by 1,000 of SP-A in binding buffer was used for the x-axis. This binding plot shows that saturation of the binding site occurs, and data are valid for determining the K_d and the capacity of the receptor.

occurred at the concentrations used, as revealed by Klotz analysis of binding (Klotz 1982) (Fig. 4, inset). Binding analysis assumed one ligand per receptor. From these experiments, the calculated K_d for SP-A binding to FRLE is 4 ± 0.2 μg/ml and the capacity is 130 ± 3 ng/10^6 cells (120,000 ± 3,000 binding sites per cell). This was calculated using the reported molecular weight of SP-A of 26-36 kDa (averaged to 31 kDa for calculations) (Wright, Borchelt, and Hawgood 1990).

Preparation and Characterization of Liposomes

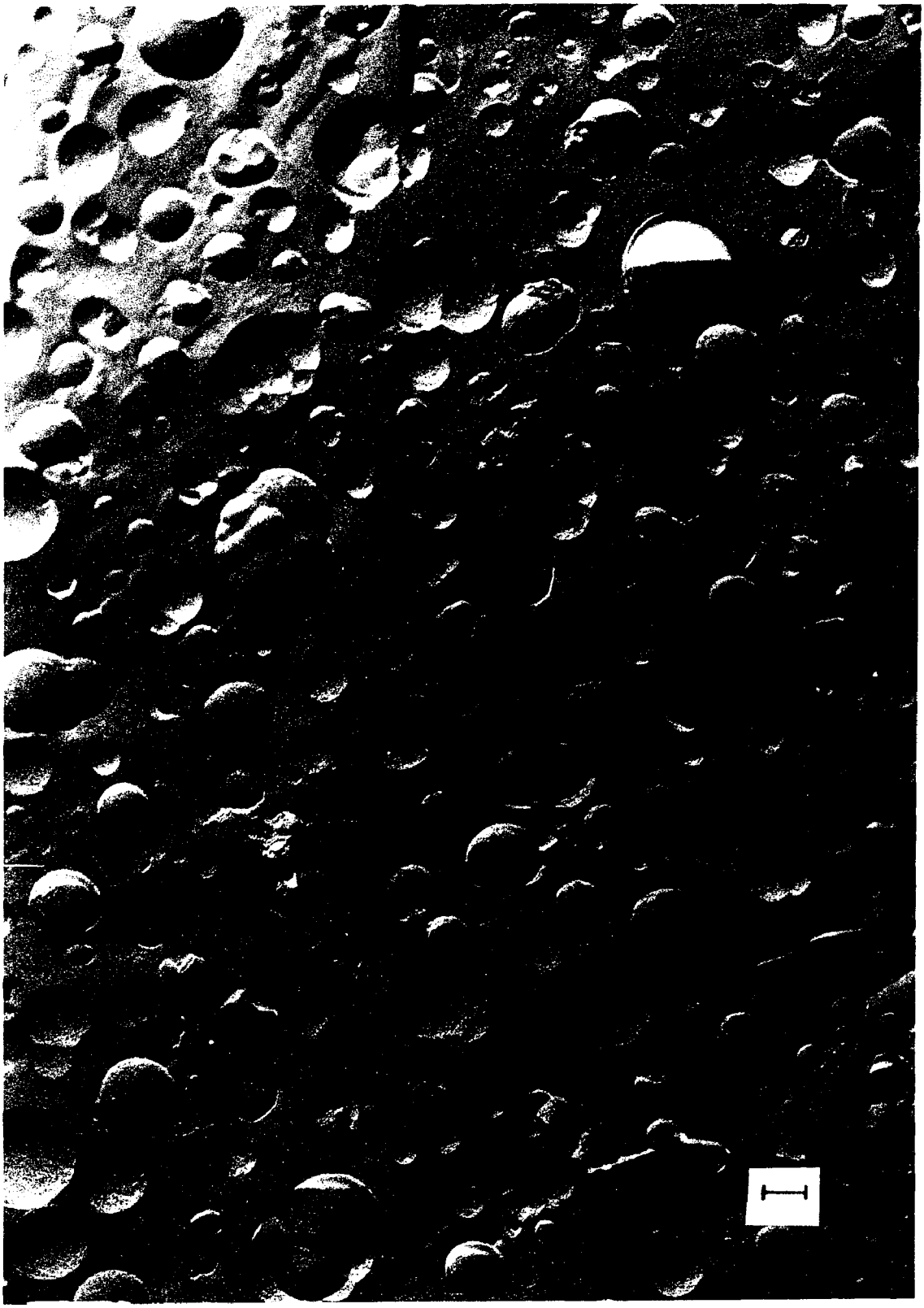
Previous approaches to entrapping enzymes in non-pH-sensitive liposomes utilized dipalmitoylphosphatidylcholine (DPPC), cholesterol (chol) , and stearylamine (SA) in a 14:7:4 molar ratio (Bulkley, Tanswell, and Freeman 1987). These were produced again in this study by reverse phase evaporation and, for comparative purposes, by extrusion. The size and aggregation state of liposomes were determined by using quasielastic light scattering. Non-pH-sensitive liposomes readily formed large aggregates when prepared with entrapped protein (Table 6). To improve efficiency of delivery of SOD into cells and avoid problematic liposomal aggregation phenomena, pH-sensitive liposomes were made by a solvent-free high pressure extrusion technique. Quasielastic light scattering showed that the extruded pH-sensitive liposomes were 200 ± 100 nm in diameter (mean ± SD), confirmed by freeze-fracture electron microscopy (Fig. 5). The size distribution of liposomes made both with and without SOD included a 20% population of liposomes under 50 nm, with 58% of the liposomes being 75-250

Table 6. Characteristics of different liposome formulations having entrapped SOD

Composition	Preparation Method	Size at 24 hours
DPPC:Chol:SA	Reverse phase evaporation	4000 ± 1000 nm
DPPC:Chol:SA	Extrusion	400 ± 200 nm
DOPE:DOSG	Extrusion	200 ± 100 nm

Values are mean ± S.D. and were obtained 24 hours after preparation and storage at 4° C by quasielastic light scattering.

Figure 5. Freeze-fracture electron micrograph of pH-sensitive liposomes. Liposomes are 1:1 DOPE:DOSG with 10 mg/ml entrapped superoxide dismutase. Bar represents 100 nm.



nm in diameter (Fig. 5). Freeze-fracture analysis also showed that liposomes were mostly unilamellar and not aggregated. The pH-sensitive liposomes did not aggregate over a period of one week at 4°C (Fig. 6). Freeze-fractured liposomes subjected to pH 3 have an irregular appearance of the liposome surface, suggesting that they tend to aggregate together and destabilize at low pH (Fig. 7). The pH-sensitive liposomes also did not exhibit any lipid oxidation, either at room temperature or at 37°C in media. After formation, liposomes stored at 4°C for 24 hr exhibited only minimal loss of contents, as indicated by calcein release.

Liposomes made with SP-A did not have an increased tendency to aggregate, with SP-A liposomes being 142 ± 60 nm in size (Table 7). The net recovery of precursor lipid into liposome membrane pellet was 85%, as calculated by the amount of recovered ^{14}C-cholesteryl oleate associated with liposome pellets. The 15% of lipid not recovered was probably lost due to less than 100% recovery of material from the extruder and the centrifuge tubes, and to incomplete sedimentation of the smaller liposomes. The amount of SOD activity incorporated into washed liposomes was 10 ± 4% of that initially added to lipid emulsions. From the average amount of SOD activity entrapped and the extent of lipid incorporation in liposome membranes, the content of SOD in liposomes was calculated to be 100 U/μmol lipid (Table 7). Liposomes were exposed to buffers of varying pH to determine the pH at which membrane destabilization occurred to induce release of contents. Liposomes having

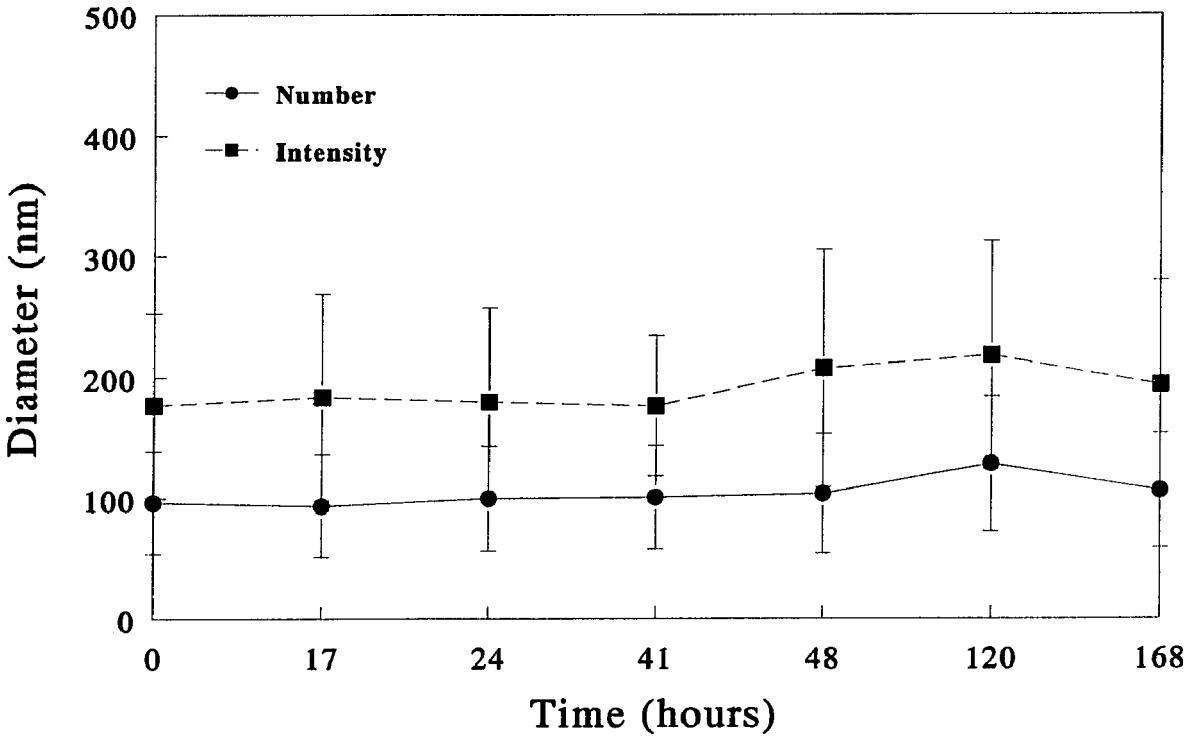


Figure 6. Size analysis of pH-sensitive liposomes over one week. The liposomes were kept at 4°C, and at each time point a small (2 μl) aliquot was removed and resuspended in 1 ml of buffer (7 mM HEPES, 0.21 M sucrose, pH 8.0). This was then analyzed using a Nicomp using number (filled circles) and intensity (filled squares) weighting for size analysis. The size of the liposomes did not significantly change over a period of one week.

