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Ortho ester-based surfactants for pH-triggered release in drug and gene delivery

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Ortho Ester-Based Surfactants for pH-Triggered Release in Drug and Gene Delivery

by

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DISSERTATION

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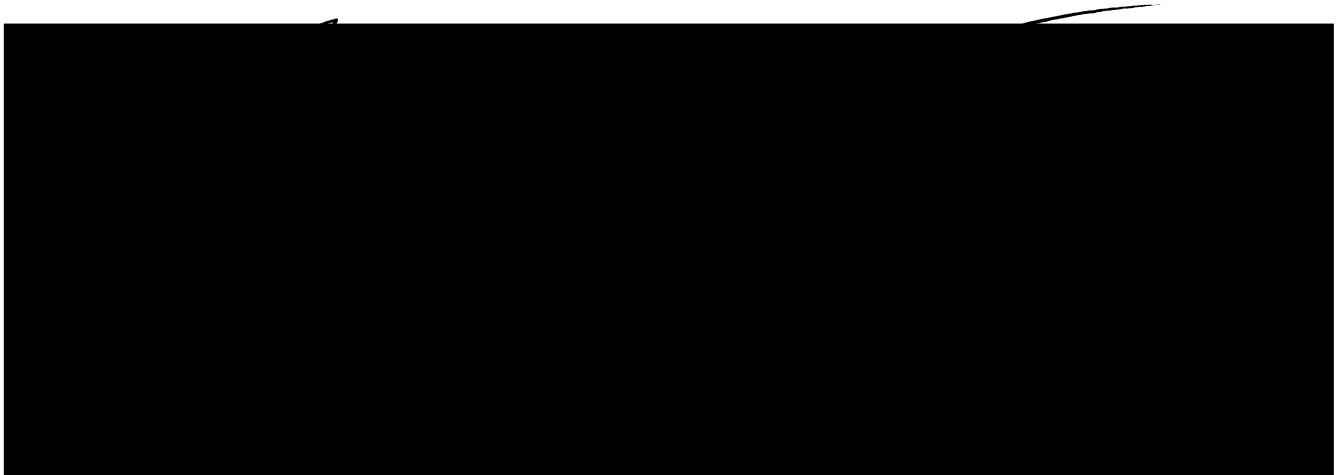

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dedicated to the Lord Almighty

and my family

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ORTHO ESTER-BASED SURFACTANTS FOR pH-TRIGGERED RELEASE IN DRUG AND GENE DELIVERY

by Xin Guo

ABSTRACT

The technology of liposomal drug delivery has made exciting progress over the last twenty years and a number of liposome formulations of drugs for tumor chemotherapy are now in the market. However, the success of liposomal triggered-release for drug and gene delivery in vivo is still limited despite the current literature that stresses its importance as well as a plethora of reported examples. The work reported in this thesis provides two chemical approaches for novel derivatives that significantly improve the pH sensitivity of liposomes for drug delivery.

Liposomal drug delivery systems must be stable in the delivery phase but rapidly release their contents in the target cell. Since liposomes are internalized into endosomes which have an acidic pH, methods have been devised to develop pH-sensitive lipid compositions. In this thesis, a number of amphiphilic surfactants that comprise a hydrophilic headgroup and a hydrophobic tail joined by an acid-labile ortho ester linker group have been designed and synthesized for use as pH-triggered drug and gene delivery systems.

The physical and chemical properties of the novel surfactants and their liposomes and lipoplexes were characterized by TLC, Photon Correlation Spectrometry and Fluorescent Spectrometry. The biological activities of these surfactants were evaluated in CV-1 cell culture and mice.

One of the conjugates, 3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(Ω-methoxy-polyethylene glycol2000-1-yl)-2,4,8,10-tetraoxaspiro[5,5]undecane (POD, **1**) contains a methoxypolyethylene glycol headgroup, a tetraoxaspiro[5,5]undecane diortho ester linker, and a distearoyl glycerol lipidic tail. POD was stable at neutral pH, 37 °C for greater than 3 hours, but degraded completely within one hour at pH 5. Liposomes composed of 10% POD and 90% of a fusogenic lipid, dioleoyl phosphatidylethanolamine (DOPE) remained stable for up to 12 hours in neutral buffer and in 75% fetal bovine serum, but aggregated and released most of their contents within 30 minutes of incubation at pH 5.5. The POD/DOPE (10/90)

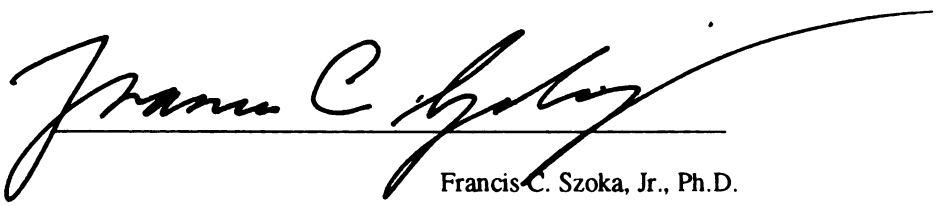
liposome is as stable as DOPE liposomes stabilized by 10% of a pH-*insensitive* PEG lipid in 75% of fetal bovine serum for up to 12 hours. Following i.v. injection into mice, the POD/DOPE (10/90) liposome has a half life of about 200 minutes in blood.

The mechanism of pH-triggered destabilization of POD/PE liposomes has been studied by fluorometric assays and can be described by the "Minimum Surface Shielding" model. The liposome leakage has two phases, a lag phase and a burst phase. During the lag phase, POD on liposome surface is hydrolyzed at a rate proportional to proton concentration and the liposome remains in a lamellar structure with a slow content leakage. When the acid-catalyzed hydrolysis lowers the mole percentage of POD on liposome surface to a critical level, inter-liposomal fusion is initiated and the leakage undertakes a much faster and fusion-dependent burst phase. Incorporation of POPE into the bilayers facilitates the mixing of the saturated and the unsaturated lipidic components and improves the kinetic profile of the resultant vesicles.

Three ortho ester-based cationic lipids were prepared in an effort to enhance the delivery of plasmid DNA by cationic liposomes. One of the synthesized lipids, *N*,*N*-dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (DOC, **2**) was combined with the fusogenic helper lipid, DOPE and used to prepare cationic liposomes. The DOC/DOPE liposomes carry excessive positive charges and spontaneously condense with plasmid DNA into cationic lipoplexes at N/P ratio of 5/1 and 3/1. Upon incubation at acidic pHs, the lipoplexes lost their positive charges as a result of DOC hydrolysis. Both in CV-1 cell culture and in CD-1 mice, lipoplexes containing DOC increased the transgene expression more than 5 fold compared with the structurally similar but pH-*insensitive* control lipid, 3β-[N-[2-(N,N-Dimethylamino)ethyl]carbamoyl]cholesterol (DC-Chol). These observations support the hypothesis that the incorporation of a pH-sensitive functionality into a cationic lipid enhances its ability to transfer DNA from endosomes into the cytoplasm, which is one of the key steps of gene delivery.

The pH-sensitive ortho ester linkers that cause a direct and immediate disassembly of the headgroup from the lipid could find a general utility in liposomal drug and gene delivery.

Thesis Committee Chairman



Francis C. Szoka, Jr., Ph.D.

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LIST OF NUMBERED COMPOUNDS

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1	3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(Ω-methyl-polyethylene glycol2000-1-yl)-2,4,8,10-tetraoxaspiro[5.5]undecane (**POD**)
2	3,9-Diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane
3	Distearoyl glycerol (**DSG**)
4	3,9-Diethyl-3-(cholest-5-en-3β-oxy)-9-(*N*,*N*,*N*-trimethylaminoethoxy)-2,4,8,10-tetraoxaspiro[5.5]undecane, chloride salt
5	3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(*N*,*N*,*N*-trimethylamino-ethoxy)-2,4,8,10-tetraoxaspiro[5.5]undecane, *p*-toluenesulfonate and chloride salts
6	*N*,*N*-Dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (**DOC**)
7	α,α–Dichloromethyl methyl ether
8	*N*,*N*,*N*-Trimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)-ammonium iodide

LIST OF ABBREVIATIONS

A_c	critical POD mole percentage; minimum POD percentage necessary to stabilize a bilayer structure
A_o	initial mole percentage of POD in the bilayer
ANTS	8-aminonaphthalene-1,2,3-trisulfonic acid
BPE	*p*-hydroxybenzamidine phosphatidylethanolamine
CHEMS	cholesteryl hemisuccinate
Chol	cholesterol
DC-Chol	3β-[*N*-[2-(*N*,*N*-dimethylamino)ethyl]carbamoyl]cholesterol
DOPE	dioleoylphosphatidylethanolamine
DOSPA	1-propanaminium, *N*-[2-[[2,5-bis[(3-aminopropyl)amino]-1-oxopentyl]amino]ethyl]-*N*,*N*-dimethyl-2,3-bis[(1-oxo-9-octadecenyl)oxy]-, salt with trifluoroacetic acid (1:1) (9CI)
DOTAP	*N*-[2,3(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate
DOTMA	1-propanaminium, *N*,*N*,*N*-trimethyl-2,3-bis[(9Z)-9-octadecenyloxy]-, chloride (9CI)
DPX	*p*-xylenebis(pyridinium) bromide
DSG	1,2-distearoyl glycerol
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-poly(ethylene glycol)-2000
HEPES	(hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid
k	hydrolysis rate constant of POD in $M^{-1}sec^{-1}$
NBD-PE	dipalmitoylphosphatidylethanolamine-NBD
OOCE	oleyl oleoyl carnitine ester (oleyl 3-oleoyloxy-4-trimethylammonium butyrate chloride)
PCS	Photon Correlation Spectrometry
PEG	polyethylene glycol
POD	polyethylene glycol 2000-diortho ester-distearoyl glycerol conjugate
POPC	1-palmitoyl-2-oleoylphosphatidylcholine
POPE	1-palmitoyl-2-oleoylphosphatidylethanolamine
POPG	1-palmitoyl-2-oleoylphosphatidylglycerol sodium salt

LIST OF ABBREVIATIONS (continued)

Rh-PE	dipalmitoylphosphatidylethanolamine-lissamine rhodamine B
RLU	relative light units
S.D.	standard deviation
t_l	lag time or length of lag phase
TLC	thin layer chromatography

ORTHO ESTER-BASED SURFACTANTS FOR pH-TRIGGERED RELEASE IN DRUG AND GENE DELIVERY

by Xin Guo

OBJECTIVE OF RESEARCH AND OVERVIEW OF THESIS

Triggered release(1) has long been an attractive concept in the design of drug and gene delivery systems since it would not only elevate the level of the therapeutic agent at the target site, leading to an improved efficacy, but also decrease toxicities from systemic drug disposition. The technology of liposomal drug delivery has made exciting progress over the last twenty years and a number of liposome formulations of drugs for tumor chemotherapy are now in the market(2). However, the success of liposomal triggered-release for drug and gene delivery in vivo is still limited despite the current literature that stresses its importance as well as a plethora of reported examples(1,3,4). Chapter one of the thesis reviews the liposomal triggered-release systems in the context of the requirements for drug or gene delivery in vivo, and serves as the background and the rationale for my research work.

Since the decrease of pH is implicated in many physiological and pathological processes such as endosome trafficking(5), tumor growth(6), inflammation(7) and ischemia(8), it has been extensively exploited to trigger the release of drugs from liposomes(9). Compared with other triggering mechanisms such as hyperthermia and photon dynamic therapy, pH-triggering is endogenous at the target site and hence does not require complex medical engineering procedures. The majority of the reported pH-sensitive liposomes are based on the neutralization of excess negative charges on

liposome surface(9), but the negative charges induce undesired interactions with serum proteins and fixes macrophages, leading to rapid elimination of the liposomes from circulation(10). The research in this thesis is focused on developing a novel category of lipid surfactants that comprise a polar headgroup and a hydrophobic tail joined together by an acid-labile, ortho ester derived linker group. By this strategy, a variety of headgroups and tails could be conjugated together to render pH-sensitive lipids of different properties. The ortho ester(11) is selected as the linker group because it is one of the most acid-sensitive functionalities known in the literature. It is hypothesized that lipids composed of such type of linkers would be relatively stable at alkaline and neutral pHs but respond quickly to small drops of pH which are relevant to biological scenarios.

One of the most important means to stabilize liposomes in blood circulations is to coat their surface with a hydrophilic polymer such as polyethylene glycol (PEG)(12). However, the grafted PEG also hinders the interaction of liposomes with the biomembranes of target cells(13). It is thus desirable to devise PEG groups that are detachable from the liposome surface at the target site. Chapter Two presents the first example of the ortho ester lipidic conjugates, 3,9-diethyl-3-(2,3-distearoyloxypropyloxy)-9-(Ω-methoxy-polyethylene glycol2000-1-yl)-2,4,8,10-tetraoxaspiro[5,5]undecane (POD, **1**), which has a detachable PEG headgroup in response to mildly acid pHs. The work of this Chapter is also reported in the March/April 2001 issue of Bioconjugate Chemistry. A "Minimum Surface Shielding" model is proposed in Chapter Two to account for the observed data.

In order to test the "Minimum Surface Shielding" model, as well as to further elucidate the mechanism of pH-triggered release of POD/PE liposomes, I carried out

extensive fluorometric studies on the kinetics of leakage from the POD/PE liposomes and the results are presented in Chapter Three. The studies strongly support the model and derived important kinetic parameters that help optimize POD liposomes for drug and gene delivery in vivo.

Cationic liposomes are the most common nonviral vector for gene delivery in vivo(14). One of the key barriers to gene delivery by this type of carrier is that most of the dose is trapped in the endosomes and subsequently processed to lyzosomes where the contents are degraded(15). In Chapter Four, novel ortho ester lipids that contain cationic headgroups are synthesized, in an effort to develop cationic liposomes that destabilize endosomes in response to the drop of pH and hence facilitate cytoplasmic translocation of the complexed DNA molecules. One of the prepared cationic ortho ester lipids, *N*,*N*-dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (DOC, **2**) mediated a more than 5-fold increase of transgene expression in CV-1 cell culture, as well as in mice following intratrachael administration, compared to a pH-*insensitive* lipid analog DC-Chol. These observations support the hypothesis that the ortho ester-based, pH-sensitive cationic lipids facilitate cytoplasmic gene delivery.

CHAPTER ONE:

Design of Triggerable Liposomal Systems for Drug and Gene Delivery: A Review of the Literature

1.1 Introduction

Since the discovery of liposomes by Bangham et al.(16) almost four decades ago, they have become paradigms for biomembranes, instructive models of self-assembling colloids and vehicles for pharmaceutical, diagnostic and cosmetic agents. There are over 18,000 papers (listed in PubMed), 600 patents (USPTO Web Patent Database), six approved liposomal drug formulations and numerous clinical trials of liposome-encapsulated agents. Cationic liposome-DNA complexes are the most studied nonviral gene delivery system in humans (Wiley's The Journal of Gene Medicine website at: www.wiley.co.uk/wileychi/genmed). In spite of this plethora of information liposomes still have not attained their full potential as drug and gene carriers.

There are two areas where chemistry can help improve liposomal drug delivery. The first is to design components that will permit the liposome to bypass the multiple anatomic and sub-cellular barriers to reach the target site. The second is to devise mechanisms for the liposomes to transfer the encapsulated drug or gene into the target site. There are other challenges that relate to the commercialization of liposomes, which will not be addressed here.

Ideally, drug-loaded liposomes should remain stable until it reaches the target site(17-19). Upon accumulation at the target site, the liposome needs to efficiently

release the drug at a high enough level for an effective therapeutic response. For the above reasons, drug release in response to a specific stimulus at the target site, i.e. triggered release, is an essential feature of more and more targeted delivery systems(1,17,20). The following sections review the physical and chemical methods that have been devised to trigger the release of liposome contents, and recent examples of triggerable liposomal systems designed for in vivo delivery.

1.2 Requirements of Liposomal Systems for Targeted In Vivo Delivery

1.2.1 *Challenges of Drug Delivery after Intravenous Administration*

The most common parenteral administration route for targeted drug delivery is intravenous (i.v.) injection. Using the i.v. route, the dose rapidly distributes throughout the vascular system. Liposomes given via this route must fulfill two requirements if they are to deliver drugs to the target cells. First, the payload needs to remain in the liposome(21). Premature leakage of the drug from the liposome will not only decrease the level of drug that finally reaches the target site, but also increase its systemic toxicity. The liposome needs to tolerate insults from plasma proteins in the vascular system(22). Secondly, once the liposomes are in the blood, they must remain there long enough to have time to accumulate in the target cells. This requires the liposomes to avoid cells of the reticular endothelial system (RES) which are located primarily in the liver and the spleen. If the target is the vascular endothelial cells, liposomes can readily reach the target site via blood circulation; for other tissues such as hepatocytes and solid tumors, the liposomes need to extravasate through the endothelial capillaries and diffuse to the target site(23). The endothelial cells that line the capillaries enforce an upper size limit of about 100 nm if the liposomes are to reach the target site(24). After reaching the target site, they must effectively deliver the drug into the target cell.

1.2.2 *Additional Barriers for Gene Delivery*

For a large number of therapeutic agents, additional mechanisms must be available to transfer them across subcellular barriers to reach their intracellular target site. These agents include DNA, RNA, oligonucleotides, proteins and peptides, which are too hydrophilic and have too large a molecular weight (>700 Dalton) to allow passive diffusion through biological membranes(25).

Delivery of plasmid DNA for gene therapy is the most formidable task among these scenarios. After reaching the target cell, DNA needs to transfer across the plasma membrane, either by fusion(26) or by endocytosis(15). Subsequent to endocytosis, DNA must be released from the endosomes into the cytoplasm(27,28) since if it trafficks to the lysosomes it will undergo extensive metabolic degradation. The molecular weight of plasmid DNA (2-10 million Dalton) is far beyond the limit to permit significant passive diffusion in the cytoplasm(29), so active transport is needed to move plasmid DNA towards the cell nucleus. Finally, in order to reach the cellular transcription machinery for expression, plasmid DNA must cross the nuclear envelope and translocate into the nucleus(30,31). Clearly translocation of high molecular weight, hydrophilic molecules across biomembranes is one of the most challenging problems in pharmaceutics.

1.2.3 *Current Status of Liposomal Drug Delivery Suggests the Need of Triggered Release*

Over the past twenty years, liposome technology has dramatically progressed and numerous new methodologies have been developed to address the above issues. Anticancer drugs can be encapsulated into liposomes at a high drug to lipid ratio by pH(32) or ammonium sulfate gradients(33). Drugs can be retained in liposomes for prolonged periods by controlling the rigidity of the lipid bilayers using saturated long acyl chain lipids and cholesterol(34). Novel polymeric amphiphiles have been reported to assemble into vesicles that can even better retain the encapsulated contents(35). Discher and coworkers(35) have recently reported on vesicles composed of a diblock copolymer of polyethylethylene and polyethylene glycol. Compared with conventional phospholipids, the copolymer (t-butyl-$[CH_2\text{-}CH(C_2H_5)]_{37}$-$[CH_2\text{-}CH_2\text{-}O]_{40}$-H) has a much larger molecular weight (number average molecular weight $\approx$ 3900 g/mol) and self-assembles into bilayers with a core thickness of about 8 nm, which are significantly larger than phospholipid bilayers (4 nm). The elastic coefficient of polymer lamellas are also 5 to 50 times larger (Ec $\approx$ 2.2 mJ/m^2) than that of phospholipid bilayers (Ec = 0.05 to 0.5 mJ/m^2). Sumida and coworkers(36,37) have reported triple-chain lipid analogues that also exhibited better long-term stability toward content leakage, compared with the corresponding diacyl phosphocholines.

An additional improvement in liposome technology was the steric stabilization of the liposome surface(38). The half life of liposomes in blood circulation can be extended to up to 24 hours by grafting their surface with hydrophilic polymers such as

polyethylene glycol as a steric barrier to minimize the opsonization of the liposomes, and hence reduce their elimination by the RES system(12). The preferential disposition of liposomes at the desired cells can be achieved either by passive targeting, in the case of solid tumors, where the liposomes accumulate at the tumor tissue due to its porous endothelial vasculature and poor lymphatic draining (know as the Enhanced Permeability and Retention phenomena) or by active targeting, whereby ligands, such as antibodies, on the liposome surface bind to cell surface receptors(39).

In contrast to the progress in the above aspects, components that are designed for optimal release at the target site have been used in only a few liposomal drug formulations and no such formulations are currently in clinical trials. The challenge here is to optimize the spatial and temporal control of drug release to treat the disease. First, the stimulus to trigger the drug release must be specific to the target site; second, the liposomes have to be sensitive enough to the trigger to yield effective release; third, the triggered release mechanism must be formulated into a delivery system while maintaining its existing properties such as drug retention, long circulation time and deposition at the target site. Recent reviews have provided persuasive arguments to support the hypothesis that a mechanism of enhanced release at the target site would greatly improve the efficacy as well as the specificity of liposomal drug delivery(17).

1.3 Strategies of Engineering Liposomes for Triggered Release

The numerous methods developed to induce liposomal leakage in response to an environmental stimulus have been the subject of several excellent reviews(1,3,9,21). The strategies used include: 1) formation of defects and channels in the bilayers, 2) lamellar-micellar phase transition, 3) lamellar-hexagonal phase transition, 4) lipid phase separation and 5) liposome fusion.

In terms of the nature of the stimuli, the triggered release liposomes can be divided into systems triggered by an externally applied stimulus such as heat or light, and those triggered by a biologically supplied stimulus such as the drop of pH in an ischemic tissue, enzymatic cleavage or change of a redox potential. The chemical components that respond to these stimuli and induce the liposome leakage include: ionizable lipids, lipids with a desired phase transition temperature (melting temperature), degradable lipids, functional polymers and peptides, ionizable detergents, cis-trans isomerization and free-radical generating compounds as photo-sensors.

This chapter will not comprehensively review the reported systems since they are adequately covered in earlier reviews but rather will discuss recent progress in liposomes that are triggerable by biologically supplied stimuli including decrease of pH, enzymatic cleavage and change of redox potential. Compared with externally applied stimuli such as heat and light, the advantage of these biological triggering mechanisms is that they do not require complicated medical engineering to devise a method for applying the stimulus after the delivery system has distributed in the body, and hence would, in theory, be much more convenient and economical. Since the stimulus is supplied by the disease site, the caregiver would only need to administer the formulation; nature would do the rest. The

1.4 pH-Sensitive Liposomes

1.4.1 *Overview of the Mechanism*

The decrease of pH is implicated in many physiological and pathological processes such as endosome trafficking(5), tumor growth(40), inflammation(7) and myocardial ischemia(8). It is not surprising that numerous pH-sensitive liposomes have been designed and intensively studied over the past twenty years(1,3,9). Compared with thermosensitive and photosensitive liposomes, the reported mechanisms of pH-triggered liposome destabilization are much more diverse and can be categorized as follows: 1) neutralization of negative lipids in the bilayers via protonation, leading to lamellar to hexagonal phase transition; 2) protonation of negative polymers or peptides, which in turn absorb to the bilayers and destabilize their structures by lysis, phase separation, pore formation or fusion; 3) acid-catalyzed hydrolysis of bilayer-stabilizing lipids into destabilizing detergents or conical lipids; 4) activation of pH-sensitive surfactants into their positive and surface-active conjugate acids.

1.4.2 *Requirements of pH-Sensitive Liposomes as Delivery Systems In Vivo*

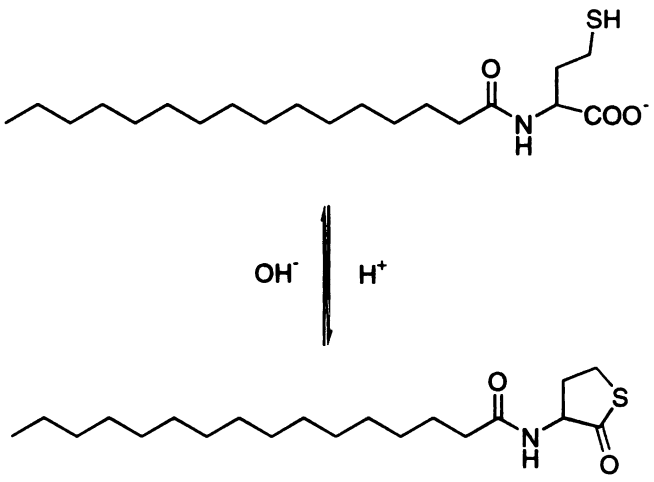
To achieve sufficient accumulation at the target site, the liposomes need to be reasonably stable in the circulation. However, the sensitivity of the liposomes to change in pH needs to be sufficient to respond to the decrease of pH at potential therapeutic sites.

For example, the transit through the endosomes in cells occurs in about 10 to 30 minutes with pH in the range of 5 to 6 before the endosomal contents are delivered into the lysosome(41,42). Therefore, it is important for pH-sensitive liposomes to respond quickly to the initial drop in pH and release their contents prior to trafficking into the lysosomal compartments, where extensive degradation takes place. At inflammatory tissues(7,43) and solid tumors(6,40), the pH is only 0.4 to 0.8 units more acidic than that of the circulation, suggesting that the liposomes need to respond to a small stimulus and release enough drugs for a therapeutic effect. It should also be noted that the requirements may vary depending on the delivered agent (see Sections 1.2.1 and 1.2.2). In the case of small molecules, triggered release at the interstitial space of the targeted tissue may be sufficient, provided that the surrounding cells can readily take up the drug; for gene delivery, however, fusion between the liposome bilayers and host cell membranes appears necessary to transfer DNA into the cytoplasm of most cell types.

1.4.3 *Neutralization of Bilayer Surfactants*

The first pH-sensitive liposome system introduced by Yatvin and colleagues(44) was composed of phosphatidylcholine and N-palmitoyl homocysteine (Scheme 1-1). Upon decrease of the pH, the carboxylate group of N-palmitoyl homocysteine is protonated and reacts with the sulfhydryl group to form a neutral thiolactone. The time dependence of this lactonization is rather slow. Since then, most of the pH-sensitive liposomes feature a surfactant with a pH-titratable carboxylate group and a fusogenic,

conical-shaped lipid such as DOPE(9). The decrease of pH results in the neutralization of the excess negative charges of the carboxylate groups. This reduces the molecular dimension of the surfactant headgroups and triggers the fusion and collapse of the PE-rich lamellae into hexagonal phases with concomitant release of the encapsulated contents(45,46). The pH-titratable, carboxylate-containing surfactants can be categorized into phospholipids, acylated amino acids, cholesterol derivatives and double-chain amphiphiles(9). The drawback of these surfactants, however, is that at neutral pH, the excess negative charges of the carboxylate groups on liposome surface induce undesired interactions with plasma proteins and fixed macrophages, leading to rapid elimination of the liposomes from circulation(10,47).



Scheme 1-1. Acid-triggered lactonization of homocysteine.

1.4.4 *Neutralization of Polyelectrolytes*

As an alternative to devising pH-titratable lipids, one can use functional polymers and peptides with titratable acidic groups to destabilize the liposomes at low pH. Tirrell and his associates published a series of studies on poly-(2-ethylacrylic acid)(PEAA) and its derivatives. At low concentrations (<20 μg/mL), PEAA induces the formation of cation-selective pores in bilayers in a pH-dependent manner(48). At high concentrations, PEAA induces the reorganization of phospholipid vesicles into small disk-shaped micelles of average thickness 55 Å and diameter 160 Å upon a pH shift from 7.0 to 6.5(49). The exact critical pH of membrane destabilization can be tuned in the range of 5.7 to 6.5 by copolymerizing 2-ethylacrylic acid with 2-methacrylic acid in appropriate composition(50). Recently, it was found that a related polymer, poly(propyl acrylic acid) (PPAAc), disrupted red blood cells 15 times more efficient than PEAA at pH 6.1 and displayed a pH-dependent hemolysis that was shifted toward higher pHs(51). The surface activity of PEAA is relatively insensitive to membrane compositions including neutral phosphatidylcholine vesicles, negatively charged phosphatidylglycerol vesicles, as well as membranes of red blood cells. It is thus conceivable that these acrylic acid-based polyelectrolytes could be co-encapsulated into serum stable liposomes with therapeutic agents and would facilitate their release from endosomes into cytoplasm following the endocytosis of the liposomes. PPAAc was found to retain its hemolytic activity even after it is conjugated with streptavidin protein in 3:1 or 1:1 polymer to protein molar ratio(52), suggesting that PPAAc can be conjugated with macromolecules

such as proteins and DNA, followed by liposome encapsulation as a means of enhancing their endosomal escape.

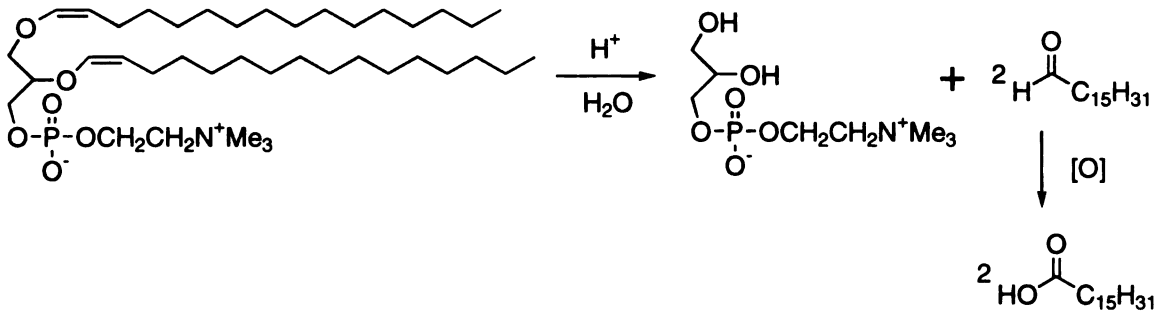
A number of other pH-sensitive and membrane-active polyelectrolytes are known in the literature; some of them, such as poly(malic acid)(53) and the GALA peptides(54,55) extensively studied by the Szoka group may have potential advantages for delivery due to their excellent biodegradability.

1.4.5 *Surfactant Hydrolysis*

One attractive approach to circumvent the problem of a negatively charged surface of pH-sensitive liposome is to design degradable surfactants with functional groups whose hydrolysis is catalyzed by acidic conditions. In this case, various headgroups could be used to generate pH-sensitive surfactants of different properties.

Thompson and associates reported a number of mono- and di-plasmenyl lipids with an acid-sensitive vinyl ether linkage between the headgroup and one or both of the hydrocarbon side chains(56,57). Upon exposure to low pH or photooxidation, the vinyl ether side chains are cleaved from the lipids (Scheme 1-2), leading to the structural defects of the bilayers and the release of liposomal contents. At 38 °C, liposomes composed of pure plasmenylcholine (1-hexadecyl-1'Z-enyl-2-palmitoyl-*sn*-glycero-3-phosphocholine) need about 4 minutes to release 50 % of their contents at pH 2.3 and about 500 minutes for 50 % release at pH 5.3(58). The incorporation of dihydrocholesterol into the bilayers for serum stability, however, greatly reduces the pH-

sensitivity. At pH 2.3, over 70 minutes are needed to effect 50 % content release in 6:4 PlsPamCho/DHC liposomes. Compared with the mono-plasmenyl analog, liposomes composed of diplasmenyl phosphocholine (DPPlsC) appeared to possess better pH-sensitivity and released 50 % of encapsulated calcein in 230 minutes at pH 5.3(59). Pure DPPlsC liposomes released 27 % of their contents after 24 hours of incubation in 50 % of heat inactivated fetal calf serum at pH 7.4, 37 °C. The incorporation of dihydrocholesterol increased the serum stability, at the price of decreasing the pH sensitivity. When KB cells were treated with folate targeted DPPlsC liposomes (DPPlsC/DSPE-PEG3350-folate = 99.5/0.5) containing propidium iodide (PI), 83 % of the PI escaped the endosomal/lysosomal compartments within 8 hours. Encapsulation of 1-β-arabinofuranosylcytosine into DPPlsC/DSPE-PEG3350-folate liposomes enhanced its cytotoxicity in KB cell culture by 6000 fold compared with the free drug. These results in cell culture demonstrate in principle that the introduction of a pH-triggering mechanism into targeted liposomes can significantly increase the efficacy of the encapsulated therapeutic agents.

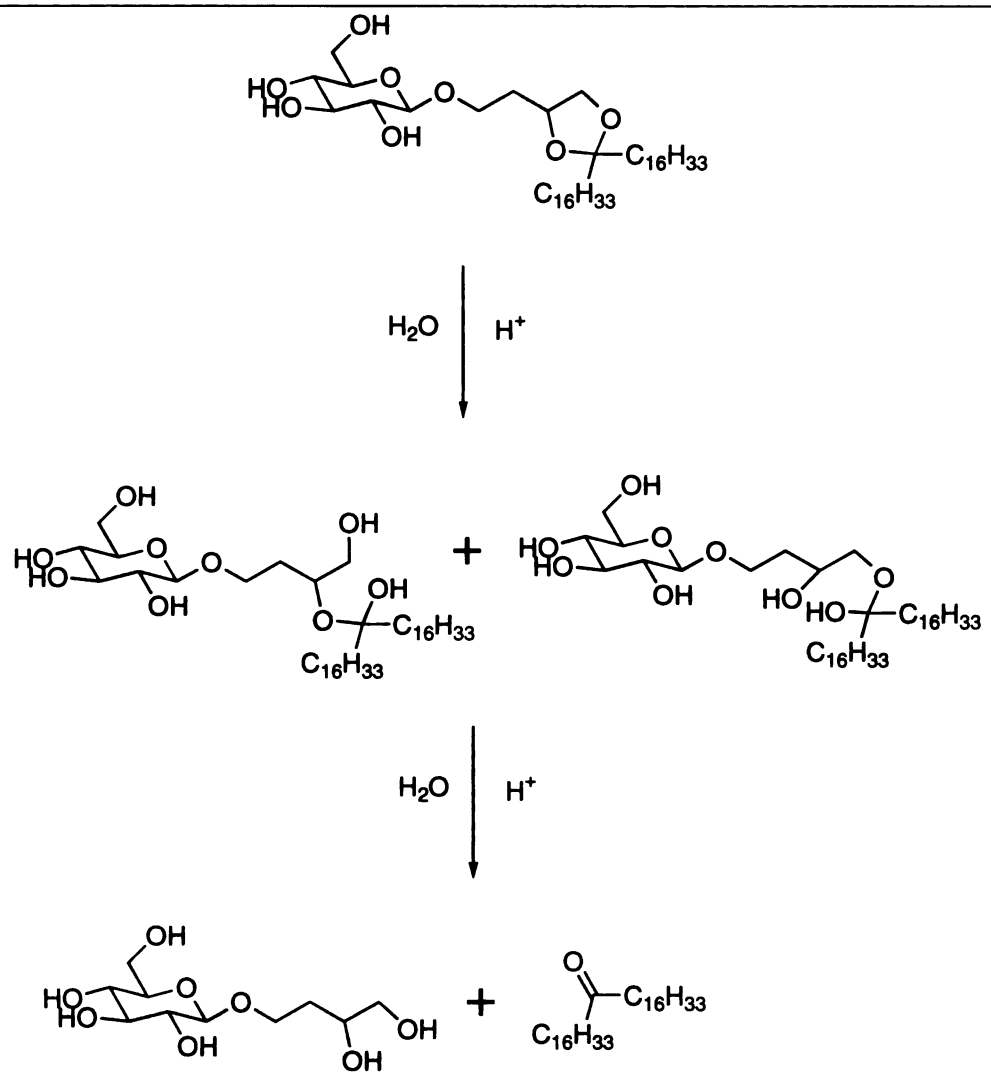


Scheme 1-2. Hydrolysis of diplasmenyl phosphocholine.

Boomer and Thompson(56) recently reported the synthesis of three novel diplasmenyl lipids based on a chiral 1,2-di-*O*-(1Z',9Z'-octadecadienyl)-*sn*-glycerol platform. The lipids displayed three different headgroups: a neutral phosphocholine group, a sterically stabilizing poly(ethyleneoxide) group and a cationic *O*-carbamoyl-*N*-diethylenetriamine group. The diplasmenyl PEG lipid, (R)-1,2-di-*O*-(1Z',9Z'-octadecadienyl)-*sn*-glyceryl-3-(ω-methoxy-poly(ethyleneglycolate[112]) (BVEP) was formulated with the nonlamellar DOPE to generate unilamellar liposomes(60). BVEP:DOPE liposomes at 3:97 molar ratio offered the best pH-triggered leakage profile, releasing 50 % of encapsulated calcein in 4 hours at pH 4.5 and 15 % at pH 7.4. Lipid mixing assays and transmission electronmicroscopy experiments suggested that the acid-triggered hydrolysis of BVEP primarily induced the content leakage and Lα to H_{II} phase transition, with membrane fusion occurring on a slower time scale. The PEG headgroup

of BVEP provides considerable promise for in vivo applications of the diplasmenyl lipid. However, the leakage rate of the BVEP/DOPE liposomes at mildly acidic pH (5 to 6) may need to be increased to yield an optimal drug release system.

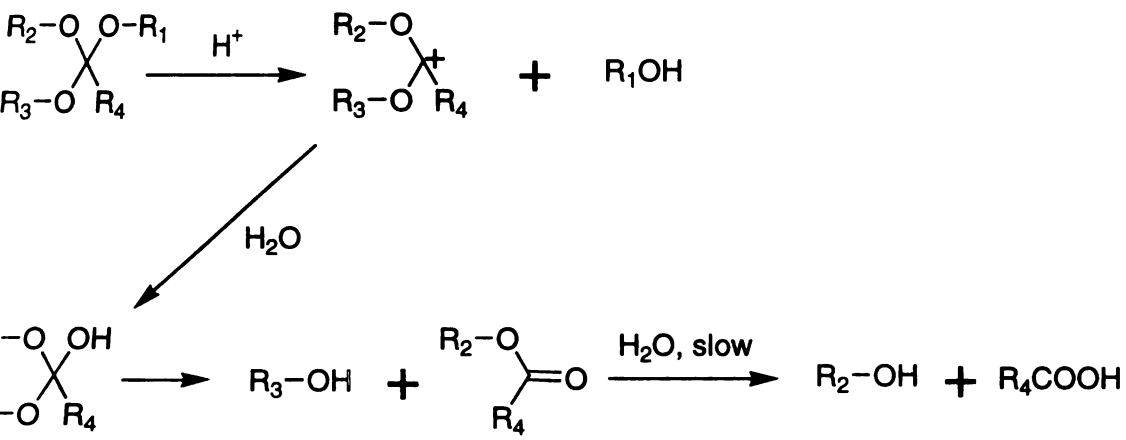
Song and Hollingsworth(61) reported a glycolipid conjugate (Scheme 1-3) of glucose and two palmityl side chains *via* an acid-labile acetal moiety. The glycol lipid self-assembles into lamellar structures in aqueous solution as indicated by confocal microscopy studies. The acetal linkage of the glycolipid was completely cleaved in ethanol solution with 0.01 % concentrated DCl (pD slightly lower than 3), but no cleavage was observed when the lipid was in ethanol with 1 % to 20 % acetic acid. It remains to be seen if the kinetics of the hydrolysis of this glycolipid is sufficient for applications in triggered drug release in vivo.



Scheme 1-3. Hydrolysis of the acetal-derived glycolipid.

In contrast with the huge diversity of pH-sensitive liposomes in the literature, little success has been reported in the use of these systems for in vivo drug or gene delivery. Such a disappointing history prompted us to re-consider the physical and pathological scenarios where the decrease of pH takes place and, hopefully, to devise a pH-sensitive liposome for use as an in vivo delivery vehicle. Therefore, I have designed and synthesized a lipid conjugate of polyethylene glycol (PEG2000) and distearoyl

glycerol *via* an acid labile diortho ester linker (see Chapter 2, Scheme 2-2, compound **1**). Scheme 1-4 shows the general mechanism for acid-catalyzed ortho ester hydrolysis. Ortho esters are expected to hydrolyze more quickly than vinyl ethers and acetals in response to pH decrease due to the stable dialkoxy cation intermediate.



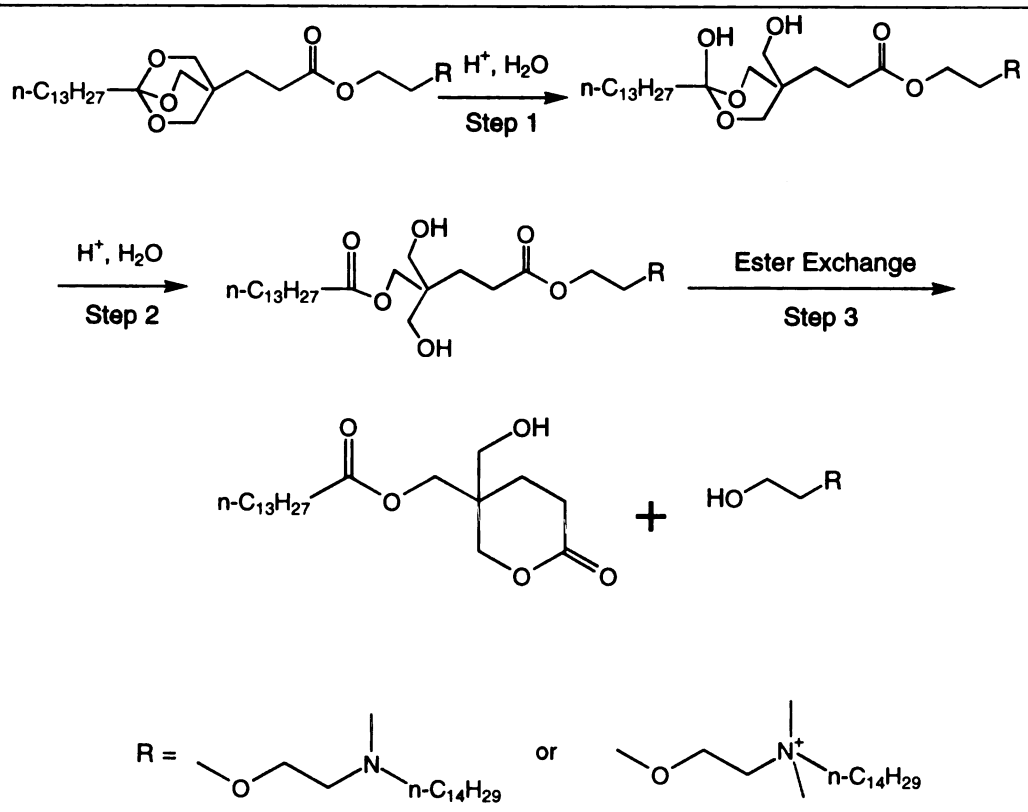
me 1-4. General mechanism of acid-catalyzed hydrolysis of ortho esters.

The PEG2000-orthoester-distearoyl glycerol conjugate (POD) was found to be ely stable for 3 hours in pH 7.4 buffer at 37 °C but degraded completely in one hen the pH was decreased to 5. POD was thus formulated with the nonlamellar, ic lipid DOPE, with the anticipation that the resultant liposomes will be stable in serum due to the steric effect of the PEG headgroup of POD, but will collapse agonal phase and release their contents at lower pH, when the hydrolysis of ortho kages removes the PEG groups from liposome surfaces. In 75 % fetal bovine

serum, the POD/DOPE liposomes (1/9 in molar ratio) are as stable as the sterically stabilized and pH-*insensitive* control liposomes (DSPE-PEG2000/DOPE = 1/9, mol/mol) for up to 12 hours. However, when POD/DOPE liposomes were incubated in acidic pH as mild as 5.5, they aggregated and released most of their contents within 30 minutes, the time it takes for endosome trafficking at this pH(41,42). The kinetics of contents release from POD/DOPE liposomes consisted of two phases, a lag phase, when a small portion of ANTS slowly leaked out, and a burst phase, when most of the ANTS leaked out in a much quicker manner. The lower the pH, the shorter the lag phase and the logarithm of the length of lag phase showed a linear relationship with the incubation pH. Mathematical modeling of the data from the ANTS leakage assay and the lipid mixing assay indicated that the PEG groups are cleaved from the surface of POD/DOPE liposomes at a rate proportional to the proton concentration, but the vesicles retain their lamellar structures until the number of surface PEG groups decrease to a critical level, at which the bilayers of different liposomes start to contact each other, leading to the fusion and content leakage of the vesicles. Upon i.v. injection into mice, liposomes composed of 10 % POD and 90 % DOPE were cleared from circulation by one-compartment kinetics with a half life of about 200 minutes, comparable with the half life of about 300 minutes in the case of the sterically stabilized but pH-*insensitive* control liposomes (DSPE-PEG2000/DOPE = 1/9, mol/mol). The uniquely fast degradation kinetics of POD at low pH and its ability to stabilize liposomes in blood circulation may provide the conjugate with considerable advantages to find applications for triggered drug release systems targeted to mildly acidic bio-environments such as endosomes, solid tumors and inflammatory tissues. We are currently carrying out studies to utilize POD-containing

liposomes for *in vivo* drug delivery and gene delivery and will report the results once the experiments are completed.

Recently, Zhu and coworkers(62) reported two acid-labile cationic lipids (Scheme 1-5) containing an ortho ester linker based on the structure of 3,5,8-trioxabicyclo[2.2.2]octane. However, due to the particular configuration of the linker, the first two fast hydrolysis steps of the ortho ester functionality do *not* fragment the cationic lipid, but rather add two hydroxy groups near the cationic headgroup region. It is only after the final slower step of the hydrolysis, which is the cleavage of an ester group, that the lipids convert to two single-chain compounds. Such hydrolysis pattern may complicate the kinetics of bilayer destabilization by these pH-sensitive lipids.

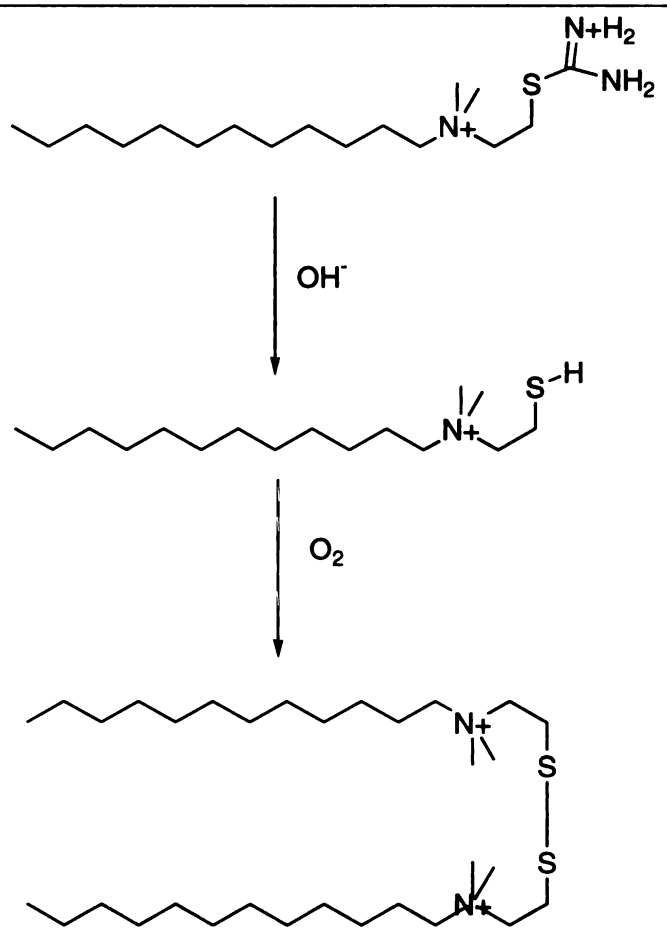


Scheme 1-5. Hydrolysis of ortho esters derived from 3,5,8-trioxabicyclo[2.2.2]octane.