Figure 7. Electron micrograph of pH-sensitive liposomes at pH 3. pH-sensitive liposomes were centrifuged one hour at 100,000 xg at 4°C. The resulting pellet was resuspended in HEPES/Sucrose, pH 3. The resulting liposome formation as seen in the electron microscope suggests that liposomes are becoming destabilized at low pH.

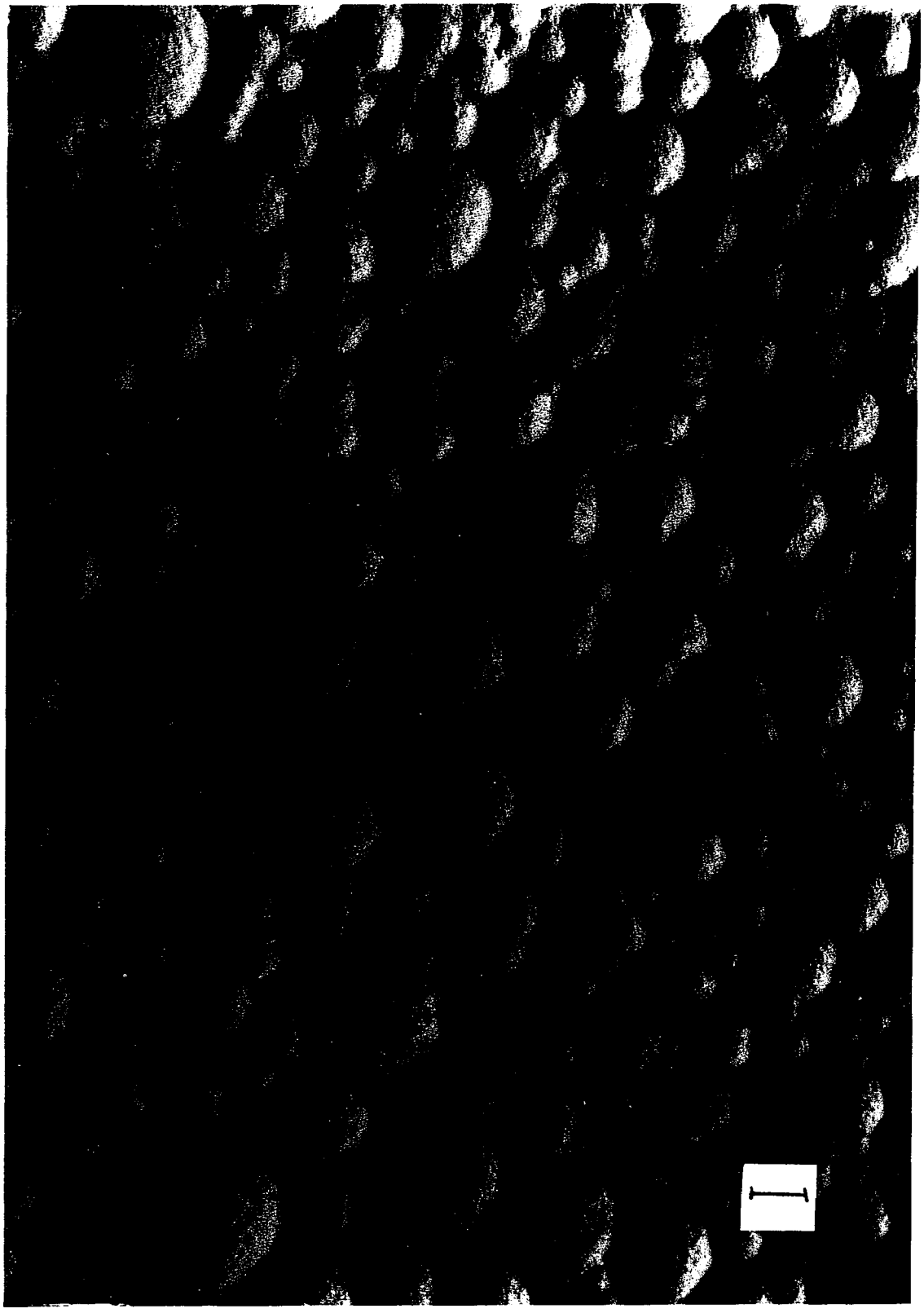

1

Table 7. Characteristics of pH-sensitive liposomes

Characteristic	Value
Mean diameter	200 ± 100 nm
Entrapment of SOD	10 ± 4%
U SOD/μmol lipid	100 U/μmol
Content release at 4° C over 24 hours	5%
Content release at 37° C over 24 hours	12%

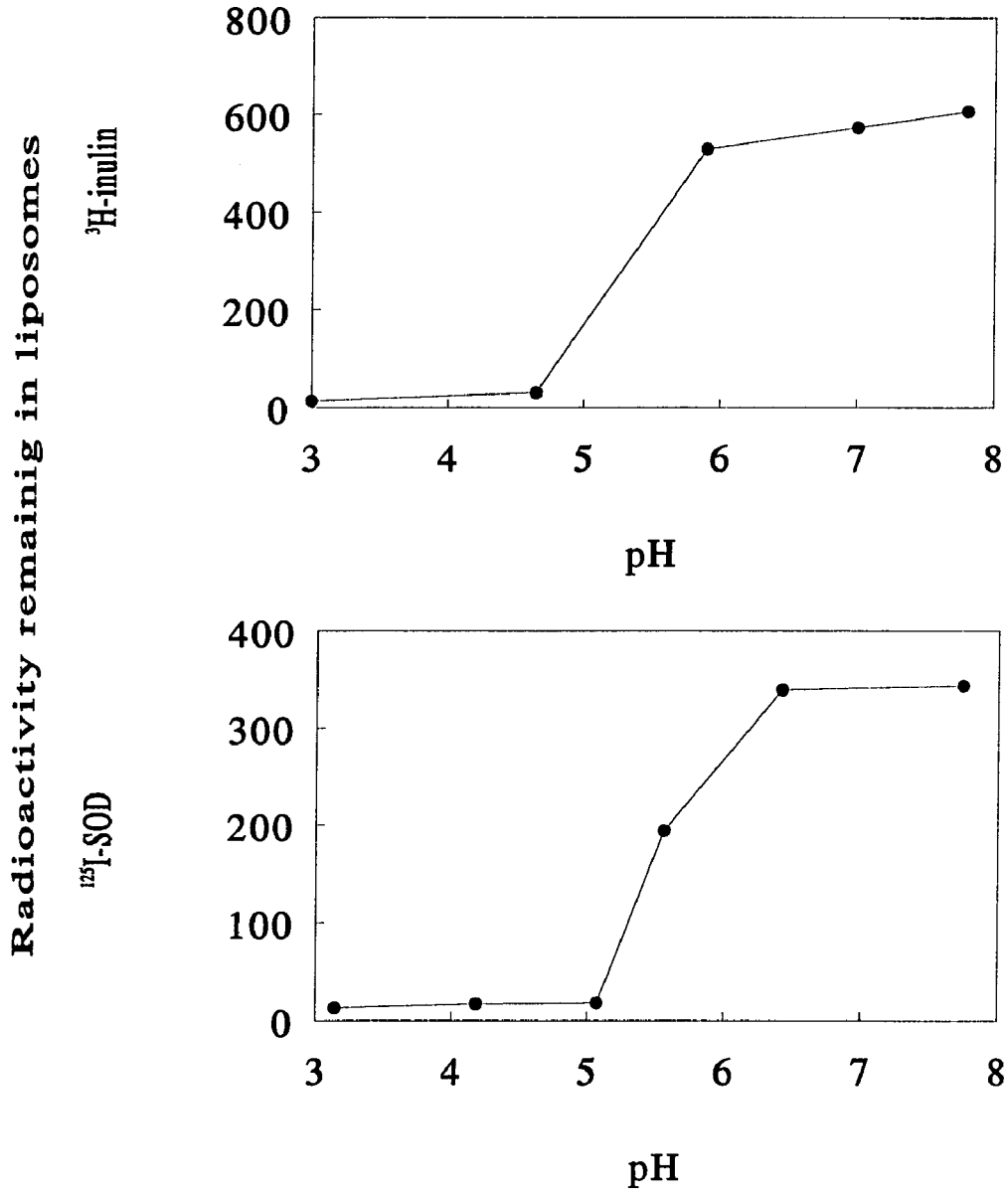
Mean diameter was determined by quasielastic light scattering and entrapment of SOD by superoxide dismutase enzymatic activity assay. Values are means ± S.D., n=3 for SOD entrapment.

entrapped bovine CuZn SOD and a mole ratio of DOPE to DOSG of 1:1 (Fig. 8, bottom) release intraliposomal contents between pH 5.5 and 4.5, similar to the pH-release profile of control liposomes having ^{3}H-inulin as an entrapped marker (Fig. 8, top). This analysis was conducted using liposomes having varying ratios of DOPE and DOSG until a compositional ratio resulting in destabilization near pH 5.0 was found.

Liposome Delivery to FRLE Cells

Liposomes prepared with entrapped SOD were administered to cultured lung epithelial cells up to a concentration of 800 nmol/cm^2 for 2 hr. Some cytotoxicity occurred at the very high doses of liposome, but no significant cytotoxicity was evident at the working concentration (400 nmol/cm^2), as determined by release of ^{14}C-adenine prelabel (Fig. 9). To determine the location where contents of pH-sensitive liposomes were delivered, the cells were first incubated one hour with the pH-sensitive liposomes having entrapped ^{125}I-SOD. All groups were washed 3 times before subsequent steps. One group was subjected to one minute of lowered pH (pH 4). This lowered pH should release the contents of the liposomes. Remaining detectable radioactivity of washed monolayers would represent that which is intracellular. The other cell group was incubated only in pH 7 media. If the radioactivity associated with pH 7 group is significantly higher, this would suggest that

Figure 8. Release of pH-sensitive liposome contents as a function of pH values. (Top) The release of ^{3}H-inulin from 1:1 molar DOPE:DOSG as determined by radioactivity in the eluent. (Bottom) The release of ^{125}I-SOD over a pH range of 3.2 to 7.8. Both pH-sensitive liposome preparations release most contents at pH between 5 and 6.5. Graphs are of representative experiments.