Although the acid-labile lipids enjoy significant versatility in the design of different headgroups, they may face the serious challenge of possessing reasonable shelf life. Suppose an acid-labile lipid has a half life of 1 hour at pH 5, then its half life at pH 8 would be 1000 hours, i.e., 42 days at the same temperature, assuming that the hydrolysis is proportional to the proton concentration. This suggests that once the pH-sensitive liposomes are prepared, it may be difficult for them to stay in aqueous buffer for 6 months (the FDA-requested shelf life for most marketed drugs at room temperature) without significant change of properties. Additional procedures such as lyophilization or storage at low temperature would be necessary in order to develop acid-degradable liposomes into pharmaceutical products.

Lipid structures that are labile to mildly alkaline conditions have also been devised, not for pH-triggered release rather for pH-triggered assembly of DNA delivery systems. Ming, Remy and Szoka(63) synthesized a series of novel cationic detergents that contain cleavable hydrophilic isothiuronium headgroups. These detergents were used to control the assembly of plasmid DNA into small stable particles with high DNA concentrations investigated. The detergents (Scheme 1-6) have alkyl chains of C8 - C12 and contain hydrophilic isothiuronium headgroups that provide relatively high critical micelle concentration (CMC) to the detergents (> 10 mM). The isothiuronium group masks a sulfhydryl group on the detergent and can be cleaved in a controlled manner under basic conditions to generate a reactive thiol group. The thiol group can undergo a further reaction after the detergents have accumulated on a DNA template to form a disulfide-linked lipid containing two alkyl chains. The pH dependent kinetics of cleavage of the isothiuronium group, the CMC of the surfactants, the formation of the

complexes, and the transfection efficiency of the DNA complexes have been investigated. Using the C12 detergent, a ~6 KB plasmid DNA was compacted into a small particle with an average diameter of around 40 nm with a ~ -13 mV zeta potential at high DNA concentration (up to 0.3 mg / mL). The compounds were well tolerated in cell culture and showed no cytotoxicity under their CMCs. Under appropriate conditions, the small particle retained transfection activity. In theory the isothiouronium group could be incorporated into a liposome and be stored for prolonged period at pH 5.0, yet generate reactive sulfhydryl groups at neutral pH and higher.

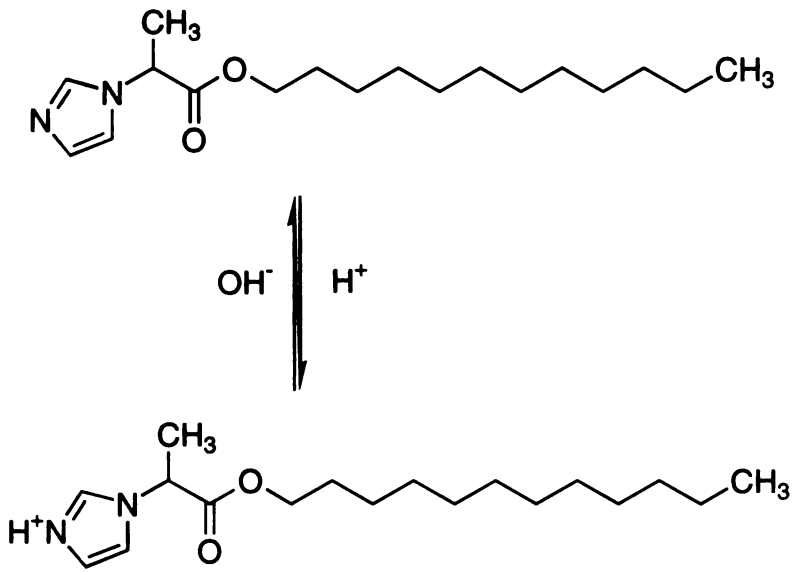


Scheme 1-6. Base-triggered cleavage and dimerization of cationic isothiuronium detergents.

1.4.6 *Ionization of Bilayer Surfactants*

In an effort to improve the gene transfection efficiency of cationic liposomes, Liang and Hughes(64,65) reported a number of biodegradable, pH-sensitive surfactants (BPSs) as potential endosomotropic agents. The surfactants (one example is shown in Scheme 1-7) possess a single 12-carbon side chain and a pH-titratable imidazole group that picks up a positive charge at acidic pH. Liposomes composed of PC and BPSs underwent fusion and content leakage that are dependent on pH and the molar ratio of BPSs to membrane lipids (R). Among the three reported BPSs, dodecyl 2-(1'-imidazolyl)propionate (DIP) at R = 0.4 showed the most pH-sensitive leakage profile, releasing more than 40 % of the encapsulated calcein at pH 5 and about 10 % at neutral pH. The incorporation of cholesterol into the lipid composition significantly enhanced the pH-sensitive fusion and leakage, whereas the presence of DOPE decreased the pH-sensitivity. Cationic liposomes composed of DOTAP, DOPE and DIP (1:1:1 molar ratio) mediated 5-fold more luciferase gene expression of pLG3 plasmid DNA in a human neuroblastoma cell line (SKnSH), compared with the control formulation of DOTAP/DOPE in 1:1 molar ratio. Since BPSs remains uncharged at neutral pH, they may also have advantages in the design of serum-stable and pH sensitive liposomes for in vivo delivery of small molecules. BPSs could be formulated into PEG stabilized and drug loaded neutral liposomes. After intravenous injection, the liposomes would remain stable in circulation, until they reach the target site with lower pH, where BPSs would become positively charged and trigger the liposomal leakage. The positive charges of BPS may also enhance the cellular attachment, endocytosis and endosomal

destabilization of the liposomes, leading to improved cytoplasmic delivery of their payloads.



Scheme 1-7. Ionization of dodecyl 2-(1'-imidazolyl) propionate (DIP).

1.5 Release Triggered by Enzymatic Cleavage

Since certain enzymes are often elevated in tumor tissues(4), a number of enzymatic mechanisms have also been exploited to create potential triggered release systems for cancer chemotherapy. For instance Davis and Szoka(66) synthesized a number of cholesteryl-based lipids with a doubly negatively charged phosphate monoester headgoup (phosphatidic acids) and used them to stabilize phosphatidylethanolamine bilayers. Two mechanisms for the triggered release of the liposomes were found-enzymatic and ionic. The first required phosphatase enzymes, either alkaline or acid phosphatases to catalyze the hydrolysis of phosphate monoesters and remove the negatively charged phosphate groups from the bilayer surface. This allowed collapse to the hexagonal phase and release of the liposome contents. A second mechanism of collapse was also found that involved divalent cations such as calcium. The rate of collapse by the two mechanisms can be modulated by altering the distance of the phosphate group from the interface of the bilayer. However, these negatively charged vesicles may be challenged by components of the immune system if administered in vivo. Other enzymes that have been investigated to trigger liposomal release include β-galactosidase(67), acetylcholine-esterase(68), sphingomyelinase(69), phospholipase C(69), phospholipase A_2(70), cathepsin B(71), elastase(72) and protease K(73). One complication of the enzymatic triggering is that steric stabilization of such liposomes with gangliosides(74,75) or PEG(76) also appears to inhibit the degradation of the vesicles by the

triggering enzymes. Therefore, in terms of applications as a delivery system, further optimizations of such liposomes are needed to find a bilayer composition that is both sensitive to the triggering enzyme and resilient to insults from the blood circulation.

1.6 Redox-Triggered Release Systems

A number of lipids that contain a disulfide bond have been reported to explore a thiolysis mechanism of triggering the liposomes. The rationale behind this approach is that the cytoplasm holds a lower redox potential and more molecules with free sulfhydryl groups than plasma. Kirpotin and colleagues(13) studied the conjugate (mPEG-DTP-DSPE) of a PEG headgroup and a DSPE via a dithiopropionyl linkage. Liposomes composed of 3 mol% mPEG-DTP-DSPE and 97 mol% fusogenic DOPE released most of their contents within 1 h of incubation with 10 mM dithiotreitol, a reductant with two sulfhydryl groups. After 36 h (37 °C) of incubation in serum, the leakage was less than 15%. A more sensitive disulfide lipid, mPEG-DTB-DSPE(77) was later reported that is labile to submillimolar concentrations of the amino acid cysteine as a milder thiolytic reagent. Tang and Hughes(78) also reported that DOGSDSO, a disulfide containing cationic lipid, mediated up to 50 times more transgene expression than that of its non-disulfide analog in cell culture.

Despite the above reports, little is known about the processing of the disulfide-containing liposomes in vivo, and the thiolytic mechanisms may be complicated. One possible mechanism is that cytosolic small molecules with free sulfhydryl groups, such as glutathione and cysteine, exist predominantly inside the cells(79) and hence would favor the triggered-release of the liposomes inside target cells. However, since liposomes are taken up by cells via endocytosis, it is questionable whether these reductive agents could encounter the liposomes in the

endosomal compartments. Another possible mechanism involves membrane-bound reductive enzymes. Ryser(80-83) and associates demonstrated that protein disulfide isomerase (PDI), a chaperone enzyme present in the ER and the plasma membrane of eukaryotic cells, plays a pivotal role in the thiolysis of molecules that do not diffuse through biomembranes. Inhibition of PDI eliminated the activity of disulfide-containing prodrugs such as diphtheria toxin(80). Similarly, disulfide-containing liposomes could be triggered by attaching to the cellular surface followed by PDI-catalyzed thiolysis. Therefore, it is important to include protein disulfide isomerase in future assays to evaluate the sensitivity of disulfide-containing liposomes towards redox-triggering. Given the observations that steric stabilization of liposomes inhibits their degradation by enzymes (see Section 1.5), more studies are needed to see if PDI-catalyzed thiolysis is a valid approach to trigger sterically stabilized liposomes.

1.7 Summary

Liposomal research has reached the stage where mechanisms of lipid phase changes are known in considerable details. Structural features of liposomes that offer prolonged circulation in the blood have also been established. Available as well, is more detailed information on the diversity of the stimuli that could be utilized for triggered release in drug delivery. Chemists can now exploit this information to design "smart" molecules that can be incorporated into environmentally sensitive liposomal formulations for improved drug and gene delivery in vivo.

CHAPTER TWO:

Steric Stabilization of Fusogenic Liposomes by a Low-pH Sensitive MethoxyPEG-Diortho Ester-Lipid Conjugate

2.1 Abstract

This chapter describes the synthesis and characterization of a pH-sensitive methoxypolyethylene glycol-diortho ester-distearoyl glycerol conjugate (POD, **1**). POD (**1**) was prepared by a one-step synthesis, and its acid sensitivity characterized by TLC. The conjugate was found to be stable at neutral pH for greater than 3 hours, but degraded completely within one hour at pH 5. Liposomes composed of 10% of POD and 90% of a fusogenic lipid, dioleoyl phosphatidylethanolamine (DOPE) were readily prepared and remained stable for up to 12 hours in neutral buffer as shown by Photon Correlation Spectrometry and a liposome contents leakage assay. However, when POD/DOPE liposomes were incubated in acidic pH as mild as 5.5, they aggregated and released most of their contents within 30 minutes. The kinetics of content release from POD/DOPE liposomes consisted of two phases, a lag phase and a burst phase. The lag phase is inversely correlated with pH and the logarithm of the length of lag phase showed a linear relationship with the buffer pH. When the POD/DOPE liposomes were incubated in 75% of fetal bovine serum at 37 °C, they remained as stable as traditional PEG-grafted liposomes for 12 hours but released 84% of the encapsulated ANTS in the following 4 hours. Upon intravenous administration into mice, liposomes composed of 10% POD and 90% DOPE were cleared from circulation by a one-compartment kinetics with a half-

life of about 200 minutes. POD is an example for the design of a novel category of pH-sensitive lipids composed of a headgroup, an acid-labile diortho ester linker and a hydrophobic tail. The uniquely fast degradation kinetics of POD at pH 5 to 6 and its ability to stabilize liposomes in serum make the conjugate suitable for applications for triggered drug release systems targeted to mildly acidic bio-environments such as endosomes, solid tumors and inflammatory tissues.

2.2 Introduction

Diacyl chain lipids represent an important class of amphipathic molecules that contain both a polar headgroup and a large hydrophobic moiety. In aqueous phase, such lipids self-assemble into colloidal particles such as liposomes, micelles and hexagonal phase. Liposomes are widely used as models of membrane bilayers and carriers of a variety of diagnostics and medicines(84). Examples of their use in drug delivery include PEG stabilized liposomes that carry cytotoxic drugs to leaky tumor tissues(18) and cationic liposomes that improve gene transfection(14).

Ideally, a targeted drug delivery system, including liposomes, should remain stable until it reaches the target site to minimize the premature loss of its payload(18,19). Upon accumulation at the target site, drug release needs to be at a high enough level for an effective therapeutic response. Since the decrease of pH is implicated in many physiological and pathological progressions such as endosome processing(5), tumor growth(6), inflammation(7) and myocardial ischemia(8), it has been extensively exploited to trigger the release of drugs from liposomal delivery systems in the past twenty years(9,44,45). Most of the reported pH-sensitive liposomes are based on the neutralization of excess negative charges on their surface upon protonation, which reduces the hydrodynamic diameter of lipid headgroups and triggers the change of lipid bilayers to hexagonal phases(9). However, at neutral pH, these excess negative charges induce undesired interactions with serum proteins and fixed macrophages, leading to rapid elimination of the liposomes from circulation(10,47). Efforts to circumvent this difficulty and provide a non-ionic pH-sensitive lipid have employed a neutral

monosaccharide as the headgroup, which is attached to long hydrophobic chains *via* an acetal moiety(61). However, these acetal analogs hydrolyzed relatively slowly at pHs 5 to 7 as found in physiological environment and need a pH less than 4 for rapid degradation. Recently, Thompson and co-workers have reported a number of acid- and light- sensitive lipids containing vinyl ether moieties(56,59,85). Although vinyl ethers represent a promising approach, their rate of hydrolysis at pH 5 is not optimal for rapid drug release. In order to develop a versatile strategy to prepare biocompatible liposomes, which are sensitive to small pH decrease, a novel class of lipids (Figure 2-1) is proposed, which possess an acid-labile diortho ester linker between the hydrophilic headgroup and the hydrophobic tail. In this case, a variety of headgroups and tails could be conjugated together to render pH-sensitive lipids of different properties.

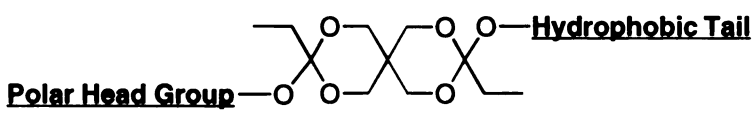
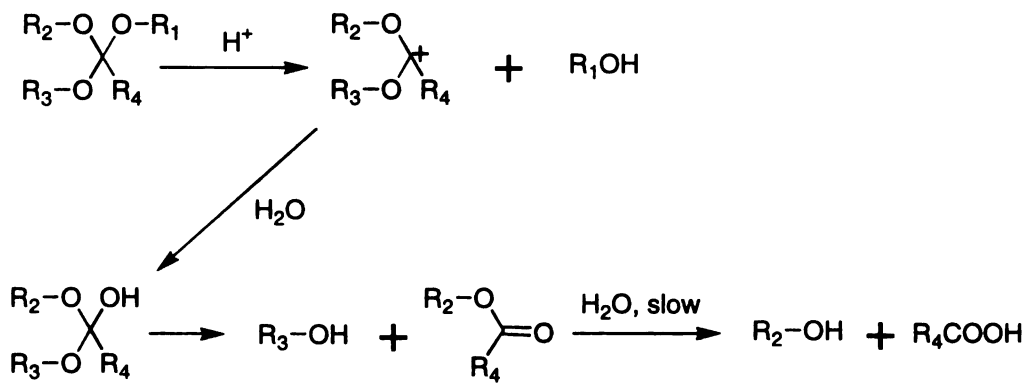


Figure 2-1: General structure of the diortho ester conjugates.

Ortho esters are relatively stable under basic and neutral conditions but hydrolyze quickly at acidic pH due to a stabilized dialkoxy carbocation intermediate (Scheme 2-1)(86-88). Heller and co-workers(89) have prepared a series of polymers based on the diortho ester moiety and exploited their pH-sensitive properties to develop sustained drug release systems. As an example of the proposed diortho ester lipidic conjugates, this chapter describes the design, preparation and characterization of a novel lipid (**1**, POD,

scheme 2-2) that consists of a methoxyPEG headgroup, a diortho ester linker and a distearoyl glycerol (**3**) hydrophobic tail. It is shown that POD can be used to prepare long circulating and acid-triggerable liposomes in combination with DOPE, a lipid that by itself does not form stable liposomes, but hexagonal phases at neutral pH.



Scheme 2-1: Mechanism of ortho ester hydrolysis.

2.3 Experimental Procedures

2.3.1 *General Techniques*

The diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane (**2**) was received as a generous gift from Dr. Jorge Heller at Advanced Polymer Systems (Redwood City, CA). Monomethyl ether of PEG (MW 2,000) was purchased from Shearwater Polymers, Inc (Huntsville, AL). Distearoyl glycerol (**3**) was purchased from Genzyme (Cambridge, MA). Triethylamine was purchased from Aldrich and redistilled under Ar before use. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylglycerol sodium salt (POPG), dioleoylphosphatidylethanolamine (DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-2000] (DSPE-PEG) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma. 8-Aminonaphthalene-,2,3-trisulfonic acid (ANTS) and *p*-Xylenebis(pyridinium) bromide (DPX) were from olecular Probes, Inc. (Junction City, OR). MilliQ water, which had a pH of roximately 8 when freshly prepared, was used to prepare all the aqueous buffers. All r chemical reagents and solvents were purchased from Aldrich or Fisher. Ratios of onents in chromatography solvent systems are in volume unless stated otherwise. of lipid components in liposomes are in mole units.

^{1}H-NMR spectra were recorded on an Oxford AS 400. Electrospray mass spectra S) were recorded on a Sciex (PE, Foster City, CA) at the Mass Spectrometry es, University of California at San Francisco. Fourier transformed infrared spectra

were measured with a Nicolet Impact 400. The FT-IR samples were applied as a $CHCl_3$ solution onto the surface of a NaCl crystal, dried under Ar, and subsequently analyzed. Elemental analysis was carried out by Microanalytical Lab at College of Chemistry, University of California at Berkeley.

2.3.2 *3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(Ω-methoxy-polyethylene glycol2000-1-yl)-2,4,8,10-tetraoxaspiro[5,5]undecane (POD, 1)*

One gram of PEG (2000) monomethyl ether (0.5 mmol) and 312.5 mg of distearoyl glycerol (**3**, 0.5 mmol) were dissolved in 5 mL of anhydrous THF under Ar. A heat gun was used to melt 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane (**2**) and 100 μL (110 mg, 0.5 mmol, d = 1.1) of the melted compound was taken by a dry syringe and injected into the THF solution. One drop (~40μL) of 0.6 mg/mL *p*-toluenesulfonic acid in anhydrous THF was added and the reaction mixture was stirred at 40 °C under Ar for 2 hours. The reaction was stopped by adding 200 μL triethylamine followed by quenching the reaction into 30-fold volume excess of 1% volume of triethylamine in methanol. Four grams of silica gel were added to the solution and the mixture was evaporated under reduced pressure. The residue was poured onto a silica gel column (45 grams) equilibrated with the eluting solvent system (triethylamine/chloroform = 1/50). The column was eluted and fractions corresponding to the product were pooled, evaporated, and dried in high vacuum to give 275 mg (20%) purified product (**1**). TLC R_f 0.4 in $CHCl_3$/MeOH/30% w/w NH_4OH (100:15:1); FTIR 2910 cm^{-1} (CH_2 and CH_3),

2850 cm^{-1} (CH_2 and CH_3), 1743 cm^{-1} (Ester C=O), 1109 cm^{-1} (PEG and ortho ester C-O); ^1H NMR (400 MHz, $CDCl_3$, chemical shifts relative to TMS signal) δ 5.22 (1H, m, glycerol methine), 3.7-4.2 (4H, m, glycerol methylene), 3.0-3.7 (~170 H, m, OCH_2 and OCH_3), 2.2-2.4 (4H, m, CH_2COO), 1.65-1.76 (4H, m, CH_2CH_2COO), 1.54-1.65 (4H, m, CH_2CH_3 on spiro rings), 1.04-1.36 (56H, m, $CH_3(CH_2)_{14}CH_2CH_2COO$), 0.82-0.97 (12H, m, CH_2CH_3); ESIMS, calcd for $[M+Na]^+$ with 36-48 CH_2CH_2O units from methoxyPEG: $C_{123}H_{240}O_{46}Na$ 2476.6, $C_{125}H_{244}O_{47}Na$ 2520.7, $C_{127}H_{248}O_{48}Na$ 2564.7, $C_{129}H_{252}O_{49}Na$ 2608.7, $C_{131}H_{256}O_{50}Na$ 2652.7, $C_{133}H_{260}O_{51}Na$ 2696.8, $C_{135}H_{264}O_{52}Na$ 2740.8, $C_{137}H_{268}O_{53}Na$ 2784.8, $C_{139}H_{272}O_{54}Na$ 2828.8, $C_{141}H_{276}O_{55}Na$ 2872.9, $C_{143}H_{280}O_{56}Na$ 2916.9, $C_{145}H_{284}O_{57}Na$ 2960.9, $C_{147}H_{288}O_{58}Na$ 3004.9, found 2478.3 (33%), 2521.1 (47%), 2565.3 (62%), 2609.3 (77%), 2654.2 (94%), 2697.3 (100%), 2741.4 (98%), 2785.3 (93%), 2830.4 (85%), 2874.4 (60%), 2917.5 (46%), 2962.4 (37%), 3006.4 (26%). Anal. Cald. for ($C_{135}H_{264}O_{52}$, C 59.62, H 9.78; $HC_{137}H_{268}O_{53}$, C 59.54, H 9.77; $C_{139}H_{272}O_{54}$, C 59.46, H 9.76; $C_{141}H_{276}O_{55}$, C 59.39, H 9.76; $C_{143}H_{280}O_{56}$, C 59.31, H 9.75; $C_{145}H_{284}O_{57}$, C 59.24, H 9.74; $C_{147}H_{288}O_{58}$, C 59.17, H 9.73) found C 59.16, H 9.57, N<0.2.

2.3.3 *Thin Layer Chromatography (TLC) Analysis of POD Degradation in Buffers of Different pHs at 37°C*

Conical polystyrene microcentrifuge tubes (0.5 mL) containing 20 µL 100 mM sodium phosphate buffer of different pHs were pre-warmed at 37°C for 30 min and silica

gel TLC plates (Whatman® from Whatman Ltd, Maidstone, Kent, England) were pre-equilibrated with the solvent system ($CHCl_3$/MeOH/ NH_4OH = 100:15:1) by running the plates without samples followed by brief air-drying. Eight milligrams of POD was dissolved into 100 μL water by vortexing and 20 μL of the solution was added into each of the pre-warmed tubes and mixed thoroughly. The tubes were incubated at 37°C and 10 μL of the solutions were aliquoted at different time points and mixed with 10 μL of 10% concentrated NH_4OH (30% w/w) in acetone to stop the hydrolysis. Five microliters of the mixture was spotted onto pre-equilibrated TLC plates. Three microliters of distearoyl glycerol (**3**), monomethyl ether of PEG2000 and POD (**1**) in 10 mg/mL $CHCl_3$ solution were also spotted as standards. TLC plates were developed, heated at 150 °C for 5 min and stained in I_2 chamber for 1 h to observe the locations of POD and its degradation products.

2.3.4 *Liposome Preparation for Aggregation and Leakage Assays*

Reverse-phase vesicles (REV) were prepared as described previously(90) in 50 mM ANTS, 50 mM DPX and 5 mM HEPES at pH 8.5. The vesicles were extruded 5 times through a 0.2-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) through a hand held extrusion device (Avestin, Ottawa, Ontario, Canada). A Sephadex G-75 column was used to separate vesicles from unencapsulated material with an elution buffer composed of 5 mM HEPES and 145 mM NaCl, pH 8.5. All freshly prepared liposomes had mean diameters ranging from 180 to 200 nm (cumulant results) and a

polydispersity index of less than 0.2 as measured by a Malvern Zeta1000 Dynamic Light Scattering Instrument using the PCS 1.32a software. The encapsulated volume was 0.25-0.3 μL/μmol total lipid. Lipid concentrations were determined based on lipid phosphorus by a modification of the Bartlett method(91).

2.3.5 *Liposome Aggregation Assay*

Conical polystyrene microcentrifuge tubes (0.5 mL) containing 300 μL of buffers of various pH (50 mM NaOAc/HOAc and 100 mM NaCl for pH < 6; 50 mM NaH_2PO_4/ Na_2HPO_4 and 100 mM NaCl for pH 6 and above) were incubated at 37 °C for 30 min. The liposome preparation was warmed to room temperature and an aliquot (100 μL) was added to each tube and mixed thoroughly. The tubes were incubated at 37 °C for different time periods and 30-40 μL of the mixtures were aliquoted and transferred into disposable polystyrene fluorimeter cuvettes containing 1.5 mL 100 mM $NaHCO_3$/50 mM NaCl buffer at pH 9. The samples were mixed by gently inverting the cuvettes 2 to 3 times.

Photon Correlation Spectrometry of the samples was then measured with a Malvern apparatus (Zeta 1000) to determine the size distribution of the liposomes. Typically, three measurements of 2-5 min were made for each sample, using the automatic algorithm mode for data analysis. The average of the three cumulant results of each sample that agreed to within 10% is reported in the paper.

2.3.6 *ANTS/DPX Leakage Assays*

The ANTS/DPX assay(46) was used to monitor leakage of ANTS from liposomes. Fluorescence measurements were made on a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source. Excitation was at 370 nm (1.25 mm slit). The 90° emission signal at 550 nm (5 mm, 5 mm slits) resulting from the dequenching of ANTS released out of liposomes was observed through a Corning 3-68 nm cutoff filter (>530 nm). One data point of fluorescent intensity was collected each second except for pH 7 and 7.4, where measurements were taken every 30 minutes and the samples were incubated in dark between the measurements to minimize the exposure of the sample to the excitation light source. The raw fluorescent data were converted into ASCII data files and processed mathematically by Microsoft Excel. The residual fluorescence of the liposomes at the starting time (t_o) of leakage experiments, F_0, was set as 0% release. At the end of each leakage experiment the liposomes were lysed with the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$) and the maximal fluorescence thus obtained, F_{100}, was taken as 100% release. The leakage of ANTS at a particular time point was then determined by the formula: Percentage of Leakage = $(Ft- F_0)/(F_{100}- F_0)$ x 100, where Ft corresponds to the fluorescence intensity observed at the time point.

In leakage experiments of POD/DOPE liposomes in aqueous buffers of different pHs, the assays were started by injecting small volumes (5-10 μL, using a Hamilton syringe) of concentrated liposome suspensions into a magnetically stirred quartz cuvette containing 2 mL of the buffer (50 mM NaOAc/HOAc and 100 mM NaCl, pH < 6; 50

mM NaH_2PO_4/ Na_2HPO_4 and 100 mM NaCl, pH 6 and above) at 37 °C. The starting time, t_o was set 5 to 10 seconds after the injection when the fluorescence signal first became stable.

In serum stability assays, aliquots of fetal bovine serum (180 μL in each tube) in 0.6 mL tubes were pre-warmed at 37 °C for 30 min. An aliquot of stock liposome solution was diluted to 2 mM of total lipid by phosphate buffered saline (50 mM NaH_2PO_4/ Na_2HPO_4 and 100 mM NaCl, pH 7.4), which had been pre-warmed at 37 °C for 30 min. An aliquot of the diluted liposome solution (60 μL) was then mixed with 180 μL pre-warmed fetal bovine serum to give a 75% final serum concentration. At different time points, a 20 μL aliquot was added into a polystyrene fluorimeter cuvette, containing 2 mL of HEPES buffered saline (5 mM HEPES and 145 mM NaCl, pH 8.5) at room temperature. The cuvette was covered with parafilm and inverted gently for 3-4 times. The fluorescence thus measured subtracted by the fluorescence of the buffer before the addition of the sample was taken as *Ft*. The *Ft* of an aliquot extracted and measured immediately after mixing a liposome sample with fetal bovine serum was taken as F_0. At the end of each assay an aliquot of the liposome-serum mixture was pipetted into 2 mL of HEPES buffered saline (5 mM HEPES and 145 mM NaCl buffer, pH 8.5), followed by adding the detergent dodecyloctaethylene glycol monoether ($C_{12}E_8$). The fluorescence measured after adding the detergent minus the fluorescence of the buffer was taken as F_{100}. Each data point of the leakage assay in fetal bovine serum represents the average and standard deviation of three independent samples.

2.3.7 *Animals*

Blood clearance, distribution and excretion studies were conducted on 4-week-old female ICR mice (about 25 grams in weight) purchased from Simonsen (Gilroy, CA). All animals were handled in accordance with protocols established by the National Institute for Health Guidelines for the Care and Use of Laboratory Animals and with the approval of the Committee for Animal Research at the University of California, San Francisco. Animals were sacrificed at stated times with a sodium pentobarbital overdose.

2.3.8 *Preparation of ^{125}I Labeled Liposomes for Animal Studies*

p-Hydroxybenzamidine phosphatidylethanolamine (BPE) was synthesized and radiolabeled with $Na^{125}I$ as previously described(92) and the purity assessed by thin-layer chromatography. The labeled lipid contained less than 0.5% of free ^{125}I. The labeled lipid, in chloroform solution, was added to POD/DOPE (1/9) or DSPE-PEG/DOPE (1/9) mixture in chloroform to yield 8.3 x 10^{11} dpm/mol total lipid. Chloroform was evaporated under reduced pressure and the resultant lipid film was placed under high vacuum for 2 hours to remove the remaining solvent. The dried lipid film was hydrated with HEPES buffered saline (10 mM HEPES and 145 mM NaCl, pH 7.4) over 20 min by intermittent agitation on a vortex mixer and the suspension extruded three times through a 0.1 μm polycarbonate membrane. The diameter of the extruded liposomes, as measured by Photon Correlation Spectrometry, was 140-160 nm. Each mouse received a

tail-vein injection of 150 μL of the extruded liposomes containing 0.9 μmol total lipid.

2.3.9 *Blood Clearance Assay*

At various time points following administration of ^{125}I labeled liposomes, animals were anesthetized with inhalation of isofluorane. Approximately 50 μL of blood was collected with a glass pasteur pipette from the orbital sinus vein, weighed and its γ-activity measured in Beckman Gamma 8000 (Fullerton, CA). The γ-activity in whole blood and hence, the percentage of dose remaining in the blood was calculated assuming 0.07 g of blood per gram of animal weight (check the following website for information: http://www.larc.ucsf.edu/Docs/PRIVATE/SpeciesSpecific/MouseCombo.cfm). The mean and standard deviation of samples collected from three animals were presented.

2.3.10 *Tissue Distribution Assay*

At various time points following administration of ^{125}I labeled liposomes, animals were anesthetized with an intraperitoneal (i.p.) injection of 100 μL anesthetics cocktail (44 mg/kg ketamine, 2.5 mg/kg xylazine and 0.75 mg/kg acepromazine). Approximately 1 mL of blood was removed *via* intracardiac puncture. One milliliter of phosphate-buffered saline (containing 0.2 g/L KH_2PO_4, 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$, 0.2 g/L KCl and 8.0 g/L NaCl) was perfused through the right cardiac ventricle. The heart, lungs, liver,

spleen, kidneys, stomach, intestines, head, mid section and tail were collected, weighed and the radioactivity counted in the Beckman Gamma 8000. The γ-activity in whole blood and hence the percentage of dose remaining in the blood was calculated assuming 0.07 g of blood per gram of animal weight. The average and standard deviation of samples from three animals were reported.

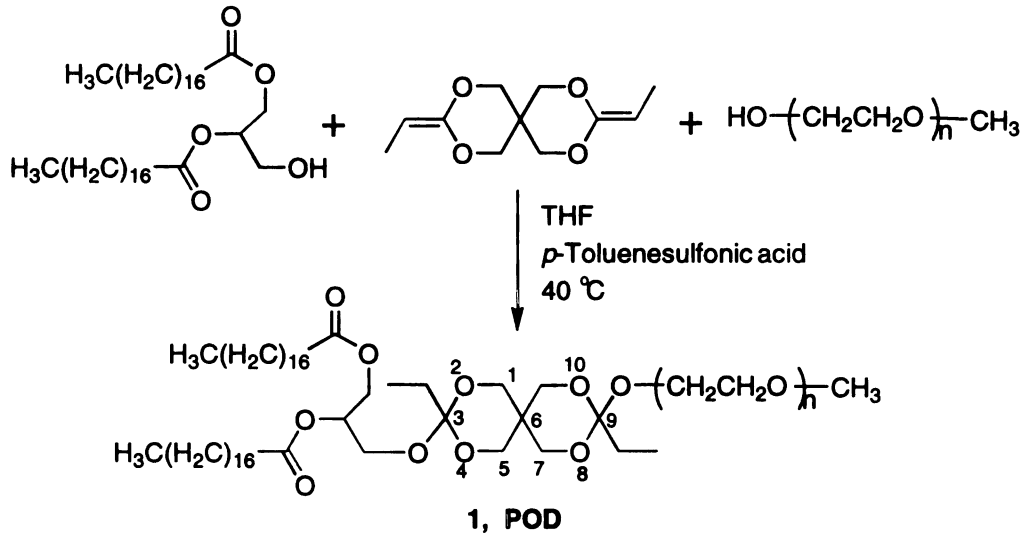
2.4 Results

2.4.1 *Design and Synthesis of MethoxyPEG-Diortho Ester-Distearoyl Glycerol Conjugate (POD, 1)*

In order to devise a lipid that degrades specifically in a low-pH environment but is inert under neutral physiological conditions, methoxyPEG is selected as the headgroup since it is one of the most stable synthetic polymers *in vivo* and liposomes coated by PEG have a prolonged circulation period(12). The monomethyl ether of PEG-2000 was used for synthesis to preclude cross-linking or polymerization. The 3,9-diethyl-2,4,8,10-tetraoxaspiro[5,5]undecane moiety was chosen as the diortho ester linker, based on previous research by Heller and co-workers(93), who showed its pH sensitivity and biocompatibility in polymeric drug delivery systems. Distearoyl glycerol (**3**), with two saturated hydrocarbon side chains, is attached to the diortho ester linker as the hydrophobic tail to anchor the conjugate into lipid bilayers(94,95). If the conjugate is incorporated into liposomes of unsaturated phosphatidylethanolamine, distearoyl glycerol (**3**) would be regenerated in the bilayers upon hydrolysis, and would favor the formation of hexagonal phases due to its conical structure(96).

POD was prepared by a one-step synthesis as illustrated in Scheme 2-2. The monomethyl ether of PEG2000 and distearoyl glycerol (**3**) were dissolved in dry THF under atmospheric Ar. The solid diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5,5]undecane (**2**) was melted with a heat gun under atmospheric Ar and

added with a syringe to the THF solution to avoid contact with air. The reaction was then initiated by adding a trace amount of *p*-toluenesulfonic acid dissolved in dry THF. The mixture was stirred at 40 °C under Ar for 2 h and the reaction was stopped by addition of triethylamine. Silica gel chromatography with a solvent system containing 2% triethylamine as a stabilizer afforded the purified conjugate (20% synthetic yield) that appears homogeneous on TLC. Although the yield of the reaction was modest, this synthetic route is chosen since the method is quick and the reagents are relatively inexpensive.



1, POD

Scheme 2-2: Synthesis of methoxyPEG-diortho ester-distearoyl glycerol conjugate (POD, **1**).

It should be noted that POD is actually a mixture of conjugates with the methoxyPEG headgroup comprising of a narrow distribution of molecular weights

around 2000, due to the polydisperse nature of the commercially available PEG2000 monomethyl ether. The electrospray mass spectrometry showed a distribution of peaks centered on 2697 dalton, corresponding to mono-sodiumated molecular cations with different numbers of ethylene glycol units. The heterogeneity of methoxyPEG as well as the stereochemistry of the conjugate also resulted in NMR peaks of POD appearing as multiplets, a feature which has been previously reported in the literature(97).

2.4.2 *Acid Sensitivity of POD*

In order to quickly estimate the sensitivity of the conjugate toward mildly acidic conditions relevant to biological scenarios, a simple TLC assay was employed to monitor the degradation of POD after it was incubated for different time periods at 37 °C in buffers of pH ranging from 4 to 7.4. As shown in Figure 2-2, within one hour of incubation at 37 °C, POD was completely degraded in buffers of pH 4 and 5 as indicated by the disappearance of the compound spot at R*f* ~0.4. However, in the buffer of neutral pH 7.4, most of the conjugate remained intact over 3 hours. In the buffer of pH 6, POD degraded with an approximate half-life of 2.5 hours. As expected from the acid-catalyzed hydrolysis illustrated in the introduction section, the degradation of POD yielded both more hydrophilic (R*f*~0.3) and more hydrophobic (R*f*~0.8) materials, presumably derivatives of PEG and distearoyl glycerol (**3**), respectively. The hydrophilic degradation products gave more intensely stained spots than the hydrophobic derivatives, because PEG derivatives are much more sensitive to iodine staining then are the saturated

acyl chains. Similar results were obtained when the TLC plates were stained with Molybdate reagent, which clearly showed the hydrophobic degradation products.

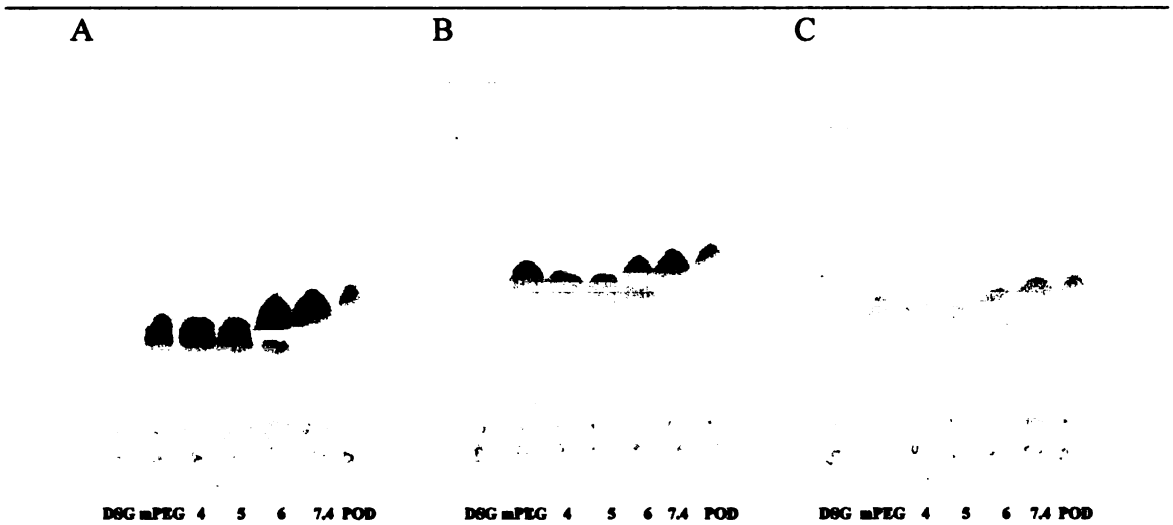


Figure 2-2: TLC monitored degradation of POD. Lipids were incubated for the period of 1 h (A), 2 h (B) and 3 h (C) in Na_2HPO_4 buffers of the specified pH. POD, mPEG and DSG in $CHCl_3$ solution were spotted alongside as standards.

2.4.3 *Liposome Formation*

Given the promising results of its degradation, POD was mixed with a high percentage of DOPE, a fusogenic lipid, in order to see if the lipid mixture can self-assemble into lamellar structures in neutral and alkaline solutions, and yet undergo phase changes upon cleavage of the methoxyPEG headgroups by hydrolysis at low pH. ANTS and DPX(46) were encapsulated into liposomes by reverse phase evaporation(90) and their leakage from the vesicles were used to monitor pH triggered release of liposomal

contents. Liposomes of defined size were prepared by extruding 5 times through polycarbonate membranes of 200 nm pore diameter. Unencapsulated ANTS/DPX was then removed by size exclusion chromatography. Liposomes composed of 10% POD and 90% DOPE were readily prepared and remained stable for up to two weeks in an alkaline HBS buffer (5 mM HEPES, 145 mM NaCl, pH 8.5) at 4 °C with no significant increase in particle size or in residual ANTS fluorescence.

2.4.4 *Acid Triggered Liposome Aggregation*

When pH-sensitive liposomes composed of phosphatidylethanolamine (PE) are subjected to low pH, the PE lipids undergo a contact-induced aggregation and a phase transition from the lamellar to the hexagonal phase(46). PEG lipids at a high enough concentration on the liposome surface can shield the PE lipids of one liposome from contacting those of another liposome, thus preventing aggregation and membrane-destabilizing phase changes. Thus one can follow the loss of the PEG coating from the liposome surface as a result of POD hydrolysis by monitoring the aggregation of POD/DOPE liposomes at different pHs with Photon Correlation Spectroscopy (PCS). However, as indicated by the TLC assays, the kinetics of the aggregation, particularly at pH below 5.5, may be too fast to be monitored by PCS, which takes about 10 to 20 min for a triplicate measurement. In order to circumvent this problem, the aggregation process was "frozen" by quenching aliquots of samples in excess buffer of pH 9. The quenching stopped the acid-catalyzed POD hydrolysis and prevented the liposomes/lipid

particles from contacting each other by inducing negative charges to their surfaces *via* deprotonation of the amine in DOPE. The "base-freezing" was found to be a valid method to prevent size increases in the aggregation products. To determine if quenching of the reaction to pH 9 resulted in dispersion of aggregates into smaller particles, aliquots of liposomes under incubation at pH 6 were diluted in the same pH 6 buffer. The particle size of the liposomes was then immediately determined by a single PCS measurement of two minutes. The size data thus obtained were not significantly different from the diameters measured after "base-freezing". Furthermore, the samples quenched at pH 9 gave the same PCS readings after being left overnight at room temperature. Thus the base quenching yielded a reproducible value that reflected the particle size distribution at the quench time. Therefore, the light scattering of the aliquots quenched at pH 9 was recorded and the particle diameter from the cumulant results was plotted against incubation time in Figure 2-3.

The aggregation process showed a pH dependent lag phase followed by a rapid increase in diameter (Figure 2-3). At pH 7.4, the POD/DOPE liposomes were relatively stable and the aggregation did not occur after more than 10 hours of incubation at 37 °C. In buffers of lower pH, the lag phase was much shorter. At pH 6.2, the liposome size started to increase after 1 hour and when the pH was decreased to 5, extensive aggregation was observed within 10 min. Thus, the kinetics of the liposome aggregation correlated with that of pH-dependent POD degradation shown by the TLC studies. These results demonstrate the ability of POD to protect the DOPE-containing liposomes from aggregating and converting into hexagonal phases until the methoxyPEG headgroups are removed from the surface of the liposomes at low pH.

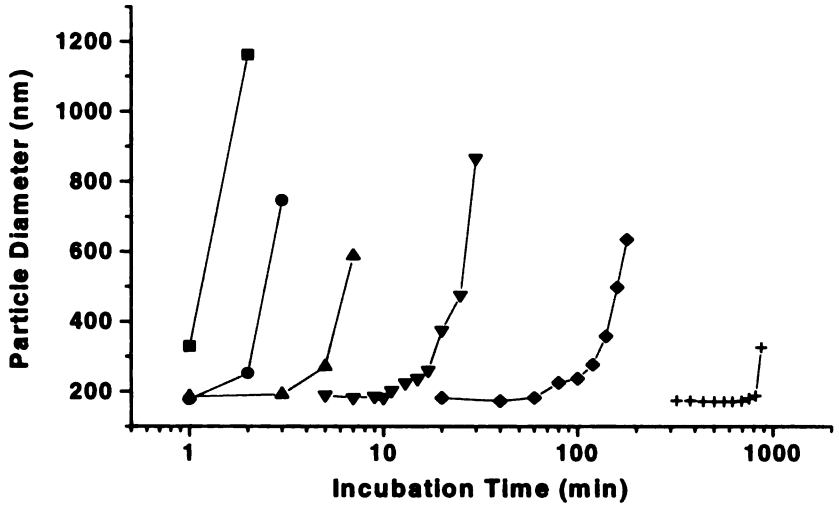


Figure 2-3: Aggregation of POD/DOPE (1/9) liposomes in buffers of different pHs. (■) pH 4; (●) pH 4.5; (▲) pH 5; (▼) pH 5.5; (◆) pH 6.2; (+) pH 7.5.

2.4.5 *Acid Triggered Content Leakage*

Since POD was designed for potential applications in liposomal drug delivery, POD/DOPE liposomes containing ANTS/DPX was used as a model system to examine if the hydrolysis of POD can trigger the release of the contents from DOPE liposomes in response to a low pH environment. The ANTS/DPX method is often used to study pH-sensitive liposomal leakage since the fluorescence of ANTS is virtually unchanged from pH 4 to 8(46). When both compounds remain encapsulated, the fluorescence of ANTS is

quenched by DPX(46). Upon leakage from the liposomes, ANTS is no longer quenched by DPX and gives an increase of fluorescent signal at 550 nm (excitation wavelength = 370 nm). As shown in Figure 2-4A, the release of ANTS at different pHs also occurs in two distinct phases, a lag phase, where a small portion of ANTS slowly leaks out of the liposomes, followed by a burst phase, when most of the ANTS is quickly released. The burst phase correlates well with the aggregation of the POD/DOPE liposomes in Figure 2-3 and the collapse of the lamellar phase. As the liposomes are subjected to more acidic environments, the lag phase shortens. At pH 7.4, the lag phase lasts approximately 12 hours; at pH 6, the lag phase is reduced to 60 min; at pH 5, the lag phase is less than 5 minutes. As a control, ANTS/DPX was encapsulated into liposomes composed of 90% DOPE and 10% of a pH-*insensitive* PEG lipid, DSPE-PEG. Less than 5% of the encapsulated ANTS was released over 12 hours when the vesicles were treated in the same way as the POD/DOPE liposomes even at the lowest pH. This confirms the role of acid catalyzed hydrolysis of the methoxyPEG-diortho ester-DSG conjugate in the leakage of the POD/DOPE liposomes.

In Figure 2-4B, the logarithm of the length of lag phase in Figure 2-4A is plotted against buffer pH from 4 to 6 and a clear linear relationship ($r = 0.984$) is observed. The lag time at pH 7.4 is not plotted in the graph since the transition is too slow to allow a precise determination. The slope of the regressed line is 0.883. This linear relationship demonstrates that the buffer pH plays a pivotal role in the kinetics of liposomal leakage. This phenomenon is discussed in detail in the discussion section, where a mathematical model is proposed to account for the data.