Radioactivity remainig in liposomes
^{3}H-inulin
800
600
400
200
0
3
4
5
6
7
8
pH
^{125}I-SOD
400
300
200
100
0
3
4
5
6
7
8
pH

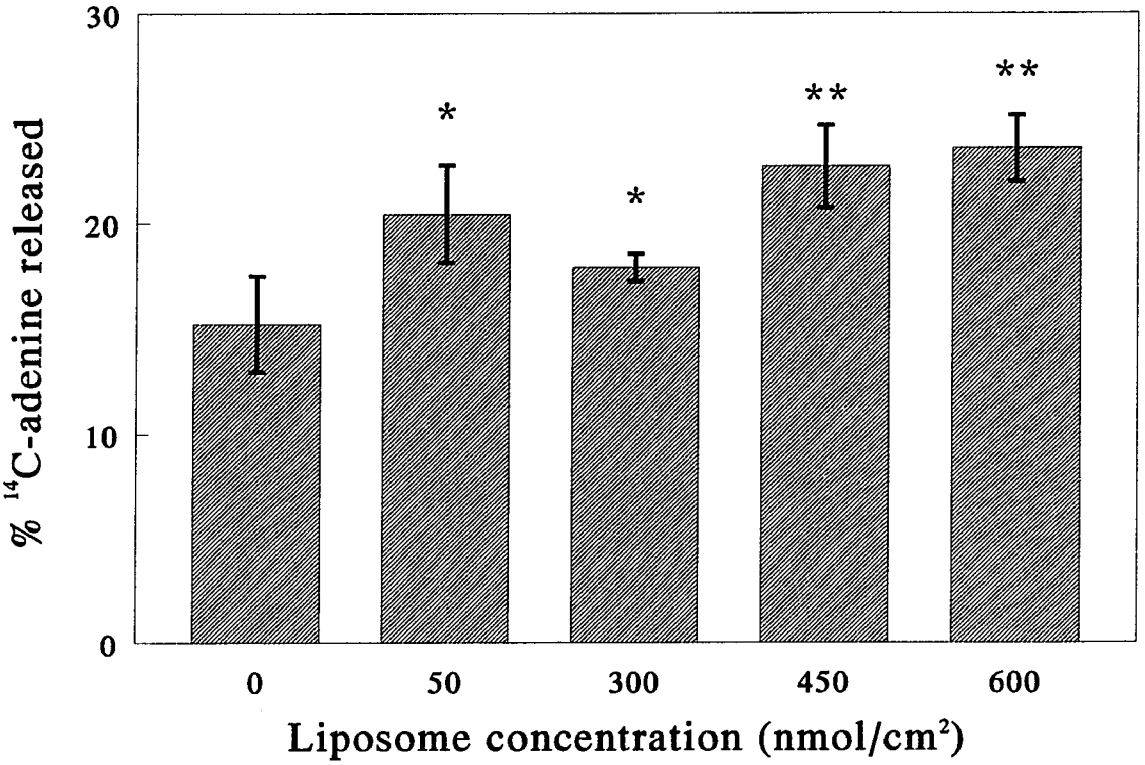


Figure 9. Effect of pH-sensitive liposomes on FRLE cells in culture. FRLE cells were incubated with ^{14}C-adenine for 2 hours, and then thoroughly rinsed with MEM. The indicated doses of liposomes were added for 2 hours, and the amount of radioactivity in media was compared to total radioactivity of cells plus media and reported as percent release of ^{14}C-adenine. pH-sensitive liposomes are slightly significantly toxic at the higher doses, as compared to control. Data represent the mean ± S.D., n=6, and one-way ANOVA performed with Student-Newman-Keuls post-hoc test, $p<0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.

there is a significant amount of extracellularly-associated liposomes. The media from these cells and the cell lysate were then counted radioactivity distribution. There was no significant difference between the groups, suggesting that cell-associated liposomes are intracellular (Fig. 10).

To enhance and target SOD delivery to specific lung cells, SP-A was incorporated into liposome suspensions before extrusion. As assayed by SOD catalytic activity, there was a significant increase in the amount of SOD delivered by SP-A-containing pH-sensitive liposomes (liposome concentration of 400 nmol/cm^2) (Fig. 11). There was a 5-fold increase (10 ± 2.5 U/mg to 50 ± 2 U/mg) in SOD activity following cell incubation with SP-A targeted liposomes (Fig. 11), and a 3-fold increase in activity following cell incubation with non-targeted pH-sensitive liposomes. When the amount of SOD delivered to cells was compared with the amount of added liposomal SOD, it was concluded that pH-sensitive liposomes delivered 3% of net contents and SP-A-targeted pH-sensitive liposomes delivered 5% of net contents.

The effect of collagen, a competitor for SP-A-receptor binding, on the delivery of SOD via pH-sensitive liposomes targeted with SP-A was determined. At 400 nmol liposomal lipid/cm^2 cells, there was significant attenuation of SOD delivery in the presence of Type IV collagen (100 μg/ml) from 52 ± 12 U/mg to 25 ± 6 U/mg (Fig. 12), similar to non-targeted SOD liposomes, indicating non-receptor enhanced delivery of liposomal SOD.

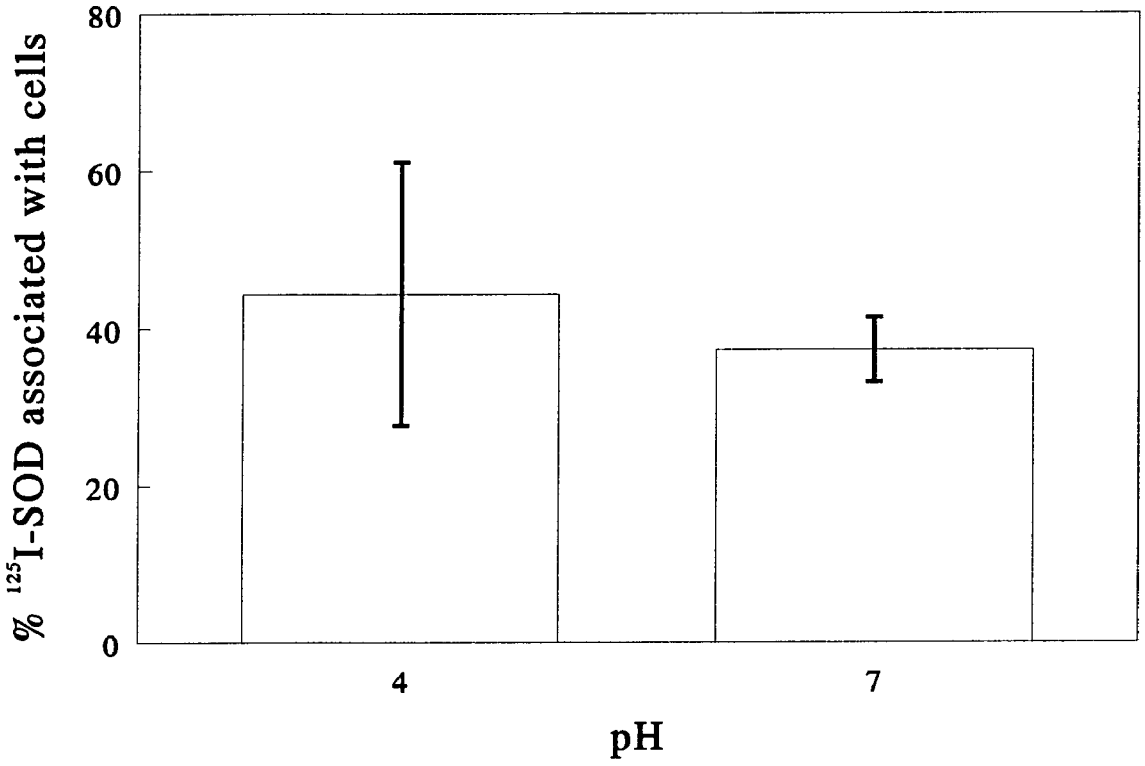


Figure 10. Cell-associated SOD at pH 7 and pH 4. FRLE cells in culture were incubated with 300 nmol/cm² pH-sensitive liposomes containing ^{125}I-SOD. After a one hour incubation, the cells were rinsed with pH 8.0 HBSS 3 times. The cells were then incubated 5 minutes in HBSS pH 7 or pH 4. The media and cells were then assayed using a gamma counter. The amounts of CPM associated with the cells at both pH values are not significantly different. Data represent mean ± S.D., n=3, and significance was evaluated using the t-test.