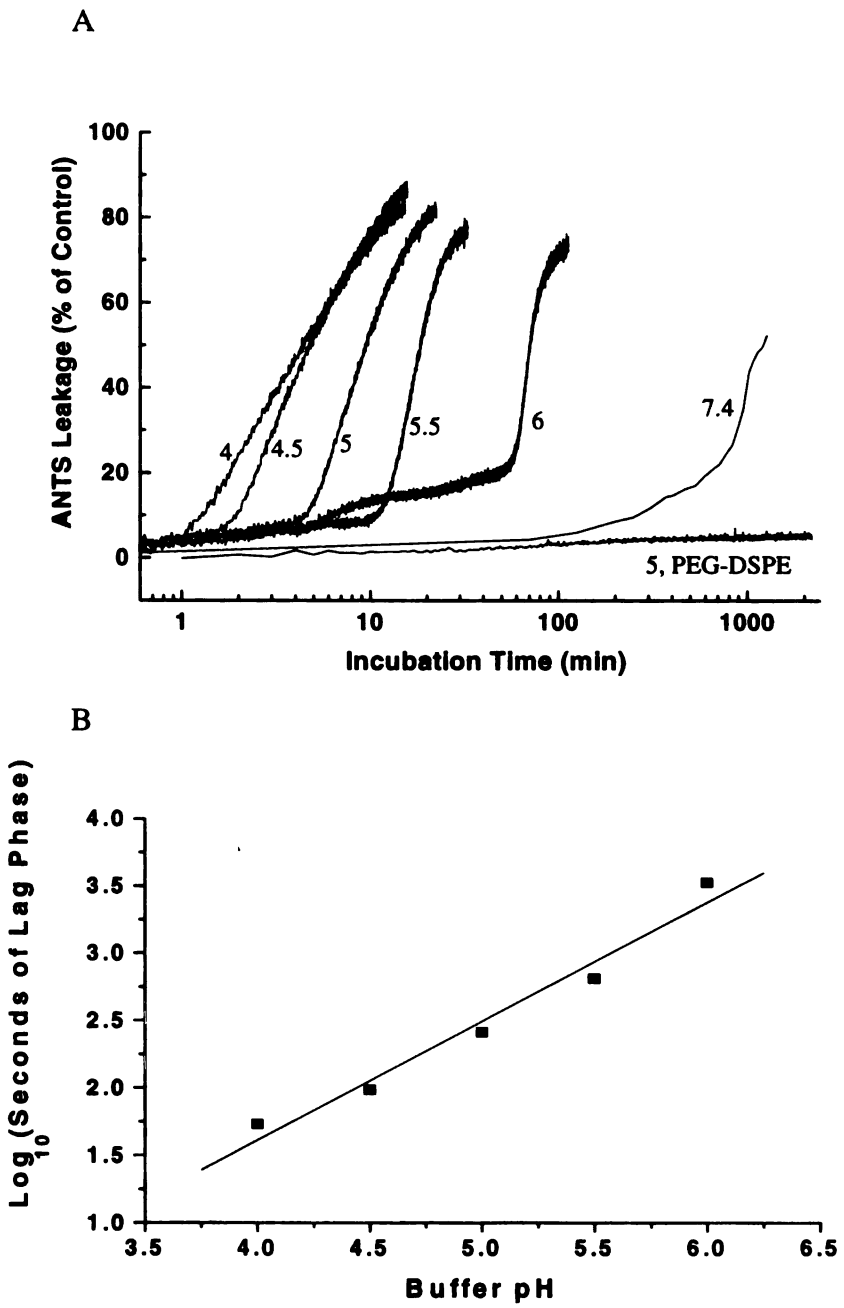


Figure 2-4: Acid triggered content release from POD/DOPE (1/9) liposomes. A. ANTS leakage in buffers of different pHs. The pH is indicated adjacent to the corresponding trace. DSPE-PEG/DOPE (1/9) liposomes were incubated at pH 5 as a control. B. Length of lag phase in buffers of different pHs. The logarithm of the length of lag phase in seconds is regressed versus the buffer pH to give: Log_{10}(Seconds of Lag Phase) = -1.92 + 0.883 x pH, r = 0.984.

2.4.6 *Stability of POD Containing Liposomes in Serum*

In order to test the idea that POD should help stabilize liposomes in circulation, an *in vitro* stability test was carried out in fetal bovine serum. POD/DOPE (1/9) liposomes containing ANTS/DPX were incubated with 75% fetal bovine serum at 37 °C and the release of ANTS was measured every 4 hours for 16 hours (Figure 2-5). For comparison, ANTS/DPX was also encapsulated into liposomes composed of DSPE-PEG/DOPE (1/9), CHEMS/DOPE (3/7), POPG/POPC/Chol (1/9/8) and the ANTS leakage was monitored in the same manner.

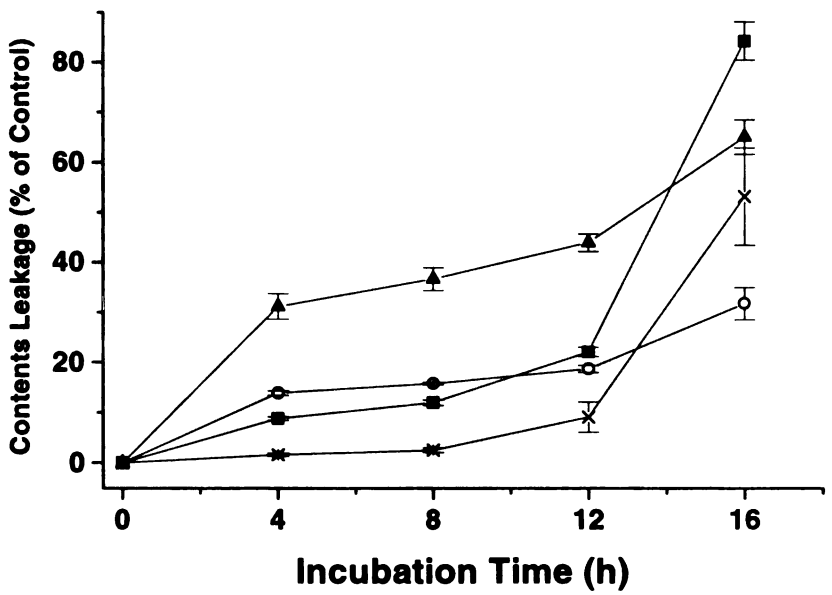


Figure 2-5: Contents leakage from liposomes of various compositions in 75% fetal bovine serum. ANTS leakage was monitored after the liposomes were incubated in 75% fetal bovine serum at 37 °C for different time periods. (■) POD/DOPE (1/9); (O) DSPE-PEG/DOPE (1/9); (▲) CHEMS/DOPE (3/7); (X) POPG/POPC/Chol (1/9/8).

POD/DOPE liposomes were found to be relatively stable over 12 hours, when less than 25% of the ANTS leaked out. However, after 16 hours of incubation, 84% of the ANTS was released, indicating a transition from the lag phase to the burst phase. The POD/DOPE liposomes were as stable as the pH-insensitive, PEG-stabilized DSPE-PEG/DOPE liposomes for the first 12 hours, but released ANTS much faster for the next 4 hours, indicating a liposome destabilization process specifically mediated by POD hydrolysis. In the first 12 hours of incubation, the POD/DOPE liposomes released 2 to 3 fold less ANTS than CHEMS/DOPE liposomes, whose excess negative charges from CHEMS tend to induce more interactions with serum components(10). The stable liposome formulation composed of POPG/POPC/Chol released less than 10% of the contents after 12 hours of incubation with serum(98). Thus, POD is able to stabilize liposomes in fetal bovine serum as effectively as traditional PEG-derived lipids for up to 12 hours, which is consistent with our findings on ANTS leakage of these liposomes in HEPES buffered saline. (Figure 2-4)

2.4.7 *Stability of Liposomes in Blood Circulation*

The steric-stabilizing effect of POD on liposomes *in vivo* was evaluated by tail-vein injection of radiolabeled liposomes composed of 10% POD and 90% of DOPE into female ICR mice and measuring their rate of elimination from the blood (Figure 2-6). For comparison, a group of control animals were injected with radiolabeled liposomes composed of 10% DSPE-PEG, a pH-insensitive lipid-PEG conjugate, and 90% DOPE. Liposomes composed of pure DOPE are not stable enough at pH 7.4 to allow their injection into animals. For both POD/DOPE and DSPE-PEG/DOPE liposome formulations, the percentage of injected dose remaining in circulation showed a single log-linear decay with a correlation coefficient constant greater than 0.995, indicating a one-compartment clearance kinetics. The blood clearance profile of the POD/DOPE liposomes is similar to the pH-insensitive, sterically stabilized DSPE-PEG/DOPE liposomes than to conventional liposomes, which usually exhibit a two-compartment elimination kinetics(99). The half-life of the POD/DOPE liposome and the DSPE-PEG/DOPE liposome is about 194 and 295 minutes, respectively. This reasonably close correspondence of the elimination rate between the two formulations is strong evidence that the methoxyPEG headgroup in the pH-sensitive ortho ester conjugate provides a steric-stabilizing effect on the fusogenic DOPE liposomes in circulation.

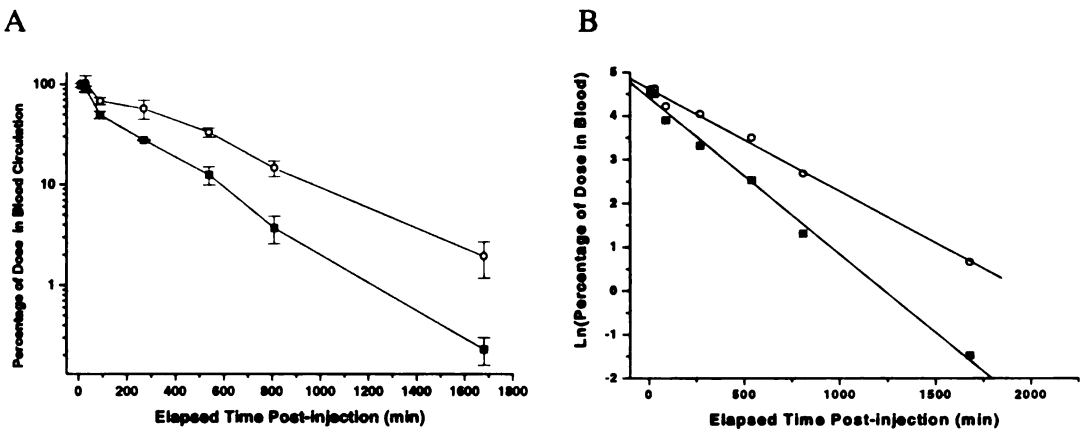


Figure 2-6: Clearance of ^{125}I-BPE POD/DOPE (1/9) liposomes (■) and ^{125}I-BPE DSPE-PEG/DOPE (1/9) liposomes (O) from circulation. A. The percentage of the injected dose remaining in blood (n = 3, mean ± S.D.) is plotted against the elapsed time post-injection. B. The natural logarithm of the percentage of dose remaining in blood is plotted against elapsed time post-injection and regressed to obtain the liposomal half-life. For POD/DOPE (1/9) liposomes, Ln(Percentage of Dose in Blood) = 4.41 - 0.00358 x (min), r = 0.997, $T_{1/2}$ = Ln2/0.00358 = 193.6 min. For DSPE-PEG/DOPE (1/9) liposomes, Ln(Percentage of Dose in Blood) = 4.62 - 0.00235 x (min), r = 0.997, $T_{1/2}$ = Ln2/0.00235 = 295.0 min.

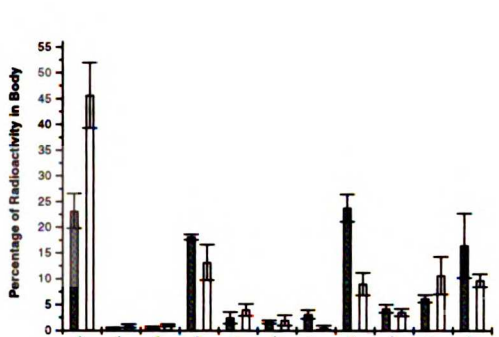
2.4.8 *Distribution and Excretion of Liposomes*

To investigate the distribution of radiolabeled POD/DOPE liposomes in more detail, the radioactivity associated with different organs of mice was measured at 270 minutes, 810 minutes and 28 hours following liposome injection. In previous studies with the ^{125}I-labeled BPE(92), it is known that this radioactive lipid label is metabolized only after being endocytosed together with the liposomes. The urine and the feces were also collected at the 28-hour time point and their radioactivity measured to determine the routes of ^{125}I excretion. At 270 minutes post-injection (Figure 2-7A), about 20% of

POD/DOPE liposomes and 45% of DSPE-PEG/DOPE liposomes remained in circulation. For both formulations, the liver and intestine were the two major organs of radioactivity disposition.

At later time points (810 minutes and 28 hours post-injection), most of the radioactivity of both formulations was excreted from the animals (Figure 2-8); the liver and intestine were the major organs for the disposition of the remaining radioactivity in the body (Figure 2-7B, 2-7C). Twenty-eight hours after injection, the residual radioactivity from POD/DOPE liposomes was distributed preferentially to the head (Figure 2-7C), probably due to an accumulation of the iodine in the thyroid followed by a slow clearance of ^{125}I from this organ. For both formulations at 28 hours post-injection, about 70% of radioactivity was excreted into urine and about 20% was found in feces (Figure 2-9). Overall, POD/DOPE liposomes were eliminated from circulation about 50% faster than DSPE-PEG/DOPE liposomes but the patterns of distribution and excretion of the two formulations were very similar.

A



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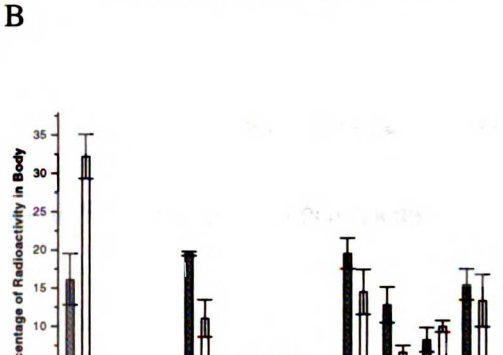

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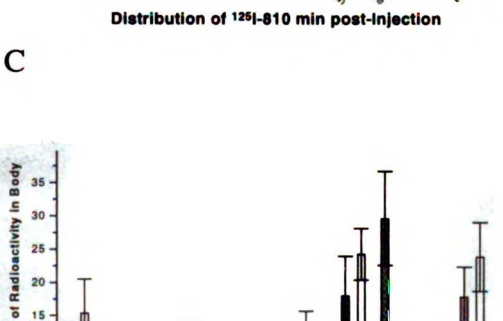


Figure 2-7: Distribution of ^{125}I radioactivity of POD/DOPE (1/9) (solid bars) and DSPE-PEG/DOPE (1/9) (open bars) liposomes in female ICR mice at 270 min (A), 810 min (B) and 28 h (C) post-injection. (n = 3, mean ± S.D.)

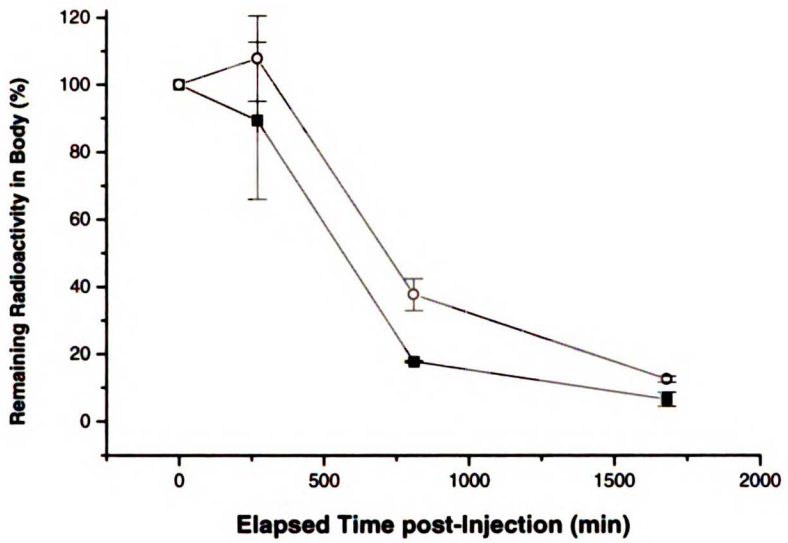


Figure 2-8: Excretion kinetics of ^{125}I radioactivity of POD/DOPE (1/9) (■) and DSPE-PEG/DOPE (1/9) (O) liposomes.

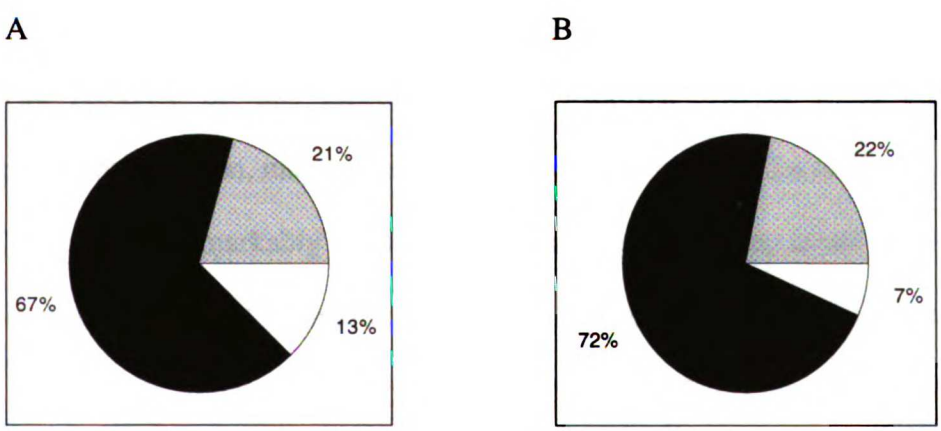


Figure 2-9: Mass balance of injected radiolabeled liposomes. ^{125}I radioactivity of POD/DOPE (1/9) (A) and DSPE-PEG/DOPE (1/9) (B) liposomes from all parts of body (white), urine (black) and feces (gray) were measured 28 hours post-injection and plotted in percentage of the total radioactivity recovered. The percentage of injected dose recovered is 110% for POD/DOPE liposomes and 93% for DSPE-PEG liposomes.

2.5 Discussion

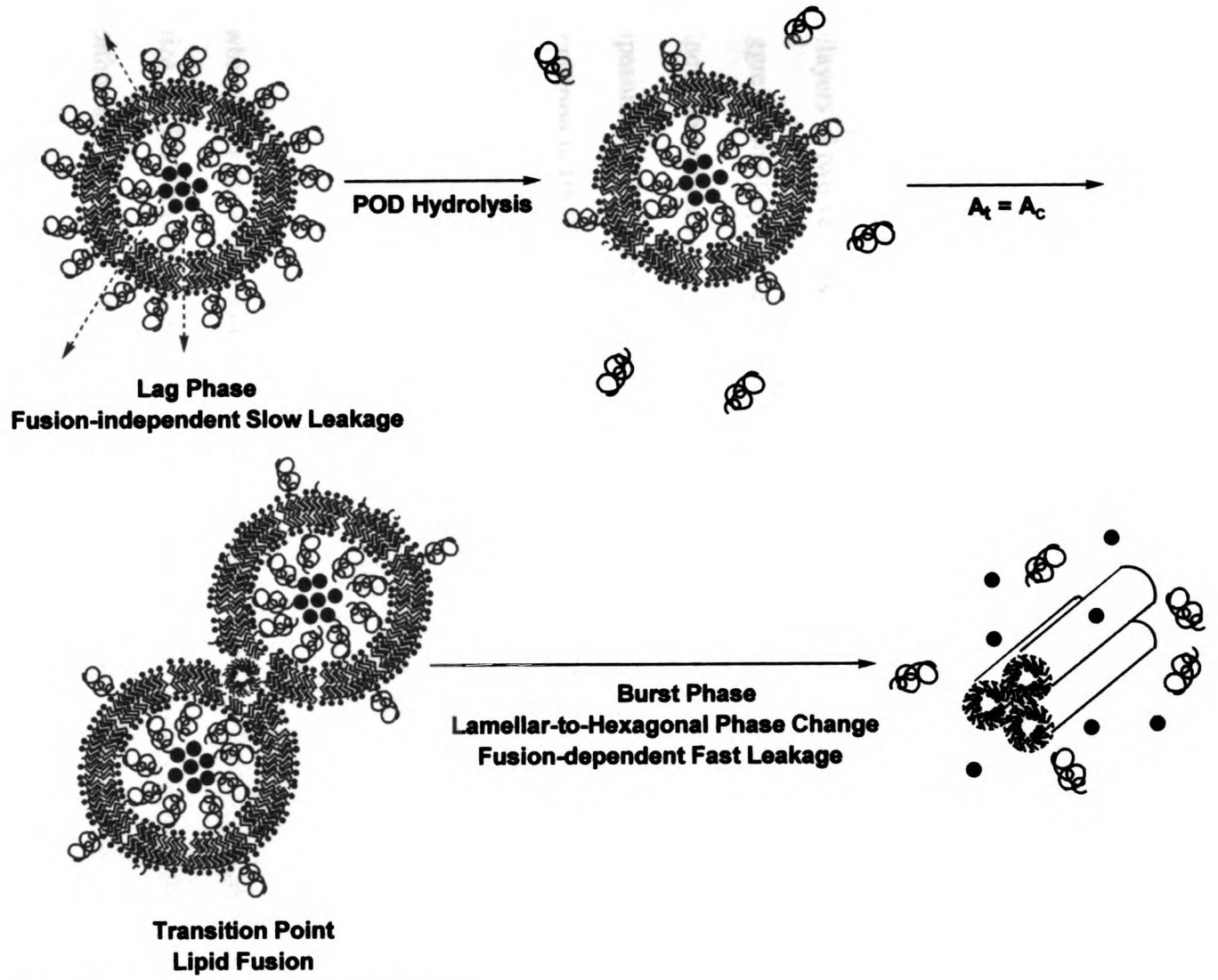
The novel pH-sensitive ortho ester lipid derivative introduced here is a useful addition to the other reported pH-sensitive linkages for modifying liposome surfaces. The synthesis is simple and versatile. The process with monomethyl ether of PEG was illustrated in this chapter but the chemistry could be used to attach various other headgroups to diacyl/steryl hydrophobic groups. For instance, anionic groups, cationic groups and even targeting ligands can be attached to a lipid anchor to create a triggerable headgroup for liposome formulations, micelles and other particulates. Modified glycerols, dendrons(100) or polyvinyl pyrrolidone(101) could also be conjugated to lipids using this chemistry.

Regarding the synthesis of POD, it should be noted that the acid-catalyzed conjugation does not produce POD as the unique product. Our preliminary TLC observations suggested that the side-products of the reaction include the conjugate of two methoxyPEG or two distearoyl glycerol (**3**) molecules on both ends of the diortho ester linker. The presence of side reactions, along with the loss of POD during its chromatography purification, may account for the moderate yield of the synthesis.

POD showed a remarkably quick degradation even at a mildly acidic pH of 5 to 6. Such sensitivity to acidic pH is significantly higher than that of acetals, vinyl ethers and polyortho esters. The higher sensitivity of POD can be attributed to the following two factors. First, the dialkoxy carbocation intermediate of ortho ester hydrolysis has four lone pairs of electrons over which to distribute the positive charge from the carbon, and hence is much more stable (Scheme 2-1) than the monoalkoxy carbocation intermediate,

stablized by two lone pairs of electrons, in the case of acetals and vinyl ethers. Secondly, the methoxyPEG headgroup of POD is more hydrophilic than the functional groups of the reported polyortho esters, allowing better hydration and faster proton transfer to the diortho ester linkage(102). When POD was incorporated into liposomes composed of a high percentage of DOPE, POD hydrolysis at pH 5 to 6 triggered the extensive aggregation and leakage of the liposomes in 10 to 100 minutes.

The rapid hydrolysis of POD at mildly acidic pH is a very useful property for drug/gene delivery systems because the decrease of pH at potential therapeutic sites may be only one pH unit or less. For example, the transit through the endosomes in cells occurs in about 10 to 30 minutes with pH in the range of 5 to 6 before the endosomal contents are delivered into the lysosome(41,42). Therefore, it is important for pH-sensitive liposomes to respond quickly to the initial drop in pH and release their contents prior to trafficking into the lysosomal compartment. The pH-dependent release profile of POD/DOPE liposomes may also be important for triggered release at inflammatory tissues(7,43) and solid tumors(6,40), where the pH is only 0.5 to 1 unit more acidic than that of the circulation.



Scheme 2-3. Minimum Surface Shielding Model.

The lag phase of ANTS leakage (Figure 2-4) corresponds quite closely to the lag time needed for liposome aggregation as measured by the increase of particle diameter (Figure 2-3). Based on these observations, the following "Minimum Surface Shielding" model (Scheme 2-3) of acid-catalyzed destabilization of POD/DOPE liposomes is proposed. Once the liposomes are subjected to an aqueous phase of mildly acidic or neutral pH, the methoxyPEG headgroups are continuously cleaved off the liposome surface. However, the lipids remain in the lamellar phase until the amount of methoxyPEG on the liposome surface decreases to a critical level, at which point the bilayers of two vesicles can come in contact. The bilayer contact is what triggers the aggregation of liposomes, the transition to the hexagonal phase and the release of liposome contents(46). The model is consistent with the known properties of PEG coated liposomes(103) and the requirement of bilayer contact to trigger the lamellar-hexagonal transition in PE liposomes(46). Assuming complete hydration and unaltered local pH at the liposome surface, the following equation holds:

$$DA_t/dt = -kA_t [H^+] \quad (1)$$

where A_t is the number of methoxyPEG groups on the surface of a liposome of certain size, $[H^+]$ is the proton concentration of the aqueous medium and k is the rate constant for ortho ester hydrolysis. In a buffer of constant pH, the equation can be integrated to give

$$A_t = A_o EXP(-k[H^+]t) \quad (2)$$

where A_o is A at an arbitrary starting point of POD hydrolysis and t is the incubation time from the starting point. At the transition point between the lag phase and the burst phase of liposomal leakage, one can have

$$A_c = A_o EXP(-k[H^+]t_l) \quad (3)$$

where A_c is the critical amount of methoxyPEG on the surface of the liposomes necessary to stabilize the lamellar structure and t_l is the length of the lag phase. If the same liposome preparation is incubated in buffers of different pHs, both A_c and A_o should be constant and one can write the following equation:

$$[H^+] \times t_l = C \quad (4)$$

where C is a composite constant composed of A_c, A_o and k. After taking the logarithm of equation (4) followed by rearrangement, the length of the lag time and the pH of the buffer would have the following relationship:

$$\log_{10} t_l = pH + C' \quad (5)$$

where C' is a constant.

Equation 5 fits closely to the observed leakage data in Figure 2-4B. The linear relationship between the logarithm of observed lag time and buffer pH, as well as the slope of the regressed line in Figure 2-4B (0.883 versus 1 in equation 5), support this

mechanistic model of acid triggered phase changes of POD/DOPE liposomes. Further biophysical studies are required to confirm this model but the simple model is consistent with previous studies on lamellar-hexagonal transition as well as the data presented in Figures 2-2, 2-3, 2-4 and 2-5.

When incubated in 75% fetal bovine serum, POD was able to stabilize POD/DOPE (1/9) liposomes for up to 12 hours, a finding consistent with the lag time for ANTS leakage of POD/DOPE liposomes in HEPES buffered saline at the same pH. Thus the stability characteristics vis-a-vis drug retention of this two component formulation are compatible for targeted delivery of encapsulated molecules given the observed intravenous elimination rate ($T_{1/2}$ = 194 min) of the formulation. Based on the distribution and the excretion data, both formulations were cleared mainly by liver from circulation, after which the ^{125}I- BPE labels were degraded and excreted into urine and bile.

The half-life of the POD/DOPE formulation in circulation is similar to other formulations that consist primarily of DOPE and that are stabilized by PEG(13,104). It is not surprising that such formulations have a shorter serum half-life than the traditional sterically stabilized formulations that consist of saturated phosphatidylcholine/Chol/PEG-DSPE(105). Such composition yields an inherently more rigid bilayer that is capable of resisting the penetration of serum components that might contribute to a more rapid elimination of a liposome even when it has a steric coat. Thus, further improvements of the serum half-life and drug release characteristics could be achieved by modification of the lipid composition such as by the inclusion of cholesterol in the formulation(98).

2.6 Conclusion

In conclusion, a methoxyPEG-diortho ester-distearoyl glycerol conjugate (POD, **1**) was designed and prepared by a one-step synthesis. POD was characterized for its pH sensitivity and ability to stabilize liposomes in serum and in blood circulation. POD was found to be highly sensitive to acidic conditions but relatively stable at neutral pH. Liposomes composed of 10% POD and 90% DOPE aggregated and released their contents at a mildly acidic pH similar to that found in an endosome. The destabilization of POD/DOPE liposomes consists of two phases, a lag phase where encapsulated contents slowly leaked from the vesicles, and a burst phase that was associated with liposome aggregation and rapid release of contents. Liposomes composed of 10% POD and 90% DOPE remained stable in 75% fetal bovine serum at 37 °C for 12 hours and had a half-life of about 200 minutes in blood circulation of female ICR mice. The fast kinetics of acid catalyzed POD hydrolysis and its ability to stabilize liposomes in serum and in blood circulation may provide important advantages for the lipid to be used for drug and gene delivery.

CHAPTER THREE:

pH-Triggered Destabilization of Sterically Stabilized POD/PE Liposomes: A Mechanistic Study

3.1 Abstract

The mechanism of pH-triggered destabilization of POD/PE liposomes has been studied using an ANTS/DPX leakage assay and a lipid mixing assay. The data was analyzed using the SAAM II statistics package to develop a kinetic model of POD hydrolysis and liposome collapse. The studies suggest the "Minimum Surface Shielding" model describe the mechanism of the pH-triggered release of POD/PE liposomes. In this model, when acid-catalyzed hydrolysis lowers the mole percentage of POD on the liposome surface to a critical level, inter-bilayer fusion is initiated, resulting in a burst of contents release. During the lag phase, contents leakage from the liposome is not due to a phase transition of a small population of less stable liposomes, but rather from a fusion-independent permeation of the contents out of the vesicles. The leakage during the burst phase is dependent on inter-bilayer contact. Incorporation of POPE into the bilayers facilitated the mixing of the saturated and the unsaturated lipidic components and improved the analysis of the kinetics of POD hydrolysis and liposome collapse of the resulting vesicles. The mechanistic studies and kinetic modeling of POD/PE liposomes provide important kinetic parameters that enable us to predict the mole percentage of POD to incorporate into the bilayer in order to obtain an optimal balance between

3.2 Introduction

In Chapters 1 and 2, I discussed how the decrease of pH is implicated in numerous physiological and pathological proceedings(5-8) and hence serves as an attractive stimulus to trigger the release of liposome-loaded therapeutic agents. Strategies to devise pH-sensitive liposomes include: 1) neutralization of negatively charged lipids in the bilayers via protonation to promote a lamellar to hexagonal phase transition(9); 2) protonation of negatively charged polymers(3) or peptides(54), which in turn absorb to the bilayers and destabilize bilayer structure by lysis, phase separation, pore formation or fusion; 3) acid-catalyzed hydrolysis of bilayer-stabilizing lipids into destabilizing detergents or conical lipids(58,61,62); 4) activation of neutral surfactants into their positive and surface-active conjugate acids(64,65).

In Chapter 2, I described an acid-labile conjugate of polyethylene glycol and distearoyl glycerol via a diortho ester linkage (POD, **1**). At neutral pH, POD was relatively stable and its presence provided a steric hindrance for DOPE-rich liposomes from fusion with one another. The steric hindrance also interfered with the interaction of serum components with the bilayer. When the pH was decreased to 6, however, POD was rapidly hydrolyzed, leading to the aggregation and leakage of POD/DOPE (10/90 in mole ratio) liposomes in 1 hour. Following intravenous injection to female ICR mice, POD/DOPE liposomes were found to be reasonably stable in blood circulation, with an apparent half life of approximately 200 minutes. The pH-sensitivity and the steric stabilization effect of POD may provide significant advantages in the design of drug delivery systems for in vivo use.

Based on the initial studies using aggregation and ANTS leakage assays, a

"Minimum Surface Shielding" model (Chapter 2, Scheme 2-3) was proposed to account for pH-dependent phase changes of POD/DOPE liposomes. Briefly, the lamellar structures of POD/DOPE vesicles are thought to remain intact until the proton-catalyzed POD hydrolysis lowered the number of PEG groups on the liposome surface to a critical level, at which the PE-rich bilayers are no longer sufficiently shielded and the liposome aggregation and leakage are triggered. However, a number of questions regarding the mechanisms of the destabilization of POD/PE liposomes have yet to be answered.

In a number of model membrane systems, bilayer destabilization has been shown to require inter-bilayer contact. Ellen and coworkers(46) designed an ANTS leakage assay and demonstrated that the contents release of diluted CHEMS/DOPE liposomes at acidic pHs can be normalized by plotting the leakage percentage versus the product of the incubation time and the lipid concentration. Hence bilayer fusion coincided with leakage in CHEMS/DOPE liposomes(46). In contrast, DOPE liposomes stabilized by BVEP(60), an acid-labile diplasmenyl lipid conjugate of PEG5000 synthesized by Thompson and associates, underwent membrane fusion on a kinetically slower time scale compared with the contents leakage and lamellar-to-hexagonal phase transition, indicating that in this particular system, contents release and phase transition are independent of inter-bilayer fusion.

Since membrane fusion plays a critical role in the intracellular delivery of liposomal contents(45,73,106,107), it is important to learn if the pH-triggered contents release requires inter-bilayer fusion in the POD liposomes.

Our previous observation on the relationship between the lag time of liposome leakage and the incubation pH (Figure 2-4) supported the "Minimum Surface Shielding"

model, but the experimental data were insufficient to determine either the rate of POD hydrolysis at the surface of POD/PE vesicles, or the minimum level of POD required to maintain the PE-rich bilayers. It is anticipated from the model that these are major parameters to determine the kinetic behavior of POD/PE liposomes, and hence would be important factors for the development of POD-containing liposomes into potential drug/gene delivery systems.

In studies in this Chapter, I address the above questions using ANTS leakage assays and lipid mixing assays based on fluorescent resonance energy transfer. I will also discuss the effect of the liposome preparation method and the acyl chain composition of PE lipids on the kinetics of acid-triggered liposome destabilization.

3.3 Materials and Methods

3.3.1 *General Techniques*

The pH sensitive lipid-PEG conjugate (POD) was synthesized as previously described (Scheme 2-2). Dioleoylphosphatidylethanolamine (DOPE) and1-Palmitoyl-2-oleoylphosphatidylethanolamine (POPE), Dipalmitoylphosphatidylethanolamine-NBD (NBD-PE) and Dipalmitoylphosphatidylethanolamine-lissamine rhodamine B (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). 8-Aminonaphthalene-1,2,3-trisulfonic acid (ANTS) and *p*-Xylenebis(pyridinium) bromide (DPX) were purchased from Molecular Probes, Inc. (Junction City, OR). MilliQ water, which had a pH of approximately 8 when freshly prepared, was used to prepare all the aqueous buffers. All other chemical reagents and solvents were purchased from Sigma (St. Louis, MO) or Fisher (Tustin, CA). Ratios of lipid components in liposomes are in mole units.

3.3.2 *Liposome Preparation*

POD/DOPE liposomes were prepared by the reverse-phase evaporation method according to Szoka and Papahadjopoulos(90). A lipid mixture (10 μmol) of POD and DOPE in an appropriate molar ratio was transferred into a Pyrex glass tube as a chloroform solution. For lipid mixing assays, the chloroform solution was mixed with NBD-PE and Rh-PE (1 mol% each in sample vesicles; 0.167 mol% each in control

vesicles) in chloroform solution. Chloroform was evaporated under reduced pressure and the resultant lipid film was placed under high vacuum for 1 hour at room temperature. The lipid film was dissolved by 1 mL of water-saturated diethyl ether, into which 400 μl of an appropriate buffer was added. The mixture was then sonicated in a bath sonicator (Laboratory Supplies Co., Hicksville, NY) for 30 seconds to form a translucent water-in-oil emulsion. A small magnetic stirring bar was placed into the emulsion and the ether was slowly evaporated with a rotary evaporator at reduced pressure (630-680 mm Hg), upon which the emulsion turned into a gel and then collapsed into a liposome solution. The pressure was further reduced to 500 mm Hg for 5 minutes to remove the residual ether in the preparation. The liposome preparation was then extruded 5 times through a 0.2-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) using a hand-held extrusion device (Avestin, Ottawa, Ontario, Canada).

POD/POPE/DOPE liposomes were prepared by the freeze-thawing method based on the procedure of Monnard et al(108). A chloroform solution of POD, POPE and DOPE in desired molar ratio (10 μmol total lipid) was added to a Pyrex brand glass tube. For lipid mixing assays, the chloroform solution was mixed with NBD-PE and Rh-PE (1 mol% each in sample vesicles; 0.167 mol% each in control vesicles) in chloroform. Chloroform was evaporated under reduced pressure (27 mm Hg) at room temperature to form a lipid film at the bottom of the Pyrex tube. The lipid film was placed under high vacuum for 1 hour to remove residual chloroform. The film was then hydrated with an appropriate aqueous buffer by 20 minutes of intermittent agitation with a vortexor at 4 °C. The tube containing the lipid suspension was then filled with argon and sealed. The lipid suspension was rapidly frozen by submergence into liquid nitrogen, followed by

melting by incubation in water at room temperature for 15 minutes. The freeze-thawing cycle was repeated 10 times and the resultant liposomes were extruded 5 times through a 0.2-μm polycarbonate membrane (Nucleopore Corp., Pleasanton, CA) with a hand-held extrusion device (Avestin, Ottawa, Ontario, Canada).

Liposomes for leakage assays were prepared using an alkaline buffer with the ANTS fluorophore (50 mM ANTS, 50 mM DPX and 5 mM HEPES at pH 8.5), and the extruded vesicles were separated from the unencapsulated material using a Sephadex G-75 column with an elution buffer composed of 5 mM HEPES and 145 mM NaCl, pH 8.5. Liposomes for lipid mixing assays were prepared in 5 mM HEPES and 145 mM NaCl, pH 8.5. Freeze-thawed liposomes for lipid mixing assays were used after extrusion without further purification. After extrusion, REV for lipid mixing assays were dialyzed 3 times with 100-fold volume excess of the alkaline buffer (5 mM HEPES and 145 mM NaCl, pH 8.5) to remove free radicals that might have been generated during the sonication process.

All freshly prepared liposomes had mean diameters ranging from 170 to 200 nm (cumulant results) and a polydispersity index of less than 0.2 as measured by a Malvern Zeta1000 Dynamic Light Scattering Instrument using the PCS 1.32a software. The automatic algorithm was employed for data analysis. Lipid concentrations were determined based on lipid phosphorus by a modification of the Bartlett method(91).

3.3.3 *Determination of Encapsulated Volume of Liposomes*

A small aliquot (20-150 nmol total lipids) of a liposome preparation for leakage assay was lysed in 3 mL of an alkaline buffer (5 mM HEPES and 145 mM NaCl, pH 8.5) supplemented with 200 μL of 1% $C_{12}E_8$ solution in water. The fluorescence at 550 nm was then measured (Excitation Wavelength = 467 nm) with the aforementioned Spex Fluorolog photon counting instrument to determine the ANTS concentration of the stock liposome preparation, using the ANTS/DPX buffer (50 mM ANTS, 50 mM DPX and 5 mM HEPES at pH 8.5) as the standard. With the anticipation that most of the ANTS in a liposome preparation is encapsulated within the vesicles and that the concentration of ANTS inside the vesicles (50 mM) does not change significantly during the encapsulation, the encapsulated volume of a liposome preparation can be determined using the equation:

$$V = (1000*C_F)/(50*C_L)$$

where V is the encapsulated volume in μL/(μmol lipids), C_F is the concentration (mM) of ANTS in the liposome solution and C_L is concentration (mM) of total lipids.

3.3.4 *Liposome Leakage Assay*

The ANTS/DPX fluorescent assay (Section 2.3.6) was used to measure the contents release of the liposomes. One data point of fluorescent intensity was collected

each second except for pH 7 and 7.4, where measurements were taken every 30 minutes and the samples were incubated in dark between the measurements to minimize the exposure of the sample to the excitation light source. The leakage assays for Figures 3-1 and 3-4 were carried out in 5 μM and 25 μM lipid concentrations, respectively. The leakage assays for Figure 3-3 were carried out in a series of lipid concentrations as specified in the Figure.

3.3.5 *Lipid Mixing Assay*

The fusion of the liposomes was monitored by a modified lipid mixing assay based on the method of Struck et al(109), employing fluorescence resonance energy transfer. Labeled vesicles were prepared to contain NBD-PE and Rh-PE (1 mol% each) in 5 mM HEPES and 145 mM NaCl, pH 8.5. Labeled and unlabeled vesicles were pre-mixed in a 1:5 molar ratio and a small aliquot (~ 20 μL) was injected with a Hamilton syringe into a magnetically stirred quartz cuvette containing 2 mL of an appropriate aqueous buffer (50 mM NaOAc/HOAc and 100 mM NaCl, pH < 6; 50 mM NaH_2PO_4/ Na_2HPO_4 and 100 mM NaCl, pH 6 and above) at 37 °C. The final concentration of the total lipids in the cuvette was 150 μM in all the lipid mixing assays. The starting time of liposome incubation (t_o) at a given pH, was set 1 to 5 seconds after the addition of the vesicles when the fluorescence signal first became stable. An increase of NBD-PE fluorescence indicates a decrease in the quenching of NBD-PE fluorescence by Rh-PE due to the dilution of the two membrane-bound probes during the lipid mixing between

labeled and unlabeled vesicles. Fluorescence measurements were made with a Spex Fluorolog photon counting instrument (Edison, NJ) using a 150-W xenon light source. Excitation was at 467 nm (1.25 mm, 1.25 mm slits). The 90° emission signal at 550 nm (5 mm, 5 mm slits) was observed through a Corning 3-68 nm cutoff filter (>530 nm). One data point of fluorescent intensity was collected per second. In the cases where pH equaled 7 or 7.4, measurements were taken every 30 minutes and the samples were incubated in the dark between the measurements to minimize the exposure of the sample to the excitation light source. In order to compensate for the interference from liposome aggregation and precipitation, I prepared the control liposomes of the same composition as that of their corresponding sample liposomes except for 0.167 mol% of NBD-PE and Rh-PE. Such mole percentages of the probes are expected when the lipid mixing of the sample liposomes reaches its theoretical maximum. The fluorescence of each control over time at different pHs was measured in the same manner as the corresponding sample and used to normalize the fluorescence of the sample to percentage of infinite probe dilution.

The raw fluorescent data were converted into ASCII data files and mathematically processed by Microsoft Excel. In order to determine the lipid mixing as a percentage of infinite probe dilution, F% is defined as the percentage of the sample fluorescence over the fluorescence of its control. F% is calculated using the following formula:

$$F\% = (Ft-Fo)/(F't-F'o) \times 100,$$

where Ft and F't are the sample fluorescence and the control fluoresence at a given

incubation time, respectively; Fo is the fluorescence of the blank buffer before adding the sample; Fo' is the fluorescence of the blank buffer before adding the control liposome. The lipid mixing as a percentage of infinite probe dilution (M%), is then determined using the following formula:

$$M\% = (F\%-F\%t_o)/(100-F\%t_o) \times 100,$$

where $F\%t_o$ is F% at the starting time of incubation (t_o).

3.3.6 *Determination of the Duration of Lag Phase*

For Figures 3-1 and 3-2, the duration of the lag phase was determined as follows. For each fluorescent trace, one line tangent to the early linear part of the lag phase and one line tangent to the steepest slope of the burst phase were drawn. The intersection of the two lines was taken as the transition point of the lag phase and the burst phase. The time from the starting point of incubation to the transition point was taken as the duration of the lag phase. For Figures 3b and 3d, the same method was employed to determine the transition point of the 5 μM trace, which was then taken as the common transition point for traces of other lipid concentrations in the same Figure.

For the leakage data in Figure 3-4, the transition point was determined by a derivation method described as follows. For each fluorescent trace, the length of the time window (t_w) for data-smoothing was set as the round number of 20% of the incubation

time (in seconds) when 50% of contents leakage was achieved. At a given time point (t) later than 0.5 x t_w, a linear regression was performed on the data points within a time window which is centered around the time point and has a length of t_w. The slope of the linear regression was taken as the derivative of the fluorescent trace at this time point. A trace of the first derivative of liposome leakage was then obtained by taking the derivative at each time point of the original fluorescent trace, except the time points that are earlier than 0.5 x t_w after the starting time point and those that are later than 0.5 x t_w before the end of the fluorescent trace. The trace of the first derivative was derivatized again in the same manner to obtain the trace of the second derivative of liposome leakage. The time point at which the second derivative reached the maximum was then taken as the transition point between the lag phase and the burst phase, and hence the duration of the lag phase between the starting time point and the transition point.

3.3.7 *Statistical Analyses Using the "Minimum Surface Shielding" Model*

Estimates for the parameters of the "Minimum Surface Shielding" Model, i.e., POD hydrolysis rate constant (k) and critical percentage of POD (A_c), were obtained by nonlinear regression using SAAM II (University of Washington Seattle, WA) software package(110). Lag time (t_l) versus initial percentage POD (A_o), was utilized from POD/DOPE and POD/POPE/DOPE preparations, and each of these data sets were taken at pH 5.0 and pH 5.5. Each of the four data sets (POD/DOPE or POD/POPE/DOPE vesicles at pH 5 or 5.5) shown in Table 3-2 was entered into SAAM II and fit to the

following equation with a fixed $[H^+]$ value (10^{-5} or $10^{-5.5}$) and FIX absolute variance:

$$t_l = (\ln A_o - \ln A_c)/(k[H^+])$$

For each types of vesicles (POD/DOPE and POD/POPE/DOPE in different molar ratios), T-tests were performed between pH 5.0 and 5.5 for both k and A_c from the data to assess the assumption that k and A_c are pH independent. To correct for multiple comparisons the cutoff significance (P=0.0125) is equal to 0.05 divided by the number of comparisons.