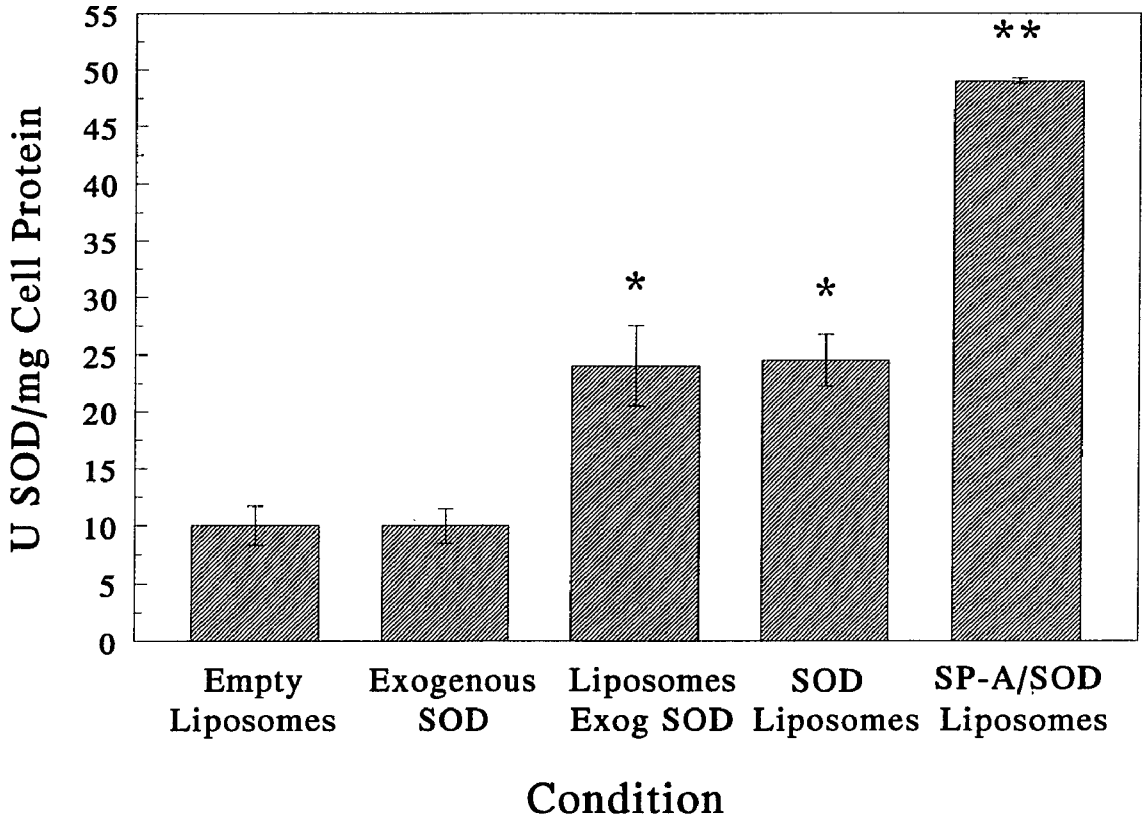


Figure 11. SOD activity delivered to FRLE cells. pH- sensitive liposomes with or without entrapped SOD were incubated with FRLE cells for one hour. Cells incubated with pH-sensitive liposomes containing buffer, or with exogenous SOD, had a background activity of 20 ± 5 U/mg cell protein. The addition of SOD containing pH-sensitive liposomes with or without exogenous SP-A (10 mg/ml) increased activity to 48 ± 9 U/mg SOD activity. However, the largest increase occurred when SP-A was added to the lipid before SOD, and before formation of liposomes through the extruder. Data were evaluated using ANOVA and Student-Newman-Keuls post-hoc test for significance. Data represent mean ± S.D., $n = 6$, $p \leq 0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.

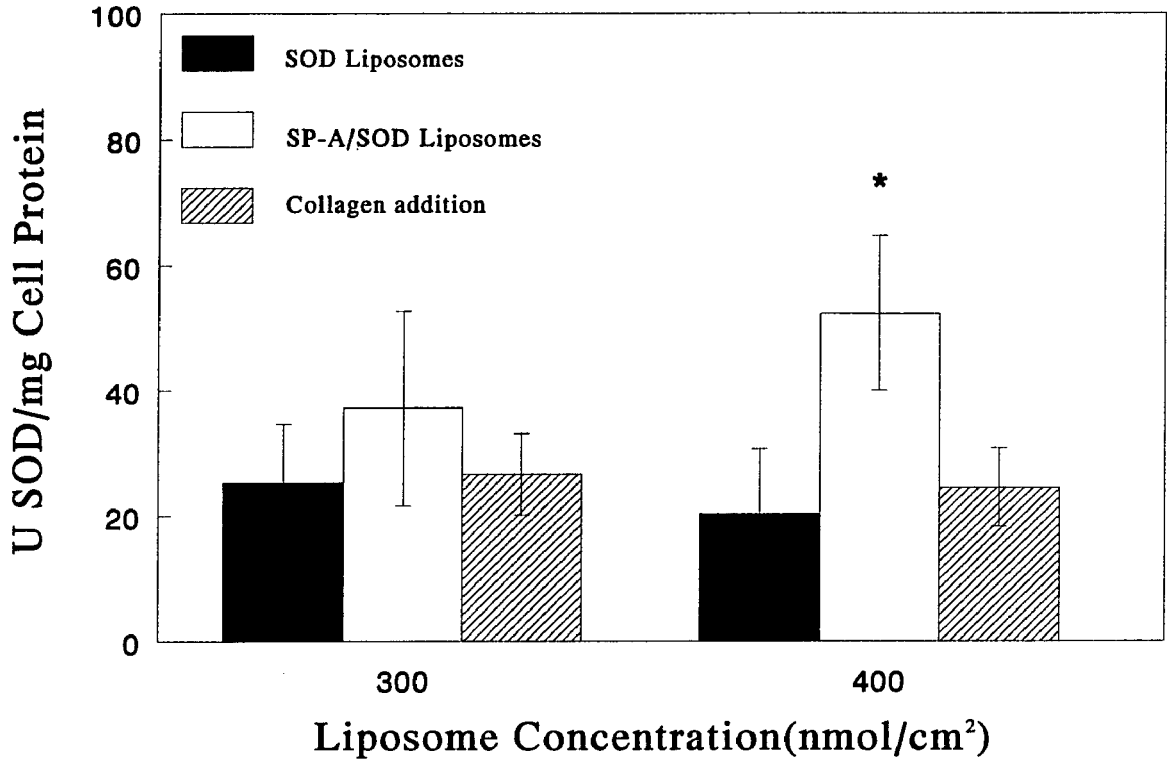


Figure 12. Inhibition of SP-A liposomal SOD cell uptake by collagen. Delivery of SOD was assessed by enzyme activity assay. At 400 nmol/cm^2 of liposome dose, 100 μg/ml collagen inhibited the targeted delivery of SOD to the cultured FRLE cells. The amount of delivery in the presence of collagen is not significantly different than control. Data were evaluated using ANOVA and Student-Newman-Keuls post-hoc test for significance. Data represent mean ± S.D., $n = 6$, $p \leq 0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.

As a prelude to experiments concerning the efficacy of antioxidant containing pH-sensitive liposomes as a drug delivery system which would protect pulmonary cells against oxidant stress, FRLE cells were incubated with liposomes, and then incubated with either 250 μM paraquat or xanthine and xanthine oxidase (X/XO). Xanthine oxidase converts xanthine into urate, and in the process forms H_2O_2 and $O_2^{\bullet -}$. This is a widely used oxidant generating system. Paraquat produces $O_2^{\bullet -}$ by becoming reduced by intracellular reductants and then autoxidizing by reducing O_2 to $O_2^{\bullet -}$ (Krall et al. 1988). The preincubation of cells with either empty liposomes or with SP-A targeted liposomes having entrapped SOD protects the cells against paraquat-induced oxidant stress (Fig. 13).

There is a XO-induced dose-dependent oxidant injury to cells (from 0.21 to 2.1 mU XO/cm^2) as determined by both detached cells and by release of ^{14}C-adenine prelabel (Fig. 14). This demonstrates a proportional correlation between a widely used approach of cytotoxicity determination (^{14}C-adenine release) and the number of monolayer-detached cells. When the cells were incubated with X/XO, cell injury was time dependent and continued to progress after the removal of the oxidant-generating system as determined by further detachment of cells (Fig. 15). Xanthine plus xanthine oxidase induced cell injury was decreased following preincubation of cells with pH-sensitive liposomes containing SOD, as indicated by a significant decrease in the

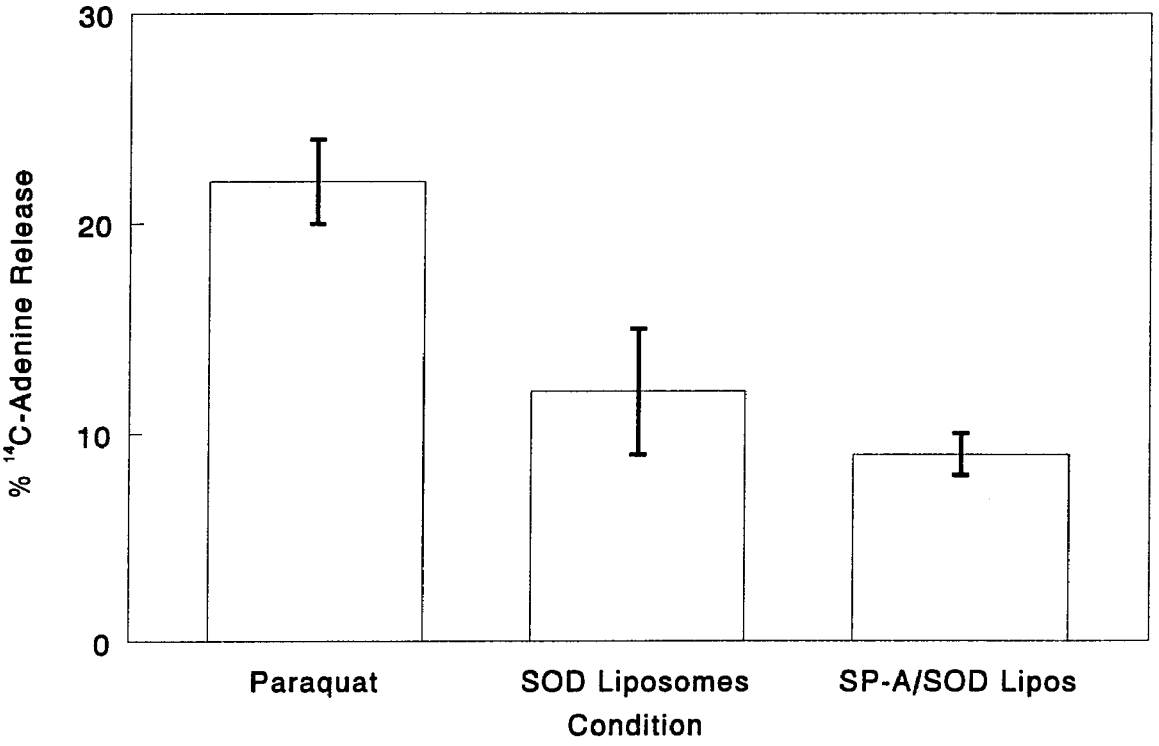
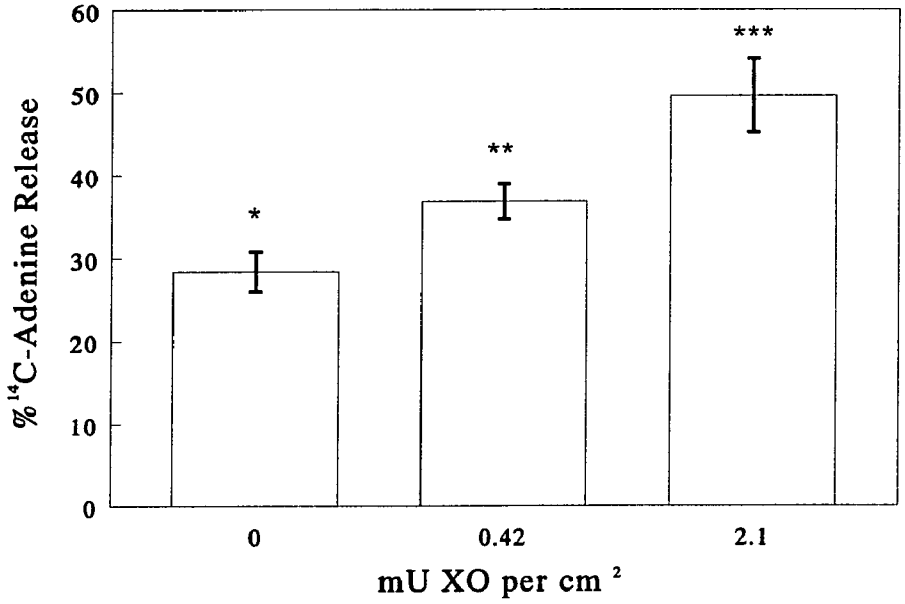
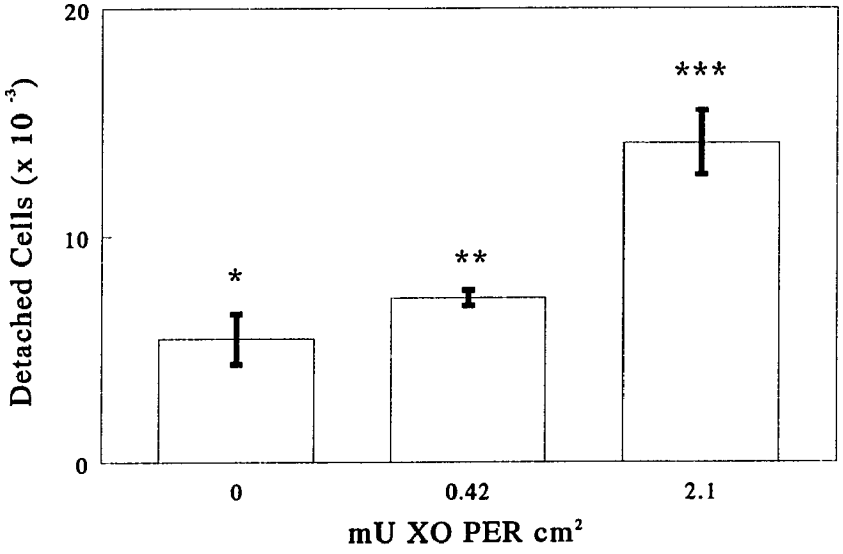


Figure 13. Inhibition of paraquat-induced FRLE cell oxidant injury by SOD-containing pH-sensitive liposomes. Cells were incubated with ^{14}C- adenine for 2 hours and, during the second hour, 400 nmol/cm^2 of indicated liposomes were added. The cells were washed, and exposed to 250 μM paraquat. The amount of ^{14}C-adenine released into the media after 4 hours was assayed. Control cells are cells that were given no liposomes, but were exposed to 250 μM paraquat; the blank liposomes group were cells exposed to paraquat and given pH-sensitive liposomes containing buffer; and the SP-A/SOD lipos group were cells exposed to paraquat and given pH-sensitive liposomes targeted with SP-A and containing SOD. Data were evaluated using ANOVA and Student-Newman-Keuls post-hoc test for significance. Data represent mean ± S.D., $n = 6$, $p \leq 0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.

Figure 14. Xanthine oxidase-induced oxidant injury to FRLE cells. (Top) Cytotoxicity of varying concentrations of xanthine oxidase as assayed by release of ^{14}C-adenine. These experiments were performed using cells cultured into 12-well plates. The indicated concentrations of xanthine oxidase were prepared and 1 ml was added along with 150 mM xanthine to each well. The counts were assayed after a 3 hr incubation. (Bottom) Same assay conditions as the ^{14}C-adenine release assay were used, except that the number of detached cells in the media was counted using a Coulter Counter. Both assays show a proportional increase with increase of oxidant stress. Data were evaluated using one-way ANOVA and Student-Newman-Keuls post-hoc test for significance. Data represent mean ± S.D., $n = 6$, $p \leq 0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.


60
50
40
30
20
10
0
% 14C-Adenine Release
*
**
***
0
0.42
2.1
mU XO per cm 2


20
10
0
Detached Cells (x 10 -3)
*
**
***
0
0.42
2.1
mU XO PER cm2

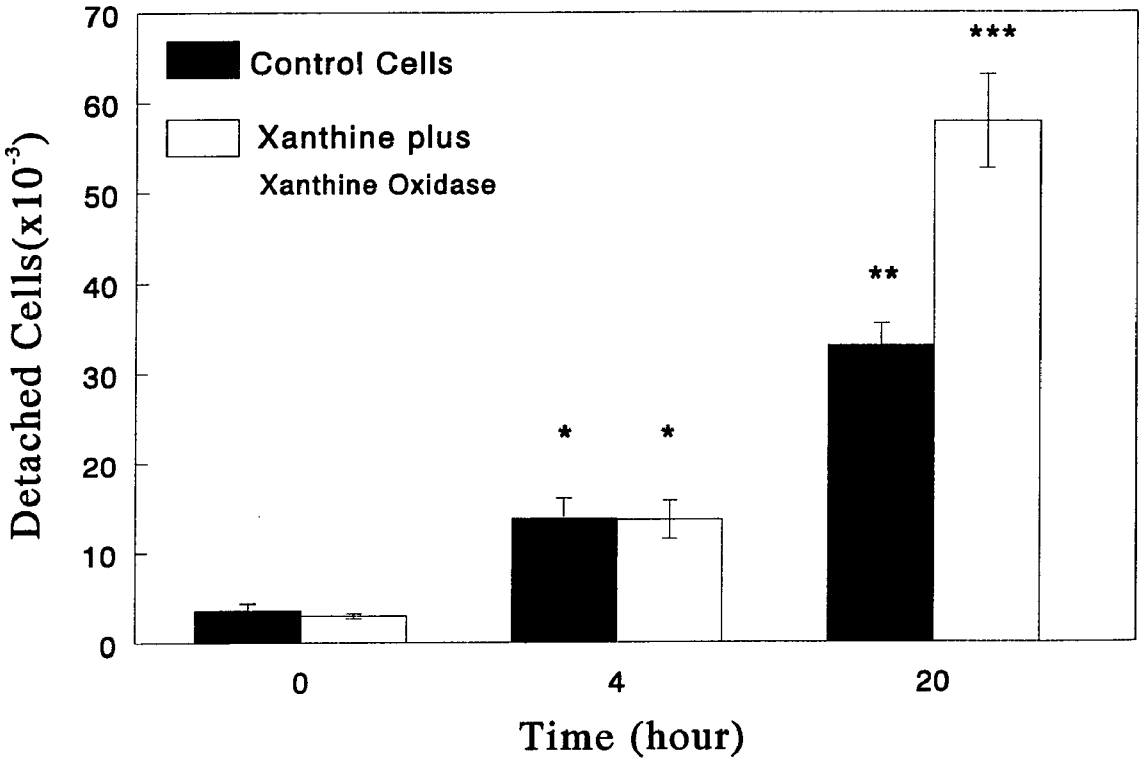


Figure 15. Time course of XO induced FRLE injury. Control cells (solid bars) show some detachment over a 20 hour period. The xanthine oxidase treated cells (open bars) were treated with 0.41 mU XO and 150 μM xanthine per well for 2 hours. Immediately after the xanthine oxidase was rinsed off, 1 ml of media was put in each well. The control is the number of detached cells observed after rinsing the xanthine and xanthine oxidase off the cells. All subsequent damage seen is after the removal of xanthine oxidase. There is a significant increase in the number of detached cells for both the control and the treated cells at 4 hours, but at 20 hours the treated cells show significantly more damage than the 20-hour control cells and both groups at 4 hours. Data were evaluated using one-way ANOVA and Student-Newman-Keuls post-hoc test for significance. Data represent mean ± S.D., $n = 6$, $p \leq 0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.

number of detached cells (Fig. 16). These are interesting observations that can be furthered studied with other oxidant stress models.