An improved estimate of the kinetic parameters (A_c and k) for each type of liposome (POD/DOPE and POD/POPE/DOPE at different molar ratios) was obtained by pooling the t_l data of each liposome preparation at pH 5.0 and pH 5.5. For each A_o, [H+] * t_l was calculated, and the values at pHs 5.0 and 5.5 were averaged. The average and standard deviation estimates of [H+] * t_l versus A_o were entered into SAAM II. The pooled data for each type of liposome (POD/DOPE and POD/POPE/DOPE at different molar ratios) were fit using an absolute variance to the following equation:

$$t_l [H^+] = (\ln A_o - \ln A_c)/k$$

3.4 Results

3.4.1 *Preparation of POD/PE Liposomes*

As an initial model of pH-sensitive liposomes composed of POD, I used the reverse-phase evaporation method(90,111) (see Section 2.3.4) to prepare vesicles composed of 10 mol% of POD and 90 mol% of the fusogenic lipid DOPE in the studies of last Chapter. However, given the tendency of POD to undergo hydrolysis in aqueous media, it is suspected that the reverse-phase evaporation method involving a sonication step may not be optimal for preserving the composition of the liposomes during the preparation. The freeze-thawing method(108,112) appears to be an attractive alternative due to the relative mildness of the procedure as well as the large encapsulation volume of the resultant vesicles. Another aspect for the improvement of the POD-stabilized fusogenic vesicles would be the lipid composition. Although DOPE has been chosen in numerous pH-sensitive liposomes to trigger lamellar-to-hexagonal phase change, both of its side chains are unsaturated with a kink from the *cis*-double bond, and hence may have difficulty in mixing with the saturated stearyol side chains of POD in bilayer structures(113). Indeed, when I attempted to prepare the POD/DOPE liposomes by freeze-thawing, the hydration of the lipid film required lengthy agitation and was often incomplete. In order to circumvent such difficulty, another lipid, POPE, was introduced into the formulation.

Since POPE has one saturated palmitoyl side chain and one unsaturated oleyol side chain, it is miscible with both POD and DOPE, and it facilitates the formation of

POD/PE vesicles with even distribution of the lipid components in the bilayer structures. The conical shape of POPE should preserve the ability of the vesicles to change from the lamellar phase to the hexagonal phase upon POD hydrolysis. The lipid films composed of POD, POPE and DOPE in the molar ratios of (7~14)/ 50/(43~36) for our studies were easily hydrated and the vesicles readily prepared following freeze-thawing and extrusion. As shown in Table 3-1, the freeze-thawed POD/POPE/DOPE vesicles and POD/DOPE REV have similar sizes of 160-200 nm in diameter, as expected after extrusion through 200 nm membranes. However, the freeze-thawed POD/POPE/DOPE liposomes possess encapsulated volumes (2.5~3.5 μl/μmol lipid) about 10 fold as large as POD/DOPE REV (0.20~0.35 μl/μmol lipid, Table 3-1), indicating that, compared with POD/DOPE REV, the freeze-thawed POD/POPE/DOPE liposomes have a considerably different structure, which is more suitable for loading hydrophilic molecules. The leakage and the fusion assays were thus carried out on both types of vesicles in order to elucidate the mechanism of their destabilization by low pH and to study the effect of the preparation method and the lipid composition on the triggering process.

Table 3-1

Physical Properties of POD Liposomes[a]

A_o[b] (mol%)	POD/DOPE = A_o/(100- A_o)			POD/POPE/DOPE = A_o/50/(50- A_o)		
	D[c] (nm)	P.I.[d]	V[e] (μL/μmol)	D[c] (nm)	P.I.[d]	V[e] (μL/μmol)
7	N.D.[f]	N.D.[f]	N.D.[f]	194	0.124	3.46
8	198	0.113	0.258	188	0.116	3.39
9	195	0.109	0.308	195	0.125	3.52
10	200	0.102	0.201	188	0.163	3.28
11	205	0.096	0.345	N.D.[f]	N.D.[f]	N.D.[f]
12	192	0.128	0.295	179	0.150	2.54
14	N.D.[f]	N.D.[f]	N.D.[f]	176	0.187	2.63

[a]POD/DOPE liposomes were prepared by reverse phase evaporation method and POD/POPE/DOPE liposomes were prepared by freeze-thawing method. All the data in the table are the mean of three measurements.
[b]The initial mole percentage of POD in the lipid mixture.
[c]The diameter of the liposomes based on the cumulant results of Photon Correlation Spectrometry measurements. The standard deviation is within 2% of the mean.
[d]Polydispersity index reflecting the homogeneity of the liposomes. The standard deviation is within 15% of the mean.
[e]Encapsulated volume of the liposomes reported as microliter of encapsulated aqueous liposome interior per micromole of lipids. The standard deviation is within 7% of the mean.
[f]Not determined.

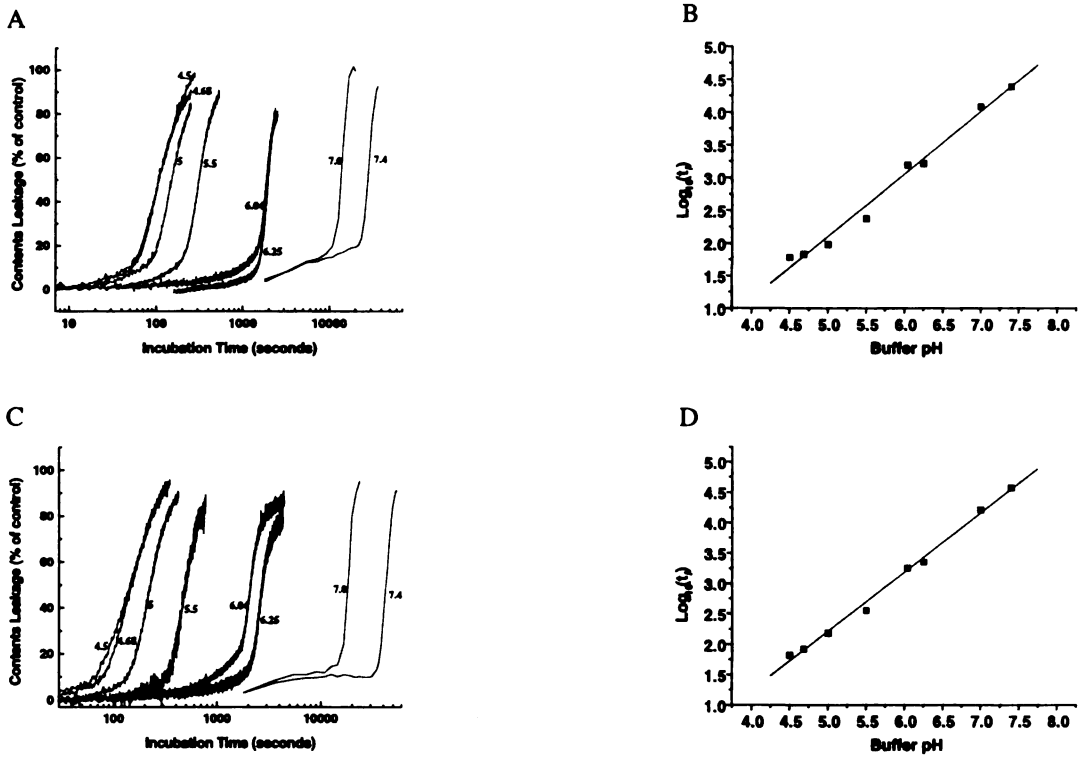


Figure 3-1. pH-dependent leakage of POD/POPE/DOPE liposomes. A and B, POD/POPE/DOPE = 10/50/40; C and D, POD/POPE/DOPE = 14/50/36. A and C, Percentage of leakage over time; B and D, Logarithm of lag time (t_l) in seconds at different buffer pH. B, $Log_{10}(t_l)$ = (0.95262±0.04655) x pH -(2.66906±0.27378), r = 0.993. D, $Log_{10}(t_l)$ = (0.97167±0.02895) x pH -(2.65037± 0.17029), r = 0.997.

3.4.2 *Effect of pH on Contents Leakage of POD/PE Liposomes*

In the previous studies (Chapter 2) on the POD/DOPE (10/90, mol/mol) liposomes, it was found that the kinetics of contents release consisted of two phases, a lag phase and a burst phase. The lag phase was inversely correlated with pH; the logarithm of the duration of lag phase showed a linear relationship with the buffer pH. I proposed a "Minimum Surface Shielding" model to account for the linearity and the value of the slope (0.883). The release profile of POD/POPE/DOPE liposomes also conforms to the model, as shown in Figure 3-1. In both POD/POPE/DOPE liposomes composed of 10% and 14% POD, respectively, the logarithm of the duration of lag phase was linear to the buffer pH, with a correlation coefficient value of higher than 0.99 (n = 8). The slope of the regressed lines for both formulations was 0.95 or greater and closer to the theoretical value of 1, compared with the POD/DOPE (10/90) liposomes.

3.4.3 *Effect of pH on Fusion of POD/PE Liposomes*

It was shown in Chapter 2 (Figures 2-3 and 2-4) that the aggregation of the POD/DOPE liposomes occurs in a pH-dependent manner which is similar to that found for release of their contents. This suggests that the leakage of POD/PE liposomes involves lipid fusion. A fluorescence resonance energy transfer assay(114) was thus carried out using the membrane probes NBD-PE and Rh-PE to confirm whether lipid mixing between POD/PE vesicles takes place during the process of pH-triggered

liposome leakage. One complication of the assay is that the fluorescent signal is influenced by light scattering due to the aggregation and the precipitation of the vesicles. This is especially evident when the PE-rich vesicles undergo a lamellar to hexagonal phase change during the fusion. To compensate for interference due to light scattering and to normalize the extent of lipid mixing to the percentage of theoretical maximum of probe dilution, I prepared control liposomes with 0.167% each of NBD-PE and Rh-PE in the bilayer. This percentage of the fluorescent probes are expected to arise when the lipids of the labeled liposome are completely mixed with 5 fold excess of the unlabeled liposomes.

The control liposomes were incubated at various pHs and their fluorescence recorded over time in the same manner as the sample liposomes. Under these conditions, the fluctuations of the fluorescent signal of the control liposomes would arise from light scattering due to colloidal aggregation and precipitation subsequent to vesicle fusion. Thus the fluorescence of the control liposomes at each time point was taken as the maximum fluorescence that would arise if there was complete lipid mixing of the sample liposomes at the corresponding time point.

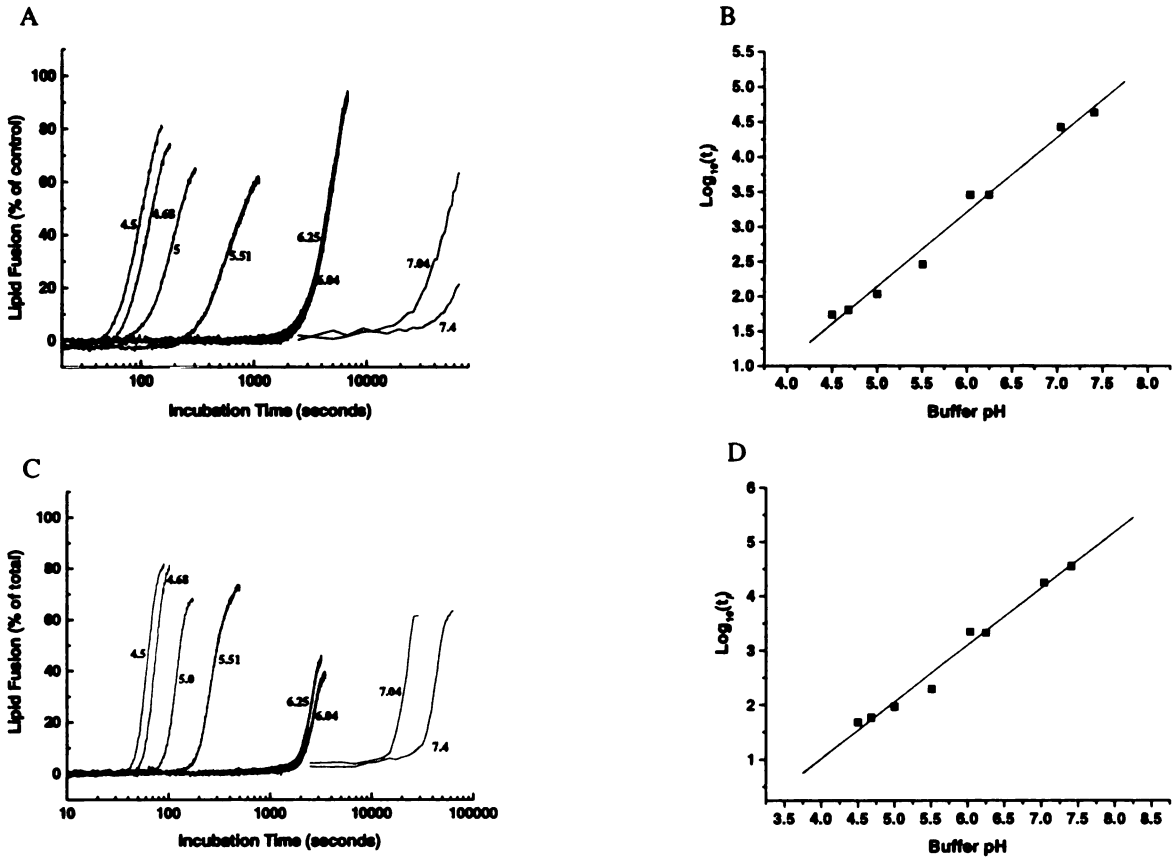


Figure 3-2. pH-dependent fusion of POD/PE liposomes. A and B, POD/DOPE = 10/90; C and D, POD/POPE/DOPE = 10/50/40. A and C, Percentage of leakage over time; B and D, Logarithm of lag time (t_l) in seconds at different buffer pH. B, $Log_{10}(t_l)$ = (1.06743±0.05395) x pH - (3.19614±0.31778), r = 0.992. D, $Log_{10}(t_l)$ = (1.04298±0.05856) x pH -(3.15916±0.34489), r = 0.991.

Both POD/DOPE (10/90) and POD/POPE/DOPE (10/50/40) liposomes (Figures 3-2a and 3-2c, respectively) exhibited lipid fusion kinetics that is comprised of two phases: a lag phase, when there was virtually no lipid mixing, and a burst phase, when up to 80% of lipid mixing was observed. This kinetic behavior was similar to that found using the contents release assay. The lag phase of both types of vesicles is inversely correlated with pH and the logarithm of the duration of lag phase (t_l) has a linear relationship with the incubation pH (Figures 3-2b and 3-2d). The slope of the regressed line for both types of vesicles is 1, which is the same within experimental error as the value predicted by the "Minimum Surface Shielding" model. These findings demonstrate that lipid fusion is involved in the pH-dependent destabilization of POD/PE vesicles and confirmed our "Minimum Surface Shielding" model of the triggering kinetics.

3.4.4 *Contact-dependent Leakage of POD/PE Liposomes at Burst Phase*

Bentz and coworkers(115) have shown by a mass action kinetic model that if the plots of leakage vs $X_o t$ (where Xo is the initial concentration of the liposomes, or in equivalent, the initial lipid concentration for a homogeneous liposome sample; t is the incubation time) lie on the same curve regardless of the lipid concentration, then the liposomes are stable until they contact with one another. Ellen and coworkers(46) have successfully utilized this theory to demonstrate that the acid-triggered leakage of CHEMS/DOPE liposomes requires interbilayer contact. Since the contents release,

aggregation and fusion of the POD/PE vesicles exhibited very similar two-phase kinetic profiles, it was hypothesized that the destabilization of such liposomes at the burst phase is also dependent on bilayer contact. The leakage assays of POD/DOPE (10/90) and POD/POPE/DOPE (10/50/40) liposomes at different concentrations were thus carried out at pH 4.5 (POD/DOPE) or pH 5 (POD/POPE/DOPE), in an effort to test the hypothesis.

For both POD/DOPE and POD/POPE/DOPE liposomes, the contents release from a given liposome sample at different concentrations possesses approximately the same duration of lag phase (Figures 3-3a, 3-3c). This conforms with the "Minimum Surface Shielding" model, which predicts that the lag time before the collapse of POD/PE bilayers should be a function of pH and the initial number of PEG groups on the liposome surface, but not a function of the liposome concentration. Thus for all of the fluorescence traces in Figure 3-3a, the transition point of the 5 μM trace (t_l = 108 seconds) is assigned as the common "transition point" between the lag phase and the burst phase and defined the incubation time at this point as t_o'. The fluorescent intensity at a given time point (t) after t_o' is normalized to percentage of leakage in the burst phase (L%') using the equation:

$$L\%'(t) = (Ft - Ft_o')/(F_{100} - Ft_o') \times 100,$$

where Ft is the fluorescent intensity of the trace at t, Ft_o' is the fluorescent intensity at t_o', F_{100} is the fluorescent intensity after the liposomes were lysed with the detergent $C_{12}E_8$. L%' was then plotted against $X_o(t - t_o')$ in Figure 3-3c. The data in Figure 3-3b were mathematically processed and replotted in the same manner in Figure 3-3d, where t_o' was

set as 95 seconds. The replotted leakage graphs for both the POD/DOPE liposome (Figure 3-3c) and the POD/POPE/DOPE liposome (Figure 3-3d) lie on the same line at 5 μM or lower concentrations, demonstrating that the bilayer contact is required for liposome leakage during the burst phase. At lipid concentrations higher than 5 μM, the curves lie lower in all values of $X_o(t - t_o')$, indicating that contents release rather than inter-liposomal contact becomes the limiting step of the dequenching of ANTS fluorescence. This is because the frequency of interbilayer contact increases proportionally with the square of lipid concentration whereas the leakage of ANTS is limited by the rate of bilayer fusion and lamellar-to-hexagonal phase changes following the contact(46).

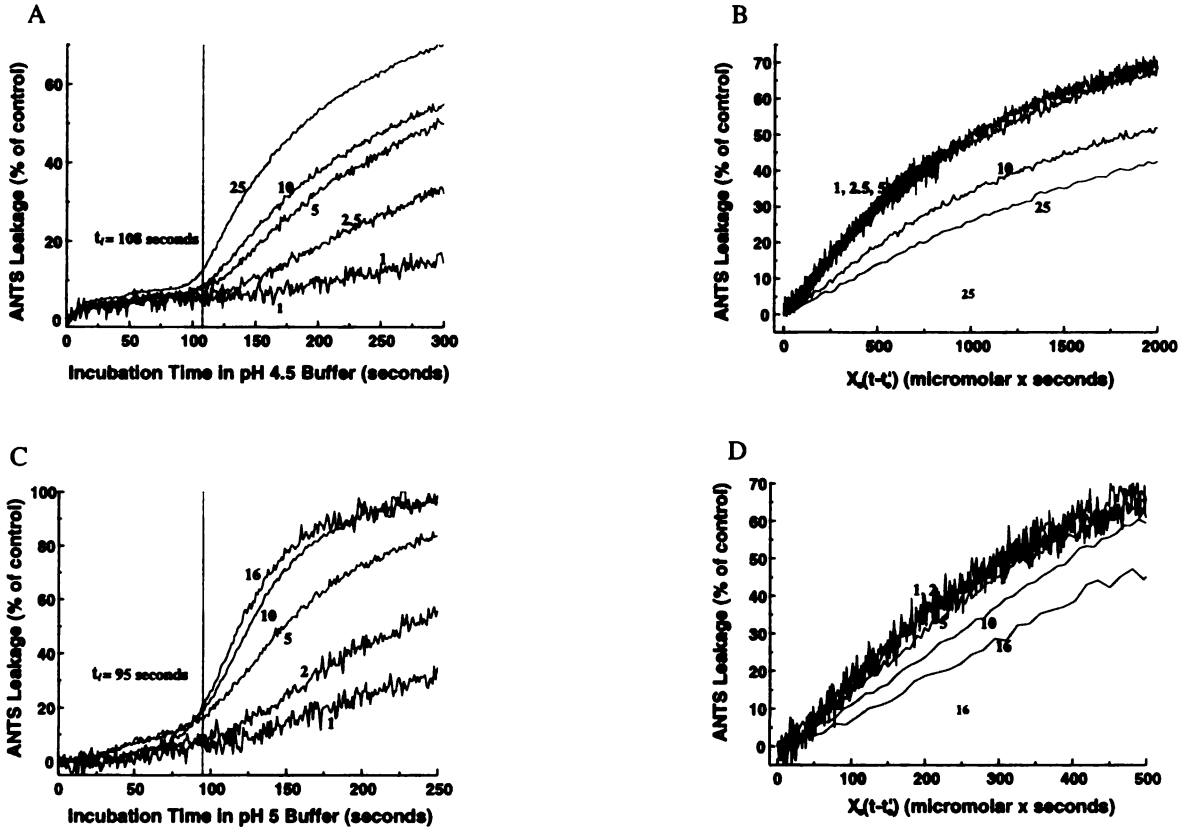


Figure 3-3. Leakage of POD/PE vesicles is dependent on inter-bilayer contact. A and B, POD/DOPE liposome (10/90); C and D, POD/POPE/DOPE liposome (10/50/40). A and C, percentage of leakage at different lipid concentrations over incubation time; B and D, leakage percentage at burst phase over the product of lipid concentration and elpased time post-transition point. The lipid concentrations (micromolar) are labeled adjacent to the corresponding fluorescent trace.

3.4.5 *Hydrolysis Rate and Minimum Stabilization Percentage of POD*

According to the "Minimum Surface Shielding" model (Section 2.5), the hydrolysis of POD on liposome surfaces at a constant pH can be described by the following equation:

$$A_t = A_o EXP(-k[H^+]t), \text{ (Eq. 3-1)}$$

where A_t is the percentage of POD on liposome surface at the incubation time t, A_o is the percentage of POD at the starting time of incubation, k is the POD hydrolysis rate constant and $[H^+]$ is proton concentration. At the transition point between the lag phase and the burst phase, the equation can be written as:

$$A_c = A_o EXP(-k[H^+]t_l), \text{ (Eq. 3-2)}$$

where A_c is the minimum percentage of POD required on liposome surface to stabilize the bilayer structures, t_l is the duration of lag phase. Taking the natural logarithm on both sides of the equation followed by rearrangements yields:

$$t_l = \ln A_o/(k[H^+]) - \ln A_c/(k[H^+]). \text{ (Eq. 3-3)}$$

Equation 3-3 shows that the length of lag time should have a linear relationship with the natural logarithm of the initial percentage of POD on liposome surface. Furthermore, the

equation predicts that by measuring the t_l of POD/PE liposomes comprising of different A_o at known pHs followed by an appropriate regression of the data, one can deduce the rate constant of POD hydrolysis (k) as well as the minimum percentage of POD (A_c) that is required to stabilize the lamellar structures. I thus prepared POD/DOPE and POD/POPE/DOPE liposomes containing different mole percentages of POD (Tables 3-1) and measured their leakage at pH 5 and pH 5.5.

Equation 3-3 also predicts that the change of t_l in response to A_o is relatively small. Therefore, a precise determination of t_l values is needed in order to obtain reasonable estimations of k and A_c. Two approaches were taken to improve the data quality for t_l values. First, more concentrated liposome samples were utilized for leakage assays (25 μM in Figure 3-4 versus 5 μM in Figures 3-1 and 3-2) to obtain smoother traces with sharper increase of the fluorescent signal at the burst phase. Second, the leakage data of improved quality allowed the use of a mathematically stricter derivation method to extract the t_l values from the fluorescent traces. Since the slow leakage during the lag phase and the fast leakage during the burst phase are reflected, respectively, by a shallow slope and a steep slope of the fluorescent trace, the transition point between the two phases can be considered as the point when the change of the leakage rate, or the change of the slope of the fluorescent trace, reaches the maximum. Mathematically, this maximum point is equivalent to the maximum point of the secondary derivative of the fluorescent trace.