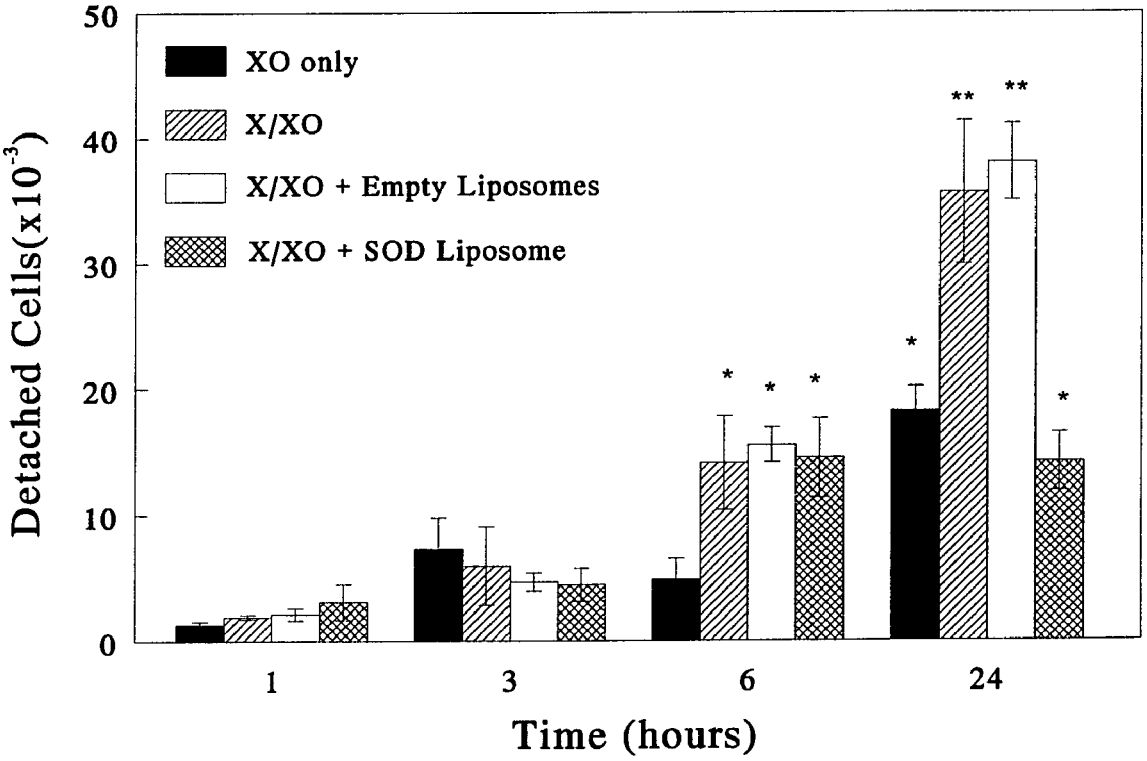


Figure 16. Attenuation of XO-induced FRLE injury by SOD-containing liposomes. Control cells (solid bars) were incubated with 0.41 mU XO per well for 2 hours and rinsed, and DMEM with 10% FCS was added for the remainder of the experiment. The xanthine oxidase group (hatched bars) are cells treated with 0.41 mU XO and 150 μM xanthine for 2 hours and rinsed, and DMEM with 10% FCS added. The empty liposome group (open bars) are cells pretreated for 1 hour with pH-sensitive liposomes containing buffer before treatment with xanthine and xanthine oxidase as described for the xanthine oxidase group. The SOD liposome group (cross-hatched bars) are cells pretreated for 1 hour with SP-A pH-sensitive liposomes containing SOD before subsequent exposure to xanthine and xanthine oxidase as described for the xanthine oxidase group. There is no significant damage seen with any group until 6 hours. The experimental protocol is the same as for figure 14. At 24 hours, cells treated with SOD are similar to control cells, whereas cells treated with xanthine oxidase with or without empty liposomes show significant damage compared to the 24 hour control and SOD treated cells. Data were evaluated using two-way ANOVA and Student-Newman-Keuls post-hoc test for significance. Data represent mean ± S.D., $n = 6$, $p \leq 0.05$. Data with the same number of asterisks are not significantly different from each other, but are significantly different from the data with a differing number of asterisks.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

The lung is critical for providing gas exchange of O_2 and carbon dioxide (CO_2) between the atmosphere and the red blood cells of the body, with the alveolar sacs of the lung providing the air-blood gas exchange surface. To facilitate gas exchange and the dynamic expansion of alveoli, the cells of the lung produce a phospholipid-enriched surfactant which lowers the surface tension of the lung to maintain alveolar expansion at end expiration.

When premature infants are delivered, they frequently have inadequate O_2/CO_2 exchange at the air-blood barrier of the lung. Thus, hyperoxic ventilation is required to maintain adequate arterial oxygen tensions or tissues will not receive an adequate supply of O_2 . If prematurity is severe or the requirement for hyperoxic O_2 lasts for more than 3 weeks, a fibrotic lung disease termed BPD often results (Northway 1992b), presumably due to the immaturity of the lung and often long exposures to hyperoxia. The intracellular concentrations of antioxidant enzymes in the premature lung are low compared to the fully developed lung of the term infant, placing additional oxidant risk on neonatal lung cells (Tanswell et al. 1989, Tanswell and

Freeman 1984b). When premature infants are given elevated oxygen levels to adequately oxygenate tissues, intracellular rates of ROS production increase, which causes an imbalance of over production of ROS compared to antioxidant defenses in the lung (Freeman and Tanswell 1985, Freeman and Crapo 1981). Damage of type I cells, type II cells, fibroblasts, and basement membrane occurs due to this increase of ROS production, which in turn causes an increase in the number of activated alveolar macrophages. Activated macrophages produce high amounts of $O_2^{\cdot-}$ (Kinnula et al. 1991), thereby contributing further to the lung damage associated with BPD.

A recently developed *in vitro* model for studying the effects of oxidant stress and delivery of antioxidants to the premature infant lung is the primary culture of fetal rat lung epithelial cells (FRLE cells) (Tanswell and Freeman 1984a). This model has advantages over using adult type II cells because FRLE cells proliferate in culture whereas adult type II cells do not. This proliferative ability makes them more amenable to *in vitro* studies. Finally, FRLE cells are a more appropriate model for work related to the premature infant lung. Using FRLE cells in culture, the determination of the oxidant-protective effects of SOD delivered by liposomes was a final goal. Before any studies of oxidant stress and delivery of SOD to cells could be accomplished, reagent development and characterization of model systems had to be performed. The procedure by which pH-sensitive liposomes were made had to be

established and the optimum conditions for liposomal enzyme delivery to cells and the methods for producing oxidant stress *in vitro* had to be developed.

At the outset of this dissertation research, significant room existed for improving liposome vehicles for antioxidant delivery studies. Liposomes used for previous antioxidant enzyme delivery studies (Buckley, Tanswell, and Freeman 1987) were synthesized by reverse phase evaporation, which involves subjecting the protein or drug to be entrapped to the denaturing effects of organic solvents. These liposomes also enhance the possibility of *in vivo* and *in vitro* exposure to residual concentrations of these organic solvents, thus potentially causing undesirable damage to tissue. With reverse phase evaporation techniques, liposomes tended to aggregate very quickly because of possible liposomal membrane interactions with exposed hydrophobic regions of denatured proteins. Additionally, there is little control over the size distribution of the liposomes made by reverse phase evaporation, making them inappropriate for therapeutic use, especially in intravenous routes of administration.

The use of pH-sensitive liposomes bypasses these problems (Torchilin, Zhou, and Huang 1993). Several types of pH-sensitive liposomes have been shown to deliver a variety of materials to the cytosol of cells (Hayashibe et al. 1990, Chu et al. 1990, Conner, Norley, and Huang 1986) and to have an increased circulating half-life (Collins, Litzinger, and Huang 1990, Liu and Huang 1990). Ammonium chloride or

EDTA blocks macromolecule delivery to cells, suggesting the process requires a low-pH environment and the presence of divalent cations. In the present experiments, pH-sensitive liposomes were used and further defined. The micro-filter pressure-extruded pH-sensitive liposomes were composed of DOPE and DOSG, were stable, unilamellar and homogenous in size, and had no residual organic solvent in final preparations.

In the studies discussed herein, the liposome-entrapped antioxidant of choice was CuZn SOD. A technique using 3-5 cycles of freezing and thawing of liposomes has been shown to increase drug entrapment (Mayer et al. 1985). However, freeze-thawing of SOD with the lipids used herein for pH-sensitive liposomes greatly decreased SOD activity, so the freeze-thawing process was bypassed during the manufacture of liposomes. This explains the lower percent entrapment of SOD in these studies compared to other reported liposomal macromolecule entrapment efficiencies. Since the SOD is exposed to a pH range of 8.5 during liposome preparation to pH 5.0 following endosomal uptake of liposomal SOD, the matter of SOD stability in these environments was considered. Superoxide dismutase is physically stable and retains activity in the pH range of 5.0 to 9.5 (Forman and Fridovich 1973). However, when the SOD is released into the cytosol, it is once again in physiologic pH.

Another step in refining the liposomes used in this study was to employ a cell-specific targeting molecule. Because it has been shown in other studies (Conner, Norley, and Huang 1986) that targeting of liposomes increases efficiency of delivery, and that it has been suggested that adult rat type II cells exhibit a SP-A receptor (Wright, Borchelt, and Hawgood 1989), it was determined whether there were SP-A receptors on the FRLE cells. The experiments described herein suggest that there is a specific receptor for SP-A on FRLE cells, with a $K_d = 4 \pm 0.2$ μg/ml and capacity of 130 ± 3 ng/10^6 cells. This is a novel report observation reporting the existence of SP-A receptors on FRLE cells.

After it was suggested that these cells expressed a receptor for SP-A, pH-sensitive liposomes were produced with SP-A. Surfactant protein A has been shown to interact with phospholipid vesicles, causing formation of MLV's, and exhibit as particulate formations on the outer face of the liposomal membranes (Williams, Hawgood, and Hamilton 1991). This strongly suggests that SP-A becomes membrane bound and orients itself to the outside of the vesicle. Consequently, pH-sensitive liposomes were made with SP-A to increase efficiency of delivery to FRLE cells by allowing possible targeting of the liposomes by SP-A interaction with SP-A receptors. Also, SP-A increases phospholipid uptake by pulmonary type II cells (Young, Wright, and Clements 1989, Wright et al. 1987), and it has been postulated that the route of type II

cell uptake of SP-A and phospholipid is by endocytosis (Fisher, Dodia, and Chander 1991). This suggests that there may be several routes of pH-sensitive liposome delivery including receptor-mediated endocytosis for targeted liposomes, non-specific endocytosis, and destabilization of liposomes with the cell plasma membrane or interstitial matrix in microenvironments of low pH. Surfactant protein A may also target SP-A-pH-sensitive liposomes for the endosomal compartment of the type II cell.

SP-A targeted liposomal delivery of SOD to FRLE cells was compared to non-targeted liposomes. Non-targeted pH-sensitive liposomes delivered 2% of their SOD to FRLE cells in culture, with incorporation of SP-A into the liposome increasing delivery of pH-sensitive liposome-entrapped SOD to 5% . Further testing of the targeting of these liposomes could be accomplished by using cells that do not have a receptor for SP-A and then determining whether the SP-A targeted liposomes deliver more efficiently than non-targeted pH-sensitive liposomes as well.

The *in vitro* studies described herein suggest that SP-A targets liposomes to FRLE cells. However, macrophages have also been shown to have SP-A receptors (Pison, Wright, and Hawgood 1992). Therefore, *in vivo*, the delivery of SP-A liposomes would increase the efficiency of delivery to type II cells and macrophages. In the case of SOD, this could prove to be beneficial, considering that in hyperoxic lung injury, a major contributor to lung injury is the oxidant production by macrophages.

After the establishment of an *in vitro* model and a liposome delivery protocol, the utility of delivering pH-sensitive liposome-entrapped SOD to FRLE cells as an approach to lend resistance to oxidant stress was tested. Two types of oxidant stress were used: an extracellular source of $O_2^{\cdot-}$ produced by xanthine oxidase, and an intracellular source produced by paraquat. Xanthine oxidase produces $O_2^{\cdot-}$ as it oxidizes purines to uric acid, and paraquat increases the intracellular production of $O_2^{\cdot-}$ (Krall et al. 1988). Only delivery of targeted liposomes containing SOD protected cells against the extracellular source of $O_2^{\cdot-}$, whereas for intracellular stimulation of $O_2^{\cdot-}$ production by paraquat, both buffer-containing and SOD-containing liposes protected the cells, though SOD still protected FRLE cell better than liposomes alone. An explanation for this observation could be that the lipids used in the pH-sensitive liposomes have only one olefin, making them less susceptible than polyunsaturated fatty acids to peroxidation and lipid peroxidation propagation reactions. Because the liposomes become cell-associated predominantly by endocytosis, the concentration of DOPE and DOSG will be transiently higher in the cellular membrane systems. Perhaps this is why liposomes without SOD protect the cells from paraquat, which causes intracellular lipid peroxidation (Sandy et al. 1986), whereas they do not protect the cells from $O_2^{\cdot-}$ produced by extracellular XO. Xanthine oxidase-derived $O_2^{\cdot-}$ can readily diffuse across the membrane, but does not diffuse far enough into the cell to

affect intracellular membranes. Since SOD is cytosolically located, it can protect oxidant sensitive proteins from the extracellularly produced $O_2^{\cdot-}$, and can provide protection for the intracellular membranes, though to a lesser degree than the addition of lipids that are not easily oxidized.

Intracellular transport of antioxidant macromolecules by pH-sensitive liposomes can be beneficial because of the cytosolic location of mitochondrial and autoxidizable small molecules and other enzymatic or autoxidizable protein sources of ROS. Liposomal delivery *in vivo* could be targeted by SP-A to SP-A receptors on alveolar macrophages and type II cells. Also, *in vivo* delivery of pH-sensitive liposome entrapped antioxidant proteins may occur extracellularly to a small extent (White et al. 1994) because of the low pH microenvironment near the cell plasma membrane (Cevc 1990) and basement membrane compartments. This microenvironment could induce liposome destabilization and release of antioxidant enzymes, thus possibly protecting the membrane of the cell and the basement membrane from extracellularly-generated oxygen radicals as well. The SP-A targeted pH-sensitive liposomes containing SOD have been shown herein to have protective effects towards cells.

In summary, the data shown herein support the hypothesis of this dissertation that the delivery of SOD by pH-sensitive liposomes to cultured epithelial cells attenuates damage caused by an oxidant-generating system. The data indicate that: (1) there may

be a receptor for SP-A on FRLE cells, (2) that SP-A targeted liposomes have a higher efficiency of delivery than non-pH-sensitive liposomes, (3) that delivery of SP-A targeted liposomes is inhibited by collagen, (4) that this delivery lowers 14C-adenine release of paraquat treated cells, and (5) this delivery lowers detachment of xanthine oxidase treated cells.

Based on the forgoing summary, the following conclusions have been drawn: (1) FRLE cells in culture are a viable *in vitro* model for studying lung physiology and pathology of the neonate, (2) FRLE cells seem to have a receptor for SP-A which facilitates delivery of SP-A targeted pH-sensitive liposomes, (3) delivery of SOD by SP-A targeted liposomes to cells is indicated to be protective against oxidant stress, and (4) SP-A targeted pH-sensitive liposomes that contain SOD may be useful in the clinical setting in attenuating lung injuries due to hyperoxia. Future experiments should now be designed to test practical clinical applications of targeted pH-sensitive liposomes, e.g. protection of adults with impaired lung function who are maintained with mechanical ventilation and hyperoxic support as well as premature infants at risk for BPD.

REFERENCES

Bray, R. C., S. Cockle, E. M. Fielden, P. B. Roberts, G. Rotilio, and L. Calabrese. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. *Biochem. J.* 139: 43-48, 1974.

Buckley, B. J., A. K. Tanswell, and B. A. Freeman. Liposome-mediated augmentation of catalase in alveolar type II cells protects against H_2O_2 injury. *J. Appl. Physiol.* 63: 359-367, 1987.

Burri, P. H. Postnatal development and growth. The Lung: Scientific Foundations, ed. R. G. Crystal, J. B. West, et al., New York, Raven Press, Ltd., pp. 677-687, 1991.

Cevc, G. Membrane electrostatics. *Biochim. Biophys. Acta.* 1031-1033: 311-382, 1990.

Chiu, D., B. Lubin, and S. B. Shohet. Peroxidative reactions in red cell biology. Free Radicals in Biology, ed. William A. Pryor, Academic Press, Inc. Vol V: 115-160, 1984.

Chu, C-J, J. Dijkstra, M-Z Lai, K. Hong, and F. C. Szoka. Efficiency of cytoplasmic delivery by pH- sensitive liposomes to cells in culture. *Pharm. Res.* 7: 824-834, 1990.

Clement, A., M. Edeas, K. Chadelat, and J. S. Brody. Inhibition of lung epithelial cell proliferation by hyperoxia: posttranscriptional regulation of proliferation-related genes. *J. Clin. Invest.* 90: 1812-1818, 1992.

Cohen, G. The fenton reaction. CRC Handbook of Methods for Oxygen Radical Research, ed. Robert A. Green, CRC Press, Inc., Boca Raton, FL, pp. 55-64, 1985.

Collins, D., D. C. Litzinger, and L. Huang. Structural and functional comparisons of pH-sensitive liposomes composed of phosphatidylethanolamine and three different diacylsuccinylglycerols. *Biochem. Biophys. Acta.* 1025: 234-242, 1990.

Conner J., N. Norley, and L. Huang. Biodistribution of pH-sensitive immunoliposomes. *Biochem. Biophys. Acta.* 884: 474-481, 1986.

DiGuiseppi, J. and I. Fridovich. The toxicology of molecular oxygen. *CRC Clin. Rev. Tox.* 12: 315-342, 1986.

Doleman, C. J. A. and A. Bast. Oxygen radicals in lung pathology. *Free Rad. Biol. Med.* 9: 381-400, 1990.

Elstner, E. F. and W. Oswald. Air pollution: involvement of oxygen radicals (a minireview). *Free Rad. Res. Comm.* 12-13: 795-807, 1991.

Fahey, R. C. and A. R. Sundquist. Evolution of glutathione metabolism. *Adv. Enz.* 64: 1-53, 1991.

Fisher, A. B., C. Dodia, and A. Chander. Alveolar uptake of lipid and protein components of surfactant. *Am. J. Physiol.* 261: L334-L340, 1991.

Fiszar-Szafarz, S. B., D. Szafarz, and G. de Murillo. A general, fast, and sensitive micromethod for DNA determination application to rat and mouse liver, rat hepatoma, human leukocytes, chicken fibroblasts, and yeast cells. *Anal. Biochem.* 110: 165-170, 1981.

Flohe, L. Glutathione brought into focus. Free Radicals in Biology, ed. William A. Pryor, Academic Press, Inc. Vol V: 223-254, 1984.

Forman, H. J. and A. Boveris. Superoxide radical and hydrogen peroxide in mitochondria. Free Radicals in Biology, ed., William A. Pryor, Academic Press, Inc., Vol. V: 65-90, 1984.

Forman, H. J. and I. Fridovich. Superoxide dismutase: a comparison of rate constants. *Arch. Biochem. Biophys.* 158: 396-400, 1973.

Freeman, B. A. and J. D. Crapo. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J. Biol. Chem.* 256: 10986-10992, 1981.

Freeman, B. A. and J. D. Crapo. Biology of disease: free radicals and tissue injury. *Lab. Invest.* 47: 412-426, 1982.

Freeman, B. A. and A. K. Tanswell. Biochemical and cellular aspects of pulmonary oxygen toxicity. *Adv. Free Rad. Biol. Med.* 1: 133-164, 1985.

Freeman, B. A., M. K. Topolosky, and J. D. Crapo. Hyperoxia increases oxygen radical production in rat lung homogenates. *Arch. Biochem. Biophys.* 216: 477-484, 1982.

Fridovich, I. Superoxide radical: an endogenous toxicant. *Ann. Rev. Pharm. Tox.* 23: 239-257, 1983.

Fridovich, I. Overview: biological sources of $O_2\cdot^-$. *Meth. Enz.* 105: 59-61, 1984. a.

Fridovich, I. Oxygen radicals, hydrogen peroxide, and oxygen toxicity. Free Radicals in Biology, ed. William A. Pryor, Academic Press, Inc. Vol I: 239-277, 1984. b.

Fridovich, I. Biological effects of the superoxide radical. *Arch. Biochem. Biophys.* 247: 1-11, 1986.

Fridovich, I. Superoxide dismutases: an adaptation to a paramagnetic gas. *J. Biol. Chem.* 264: 7761-6674, 1989.

Gregoriadis, G. and A. T. Florence. Liposomes in drug delivery: clinical, diagnostic and ophthalmic potential. *Drugs.* 45: 15-28, 1993.

Grisham, M. B., J. M. McCord, and J. T. Sylvester. Chemistry and cytotoxicity of reactive oxygen metabolites. Physiology of Oxygen Radicals, eds. A. E. Taylor, S. Matalon, P. A. Ward. American Physiological Society, pp. 1-18, 1986.

Halliwell, B., J. M. C. Gutteridge, and C. E. Cross. Free radicals, antioxidants, and human disease: where are we now? *J. Lab. Clin. Med.* 119: 598-620, 1992.

Hayashibe, H, K. Asayama, K. Dobashi, and K. Kato. Prenatal development of antioxidant enzymes in rat lung, kidney, and heart: marked increase in immunoreactive superoxide dismutases, glutathione peroxidase, and catalase in the kidney. *Ped. Res.* 27: 472-475, 1990.

Imlay, J. A. and L. Stuart. DNA damage and oxygen radical toxicity. *Science.* 240: 1302-1309, 1988.

Jassal, D., R. N. Han, I. Caniggia, M. Post, and A. K. Tanswell. Growth of distal fetal rat lung epithelial cells in a defined serum-free medium. *In Vitro Cell Dev. Biol.* 27A: 625-632, 1991.

Kinnula, V. L., J. I. Everitt, A. R., Whorton, and J. D. Crapo. Hydrogen peroxide production by alveolar type II cells, alveolar macrophages, and endothelial cells. *Am. J. Physiol.* 261: L84-L91, 1991.

Klimov, A. N., V. S. Gurevich, A. A. Nikiforova, L. V. Shatilina, A. A. Kuzmin, S. L. Plavinsky, and N. P. Teryukova. Antioxidative activity of high density lipoproteins *in vivo*. *Atherosclerosis.* 100: 13-18, 1993.

Klotz, I. M. Numbers of receptor sites from Scatchard graphs: facts and fantasies. *Science.* 217: 1247-1249, 1982.

Kobayashi, Y., S. Okahata, T. Kyoji, and T. Usui. Use of logit paper in determination of superoxide dismutase activity in human blood cells. *J. Immunol. Meth.* 24: 75- 78, 1978.

Kono, Y., and I. Fridovich. Superoxide radical inhibits catalase. *J. Biol. Chem.* 257: 5751-5754, 1982.

Krall, J., A. C. Bagley, G. T. Mullenbach, R. A. Hallewell, and R. E. Lynch. Superoxide mediates the toxicity of paraquat for cultured mammalian cells. *J. Biol. Chem.*263: 1910-1914, 1988.

Krinsky, N. I. Mechanism of action of biological antioxidants. *P. S. B. E. M.* 200: 248-254, 1992.

Kuroki, Y., R. J. Mason, and D. R. Voelker. Alveolar type II cells express a high-affinity receptor for pulmonary surfactant protein A. *Proc. Nat. Acad. Sci. (USA).* 85: 5566-5570, 1988.

Kwong, M. S., E. A. Egan, R. H. Notter, and D. L. Shapiro. Double-blind clinical trials of calf lung surfactant extract for the prevention of hyaline membrane disease in extremely premature infants. *Pediatrics.* 76: 585-592, 1985.

Liu, D and Huang, L. pH-sensitive, plasma-stable liposomes with relatively prolonged residence in circulation. *Biochim. Biophys. Acta.* 1022: 348-354, 1990.

Mao, G. D., P. D. Thomas, G. D. Lopaschuk, and M. J. Poznasky. Superoxide dismutase (SOD)-catalase conjugates. *J. Biol. Chem.* 268: 416-420, 1993.

Matalon, S., and E. A. Egan. Effects of 100% O_2 breathing on permeability of alveolar epithelium to solute. *J. Appl. Physiol.* 50: 859-863, 1981.

Mayer, L. D., M. J. Hope, P. R. Cullis, and A. S. Janoff. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta.* 817: 193-196, 1985.

McCord, J. M. and I. Fridovich. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049-6055, 1969.

Menzel, D. B. The role of free radicals in the toxicity of air pollutants (nitrogen oxides and ozone). Free Radicals in Biology, ed. William A. Pryor, Academic Press, Inc. Vol II: 181-202, 1984.

Merritt, T. A., M. Hallman, B. Bloom, C. Berry, K. Benirschke, D. Sahn, T. Key, D. Edwars, A-L. Jarvenpaa, et al. Prophylactic treatment of very premature infants with human surfactant. *New Eng. J. Med.* 315: 785-790, 1986.

Morley, C. J. Surfactant treatment for premature babies - a review of clinical trials. *Arch. Dis. Child.* 66: 445-450, 1991.

Mudd, J. B. The role of free radicals in the toxicity of air pollutants (peroxyacylnitrates). Free Radicals in Biology, ed. William A. Pryor, Academic Press, Inc. Vol II: 1984.

Muller, F. Flavin radicals: chemistry and biochemistry. *Free Rad. Biol. Med.* 3: 215-230, 1987.

Northway, W. H., Jr. An introduction to bronchopulmonary dysplasia. *Clin. Perinatology.* 19: 489-495, 1992a.

Northway, W. H., Jr. Bronchopulmonary dysplasia: twenty-five years later. *Pediatrics.* 89: 969-973, 1992b.

O'Brodovich, H. M. and R. B. Mellins. Bronchopulmonary dysplasia. Unresolved neonatal acute lung injury. *Am. Rev. Resp. Dis.* 132: 694-789, 1985.

Olszewski, A. J. and K. S. McCully. Homocysteine metabolism and the oxidative modification of proteins and lipids. *Free Rad. Biol. Med.* 11: 683-693, 1993.

Omar, B. A., S. C. Flores, and J. M. McCord. Superoxide dismutase: pharmacological developments and applications. *Adv. Pharm.* 23: 109-161, 1992.

Omar, B. A., N. M. Gad, M. C. Jordan, S. P. Striplin, W. J. Russell, J. M. Downey, and J. M. McCord. Cardioprotection by Cu,Zn-superoxide dismutase is lost at high doses in the reoxygenated heart. *Free Rad. Biol. Med.* 9: 465-471, 1990.

Palamada, J. R., and J. P. Kehrer. Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione. *Lipids.* 28: 427-431, 1993.

Panus, P. C., S. A. Wright, P. H. Chumley, R. Radi, and B. A. Freeman. The contribution of vascular endothelial xanthine dehydrogenase/oxidase to oxygen-mediated cell injury. Arch. Biochem. Biophys. 294: 695-702, 1992.

Pison, U., J. R. Wright, and S. Hawgood. Specific binding of surfactant apoprotein SP-A to rat alveolar macrophages. *Am. J. Physiol.* 262: L412-L417, 1992.

Radi, R., J. F. Turrens, L. Y. Chang, K. M. Bush, J. D. Crapo, and B. A. Freeman. Detection of catalase in rat heart mitochondria. *J. Biol. Chem.* 266: 22028-22034, 1991.

Reilly, P. M., H. J. Schiller, and G. B. Bulkley. Pharmacologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites. *Am. J. Surg.* 161: 488-503, 1991.

Sandy, M. S., P. Moldeus, D. Ross, and M. T. Smith. Role of redox cycling and lipid peroxidation in bipyridyl herbicide toxicity. Studies with a compromised isolated hepatocytes model. *Biochem. Pharm.* 35: 3096-3101, 1986.

Shirhatti, V. and G. Krishna. A simple and sensitive method for monitoring drug-induced cell injury in cultured cells. *Anal. Biochem.* 147: 410-418, 1985.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150: 76-85, 1985.

Southal, D. P. and M. P. Samuels. Bronchopulmonary dysplasia: a new look at management. *Arch. Dis. Child.* 65: 1089-1095, 1990.

Stark, G. The effect of ionizing radiation on lipid membranes. *Biochim. Biophys. Acta.* 1071: 103-122, 1991.

Tanswell, A. K. and B. A. Freeman. Differentiation-arrested rat fetal lung in primary monolayer cell culture. III. Antioxidant enzyme activity. *Exp. Lung Res.* 6: 149-158, 1984. a.

Tanswell, A. K. and B. A. Freeman. Pulmonary antioxidant enzyme maturation in the fetal and neonatal rat. I. Developmental profiles. *Ped. Res.* 18: 584-587, 1984. b.

Tanswell, A. K. and B. A. Freeman. Liposome-entrapped antioxidant enzymes prevent lethal O_2 toxicity in the newborn rat. *J. Appl. Physiol.* 63: 347-352, 1987.

Tanswell, A. K., D. M. Olson, and B. A. Freeman. Liposome-mediated augmentation of antioxidant defenses in fetal rat pneumocytes. *Am. J. Physiol.* 258: L165-L175, 1990.

Tanswell, A K, L. Wong, F. Possmayer, and B. A. Freeman. The preterm rat: a model for studies of acute and chronic neonatal lung disease. *Ped. Res.* 25: 525-529, 1989.

Thomas, C. E., L. A. Morehouse, and S. D. Aust. Ferritin and superoxide-dependent lipid peroxidation. *J. Biol. Chem.* 260: 3275-3280, 1985.

Thomas, J. P., P. G. Geiger, and A. W. Girotti. Lethal damage to endothelial cells by oxidized low density lipoproteins: role of selenoperoxidases in cytoprotection against lipid hydroperoxide- and iron-mediated reactions. *J. Lipid Res.* 34: 479-490, 1993.

Torchilin, V. P., A. L. Klibanov, L. Huang, S. O'Donnell, N. D. Nossiff, and B. A. Khaw. Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium. *FASEB J.* 6: 2716-2719, 1992.

Torchilin, V P, F. Zhou, and L. Huang. pH-sensitive liposomes. *J. Liposome Res.* 3:201-255, 1993.

Walther, F. J., F. L. Nunez, R. David-Cu, and K. E. Hill. Mitigation of pulmonary oxygen toxicity in rats by intratracheal instillation of polyethylene glycol-conjugated antioxidant enzymes. *Ped. Res.* 33: 332-335, 1993.

White, C. R., T. A. Brock, L. Y. Chang, J. Crapo, P. Briscoe, D. Ku, W. A. Bradley, S. H. Gianturco, J. Gore, B. A. Freeman, and M. M. Tarpey. Superoxide and peroxynitrite in atherosclerosis. *Proc. Nat. Acad. Sci. (USA).* 91: 1044-1048, 1994.

Williams, M. C., S. Hawgood, and R. L. Hamilton. Changes in lipid structure produced by surfactant proteins SP-A, SP-B, and SP-C. *Am. J. Resp. Cell Mol. Biol.* 5: 41-50, 1991.

Wright, J. R., J. D. Borchelt, and S. Hawgood. Lung surfactant apoprotein SP-A (26-36 kDa) binds with high affinity to isolated alveolar type II cells. *Proc. Nat. Acad. Sci. (USA).* 86: 5410-5414, 1989.

Wright, J. R., R. E. Wager, S. Hawgood, L. Dobbs, and J. A. Clements. Surfactant apoprotein Mr=26,000-36,000 enhances uptake of liposomes by type II cells. *J. Biol. Chem.* 262: 2888-2894, 1987.

Yam, J., L. Frank, and R. J. Roberts. Oxygen toxicity: comparison of lung biochemical responses in neonatal and adult rats. *Ped. Res.* 12: 115-119, 1978.

Young, S. L., J. R. Wright, and J. A. Clements. Cellular uptake and processing of surfactant lipids and apoprotein SP-A by rat lung. *J. Appl. Physiol.* 66: 1336-1342, 1989.

Zhu, N., D. Liggitt, Y. Liu, and R. Debs. Systemic gene expression after intravenous DNA delivery into adult mice. *Science.* 261: 209-211, 1993.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Page T. Briscoe

Major Subject Biochemistry

Title of Dissertation Delivery of Superoxide Dismutase to Fetal Rat Lung Epithelial Cells by pH-sensitive Liposomes

Dissertation Committee:

B A Freeman, Chairman

Director of Graduate Program Jeffrey A. Engler

Dean, UAB Graduate School John L Loden

Date 9/28/95