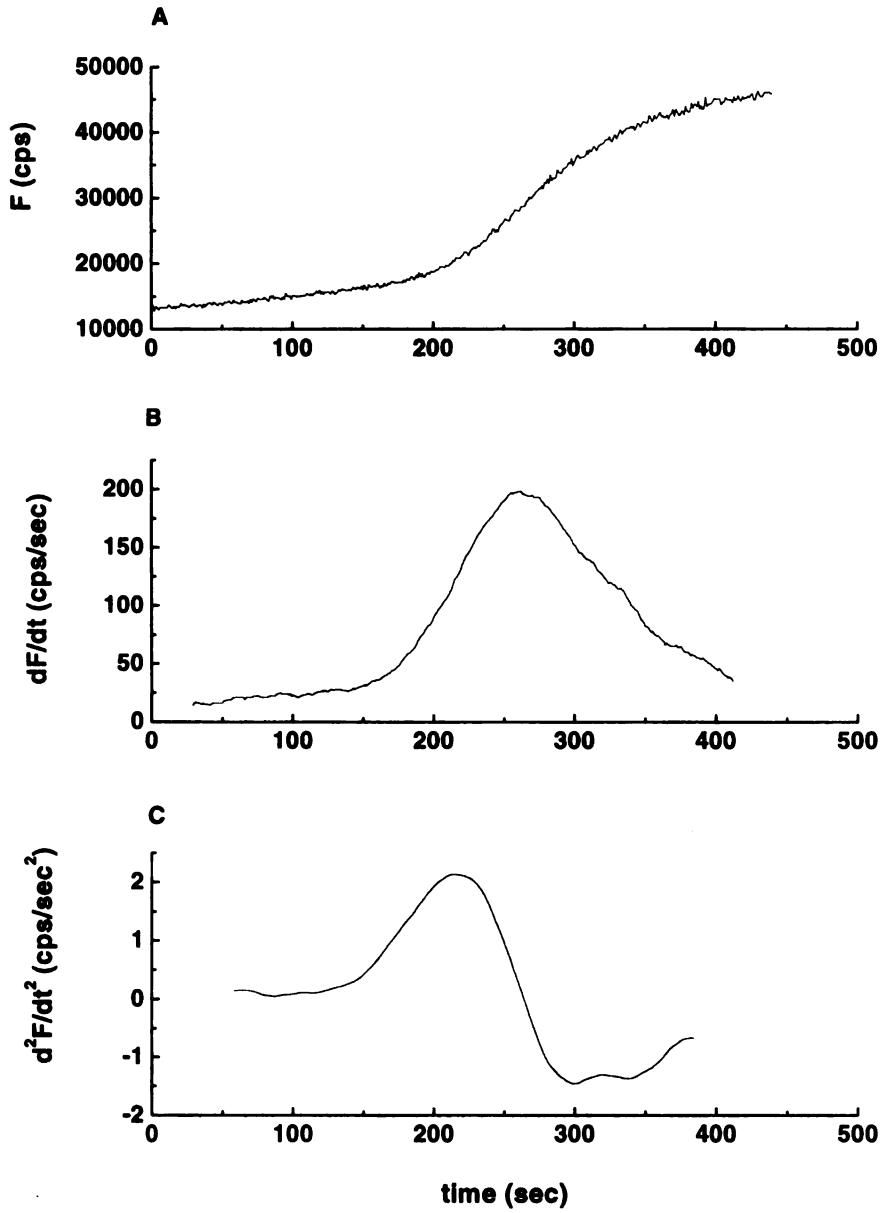


Figure 3-4. Determination of lag time (t_l) by the derivation method. As an example, the processing of the leakage of POD/POPE/DOPE (10/50/40) liposome at pH 5.5 is shown. A. The fluorescent intensity F (counts per second) over incubation time. B and C. The first and the second derivatives of the fluorescent trace in A, respectively. The maximum of d^2F/dt^2 is 2.133 cps/sec^2 at 214 seconds.

After testing a range of different time windows, one fifth of the time for 50% leakage was used as the window for data smoothing because it takes into account the different leakage kinetics of each fluorescent trace and gives smooth derivative traces with sharp peaks. A typical example (POD/POPE/DOPE = 10/50/40, pH 5.5) of determining the lag time (t_l) using the derivation method is shown in Figure 3-4. The lag times as determined by the derivation method are listed in Table 3-2 and then plotted against the natural logarithm of POD mole percentage in Figure 3-5.

Table 3-2

Lag Times for Contents Leakage from POD/PE[a] Liposomes

as Determined by the Derivation Method

A_o[b] (mol%)	POD/DOPE = A_o/(100- A_o)		POD/POPE/DOPE = A_o/50/(50- A_o)	
	pH 5.0 (seconds)	pH 5.5 (seconds)	pH 5.0 (seconds)	pH 5.5 (seconds)
7	N.D.[c]	N.D.[c]	87	180
8	123	303	95	211
9	138	366	94	224
10	165	426	97	214
11	149	399	N.D.[c]	N.D.[c]
12	174	398	118	287
14	N.D.[c]	N.D.[c]	122	332

[a]POD/DOPE liposomes were prepared by reverse phase evaporation method and POD/POPE/DOPE liposomes were prepared by freeze-thawing method. The leakage assays were carried out at 25 μM lipid concentration. The data are also plotted in Figure 3-5 and used for statistical analyses in Table 3-3.
[b]The initial mole percentage of POD in the lipid mixture.
[c]Not determined.

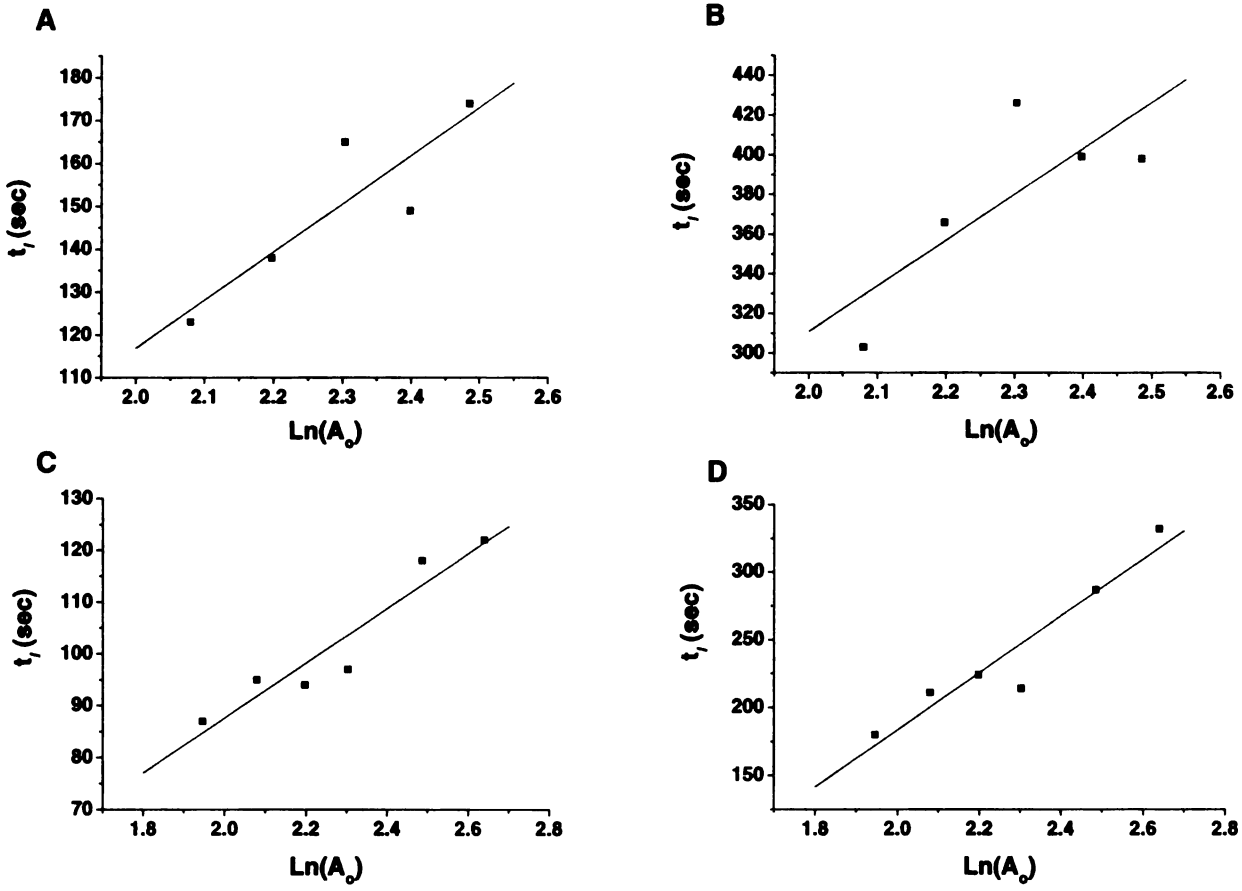


Figure 3-5. Dependence of lag time (t_l) of POD/PE liposomes on the initial mol% of POD. A and B, POD/DOPE = A_o/(100- A_o); C and D, POD/POPE/DOPE = A_o/50/(50- A_o). A and C, pH 5; B and D, pH 5.5. A, r = 0.881, P = 0.048; B, r = 0.782, P = 0.118; C, r = 0.951, P = 0.004; D, r = 0.953, P = 0.003.

For both POD/DOPE and POD/POPE/DOPE liposomes, the duration of the lag time showed a linear relationship with the natural logarithm of the starting POD mole percentage at pH 5.5 and pH 5, demonstrating again that the "Minimum Surface Shielding" model appropriately describes the mechanism of the pH-triggered release of POD/DOPE liposomes. The linear relationship is much more significant in POD/POPE/DOPE liposomes ($r = 0.951$, $P = 0.004$ at pH 5 and $r = 0.953$, $P = 0.003$ at pH 5.5) than in POD/DOPE liposomes ($r = 0.881$, $P = 0.048$ at pH 5 and $r = 0.782$, $P = 0.118$ at pH 5.5), indicating that the freeze-thawed POD/POPE/DOPE liposomes produced a more predictable kinetic profile than the POD/DOPE REVs.

To estimate the kinetic parameters (k and A_c) without the characteristic bias of linear transformation, nonlinear regression was performed using the t_l data in Table 3-2. The results from each of the four leakage data sets (POD/DOPE and POD/POPE liposomes at pH 5 and 5.5) are shown in tables 3-3. The "Minimum Surface Shielding" model assumes that these constants are independent of the pH; therefore, it was expected that the parameters derived from the pH 5.0 and 5.5 experiments would not differ significantly. In table 3-3 the parameters of the same type of vesicles (POD/DOPE or POD/POPE/DOPE) derived from data sets of different pH were compared by t-test and no significant difference was observed. Since the parameters are independent of pH, an improved estimate of Ac and k was derived from both pH 5 and pH 5.5 leakage data sets by regressing t_l against the average of $k[H^+]$ as described in the methods. For the POD/DOPE liposomes, the improved estimate for k is 1015 $sec^{-1}M^{-1}$ and for A_c is 2.524 mol%. For the POD/POPE/DOPE liposomes, the estimate for k is 1666 $sec^{-1}M^{-1}$ and for A_c is 2.180 % (Table 3-3).

Table 3-3

Statistical Estimation of Kinetic Parameters[a] of POD Liposome[b] Leakage

	POD/DOPE = $A_o/(100 - A_o)$		POD/POPE/DOPE = $A_o/50/(50 - A_o)$	
	k[c]	A_c[d]	k[c]	A_c[d]
pH 5	853 ± 238 $sec^{-1}M^{-1}$	2.755 ± 0.965 mol%	1980 ± 692 $sec^{-1}M^{-1}$	1.310 ± 0.900 mol%
pH 5.5	1217 ± 486 $sec^{-1}M^{-1}$	2.297 ± 1.320 mol%	1615 ± 344 $sec^{-1}M^{-1}$	2.861 ± 0.705 mol%
T-value[e]	0.67	0.28	0.47	1.36
P-value[e]	0.53	0.79	0.65	0.21
Comb. Est.	1015 $sec^{-1}M^{-1}$	2.524 mol%	1666 $sec^{-1}M^{-1}$	2.180 mol%

[a]The estimates are from the non-nonlinear regression of the corresponding data sets in Table 3-2 and expressed as mean ± standard error.
[b]POD/DOPE liposomes were prepared by reverse phase evaporation method and POD/POPE/DOPE liposomes were prepared by freeze-thawing method.
[c]The hydrolysis rate constant of POD in $sec^{-1}M^{-1}$.
[d]The minimum mole percentage (mol%) of POD needed to stabilized the PE-rich bilayer structure.
[e]The T-values and P-values are from comparison of the parameters (k or A_c) of the top two cells of the same column. To correct for multiple comparisons the cutoff significance (P=0.0125) is equal to 0.05 divided by the number of comparisons.

3.5 Discussion

The principal objective of experiments described in this chapter was to test the "Minimum Surface Shielding" model which I proposed to account for the kinetic behavior of contents release observed in the POD/PE vesicles as a function of pH (see Chapter 2). The minimum surface shielding model is based upon the observation in a number of model systems that PE bilayers must come into contact with another PE bilayer in order to transform into the hexagonal phase(46). The model postulates that the PE bilayers are kept from coming into contact by the PEG polymers attached to the liposome surface. When a sufficient amount of PEG is removed from the vesicle surface, the surface becomes accessible to contact with another PE surface; when contact is made between two bilayers, they rapidly convert into a hexagonal phase and the encapsulated contents are rapidly released.

In this model, the stability of POD/PE liposomes is a function of the initial mole fraction of the POD in the liposome bilayer and the hydrolysis rate of the POD. This model assumes that the fraction of POD on the outer monolayer is the same as the fraction of POD in the initial lipid mixture, that the POD mixes ideally with the other lipids and that the collapse of the bilayer occurs with similar properties regardless of the lipid composition of the bilayer. When I measured the lag time preceding contents leakage at various pH values (Figure 3-1) and the lag time preceding leakage from liposomes containing various POD mole percentages at a constant pH (Figure 3-5), I could compute the hydrolysis rate of POD and the critical POD concentration needed to stabilize PE in the lamellar phase.

I tested the assumption that bilayer collapse is responsible for contents release by measuring lipid mixing with a fluorescent resonance energy transfer fusion assay(109) (Figure 3-2). The slope of the lag time for fusion as a function of pH was 1 and the correlation coefficient was 0.99 for both lipid compositions tested. The lag time for fusion was highly correlated with the lag time for rapid release; thus hydrolysis of the ortho ester results in bilayer collapse, not increased permeability through the bilayer. During the lag phase, the POD/PE liposomes released up to 20% of the encapsulated contents whereas there was virtually no lipid fusion during the lag phase (compare Figure 3-1a with Figure 3-2c). This observation suggests that the slow leakage during the lag phase does not come from the collapse of a small population of less stable liposomes, but rather occurs by a fusion-independent mechanism, most probably due to the permeability of the bilayers to hydrophilic molecules(116).

In Figures 3-3b and 3-3d, the leakage of POD/PE liposomes during the burst phase was extracted from that during the lag phase by "resetting" the zero percent leakage as well as the zero incubation time at the transition point. Redrawn as the product of lipid concentration and the modified incubation time, the leakage at different vesicle concentrations up to 5 μM overlapped with each other, demonstrating that most, if not all of the liposome leakage at the studied pHs is fusion-dependent during the burst phase(46).

Indeed, the leakage data fits this model quite satisfactorily. The linear correlation coefficient for contents leakage is 0.88 for the DOPE vesicle composition and 0.99 for the POPE/DOPE composition (Figure 3-5). Likewise, the linear relationship in the fusion data (Figures 3-2) is 0.99 for both vesicle compositions. Based upon these data and the

data in Table 3-2, the computed rates of hydrolysis of POD was about 1000 $sec^{-1}M^{-1}$ in DOPE liposomes and about 1700 $sec^{-1}M^{-1}$ in the POPE/DOPE composition (Table 3-3). The computed critical POD percentage was 2.5 mol% for DOPE and 2.2 mol% for the POPE/DOPE (Table 3-3).

For the critical surface coverage there was no significant difference between the A_c values in the two compositions. The slight difference in the apparent hydrolysis rate of POD in the two compositions may be due to differences in interfacial properties of the two bilayer surfaces. POD contains a double saturated C18 acyl chains and would perferentially interact with POPE rather than DOPE. Thus the environment surrounding the POD in the POPE containing bilayer would be more rigid and the ortho ester might be oriented into the interfacial water. Whereas in the DOPE bilayer, with the double unsaturated acyl chains, the ortho ester might be oriented into the bilayer where the activity of water would be reduced, hence the hydrolysis rate decreased.

An alternative explanation for the slight difference in hydrolysis rate could be related to the preparation of the POD/DOPE vesicles. An assumption in the model is that the percentage of POD on the liposome surface is the same as the percentage of POD in the initial lipid mixture used for the vesicle preparation. POD needs to mix evenly with other lipid components and to undergo negligible degradation during the liposome preparation. This may not have been the case in the DOPE liposomes. First it was difficult to re-hydrate the POD/DOPE mixture and the encapsulation volume of the resulting liposome preparation was significantly less than the encapsulation volume of the POPE/DOPE vesicles (Table 3-1). Second the DOPE vesicles were prepared using the REV method. Thus during the rehydration of the DOPE composition there might

have been an unequal distribution of the POD between the monolayers. If the POD was enriched in the outer monolayer of the POD/DOPE vesicles, then the hydrolysis rate would be underestimated.

I developed the indirect contents leakage method to determine the parameters of k and A_c for two reasons: 1) it is difficult to prepare liposomes with the critical density of PEG on their surface, 2) analytical techniques used to determine the hydrolysis rate of POD require the separation of surfactants and could introduce complications from the degradation of POD during its isolation and quantification. Although we had directly estimated the pH dependent hydrolysis rate of POD using thin layer chromatography in Chapter 2, the precision of this assay was insufficient to obtain more than an order of magnitude estimate of the rate constant.

The important point is that both data sets obtained using the indirect method are consistent with the model and the parameters derived from the fit (Table 3-3) permit us to predict the lag time before which a POD/PE liposome preparation will release its contents if we know the pH. Moreover, at any pH, this lag time can be adjusted by altering the initial percentage of POD in the lipid composition. This is an important advantage when incorporating the ortho ester PEG lipids into liposomes for in vivo drug delivery where the pH of the blood is 7.4.

An interesting consequence arising from these experiments relates to the minimal surface area on two PE bilayers that must come in contact in order for the PE to undergo a lamellar-hexagonal transition(103,117). A lower bound for this value can be computed assuming a uniform surface coverage of the PE bilayer by POD. If the critical surface coverage is 2.2 mol%, about 1/45 lipid molecules contains a PEG chain. Since two

liposomes must come in contact, the average surface density of PEG2000 is one polymer for every 23 lipids. Thus 23 lipids would constitute the minimum number of PE on one surface required to initiate a lamellar to hexagonal transition. The PEG attached to POD had a Mw ≈ 2,000 and, assuming it packs as a sphere on the vesicle surface, the projected area of this sphere is 438 $Å^2$. Since each diacyl PE occupies a projected area of approximatedly 70 $Å^2$, 23 lipids would occupy 1610 $Å^2$, and at the critical area, two liposomes would have 438 ÷ 1610 x 100% = 27.2% of the available bilayer surface occluded by a PEG polymer chain. The minimal surface area on two PE bilayers that must come in contact in order for the PE to undergo a lamellar-hexagonal transition would be approximately 1610 $Å^2$ x (1 – 27.2%) = 1172 $Å^2$. These calculated values are consistent with the findings of other groups(103,117) on the mechanisms of the fusion of PE bilayers and the shielding effect of PEG polymers.

A number of pH sensitive liposome systems have been introduced over the years to provide a triggered release system. This feature of rapid all or none release of contents from POD/PE liposomes is similar to what has been observed with the CHEMS/DOPE vesicles(46), oleic acid/PE liposomes(118) and homocystein/PE liposomes(45). The pH-induced neutralization of the CHEMS/DOPE liposomes immediately increases the exposure of the PE headgroups, leading to fusion-dependent leakage kinetics. The leakage of CHEMS/DOPE liposomes has no lag phase, because the protonation of CHEMS headgroups is almost instantaneous compared with the rate of lipid fusion. However, the destabilization kinetics of POD/PE vesicles differs from that found with the BVEP/DOPE vesicles(60), whose leakage occurs at a much faster rate compared with the fusion upon acid-catalyzed hydrolysis of BVEP. This difference between the POD/PE

and BVEP/DOPE liposomes may be attributed to the different hydrolysis patterns of POD and BVEP. The hydrolysis at either of the two ortho ester groups of POD leads to the immediate cleavage of the PEG2000 headgroup from the liposome surface and the exposure of more PE headgroups. In the case of BVEP, the hydrolysis of one of the two vinyl ether linkages generates an alkyl aldehyde and a conjugate of PEG5000 and a single alkyl side chain. Thus, the PEG5000 headgroup would be retained on the liposome surface until the hydrolysis of the second vinyl ether group takes place. Therefore, during the early stage of BVEP hydrolysis, the resultant single-chain detergents may be sufficient to induce structural defects in the bilayers, leading to content release and even the collapse of the vesicles. However, the retained PEG5000 groups on their surfaces still protect the colloids from fusing with one another. Similar to the POD hydrolysis, the neutralization of CHEMS/DOPE liposomes immediately increases the exposure of the PE headgroups, leading to the fusion-dependent leakage kinetics. The leakage of CHEMS/DOPE liposomes has no lag phase, because the protonation of CHEMS headgroups is almost instantaneous compared with the rate of lipid fusion.

Recently, Zhu and coworkers(62) reported two acid-labile cationic lipids (Scheme 1-5) containing an ortho ester linker based on the structure of 3,5,8-trioxabicyclo[2.2.2]-octane. Due to the particular configuration of the linker, the initial two fast hydrolysis steps of the ortho ester functionality do not fragment the cationic lipid, but rather add two hydroxy groups near the cationic headgroup region. It is only after the final slower step of the hydrolysis, which is the cleavage of an ester group, that the lipids convert to two single-chain, membrane-destabilizing detergents. Such a hydrolysis pattern may complicate the kinetics of bilayer destabilization by these pH-sensitive lipids.

3.6 Conclusion

The mechanism of pH-triggered destabilization of POD/PE liposomes has been studied by the ANTS/DPX leakage assay and the lipid-mixing assay. The data generated from these studies were analyzed using the "Minimum Surface Shielding" model. The model adequately describes the mechanism of the pH-triggered release of POD/PE liposomes and indicates that a critical surface coverage of A_c = 2.2 mol% PEG 2000 is required to stabilize the PE bilayer. Based upon this model the rate of hydrolysis of the ortho ester was about 1000-1700 $sec^{-1}M^{-1}$. These values can be used to predict the stability of PE liposomes as a function of initial mol% of POD in the lipid composition and the pH of the environment.

CHAPTER FOUR:

Ortho Ester-Based Acid-Labile Cationic Lipids for Gene Delivery

4.1 Abstract

The majority of the lipoplexes delivered to cells traffic from the endosomal compartment into the lysosomes before DNA can be released from the cationic lipids. The research described in this chapter aims to enhance the delivery of plasmid DNA by the design of novel pH-sensitive cationic lipids that would hydrolyze at the pH found in the endosomes and release the DNA from the lipoplexes. Three ortho ester-based cationic lipids were prepared, all comprising a cationic headgroup, a hydrophobic group and an acid-labile linker containing an ortho ester moiety.

One of the synthesized lipids, *N*,*N*-dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (DOC, **2**) was combined with a fusogenic helper lipid, DOPE and used to prepare cationic liposomes. The DOC/DOPE liposomes at N/P ratios of 5/1 and 3/1 carry excessive positive charges and spontaneously condense with plasmid DNA into cationic lipoplexes. Upon incubation at acidic pHs, the lipoplexes lost their positive charges as a result of DOC hydrolysis. Both in CV-1 cell culture and in CD-1 mice, lipoplexes containing DOC increased the luciferase gene expression more than 5 fold compared with the structurally similar but pH-insensitive control lipid, 3β-[N-[2-(N,N-dimethylamino)ethyl]carbamoyl]cholesterol (DC-Chol). These observations support the hypothesis that the incorporation of a pH-sensitive functionality into a cationic lipid enhances its ability to deliver DNA into the cytoplasm. The improved gene transfection also opens considerable opportunities for the development of more efficient

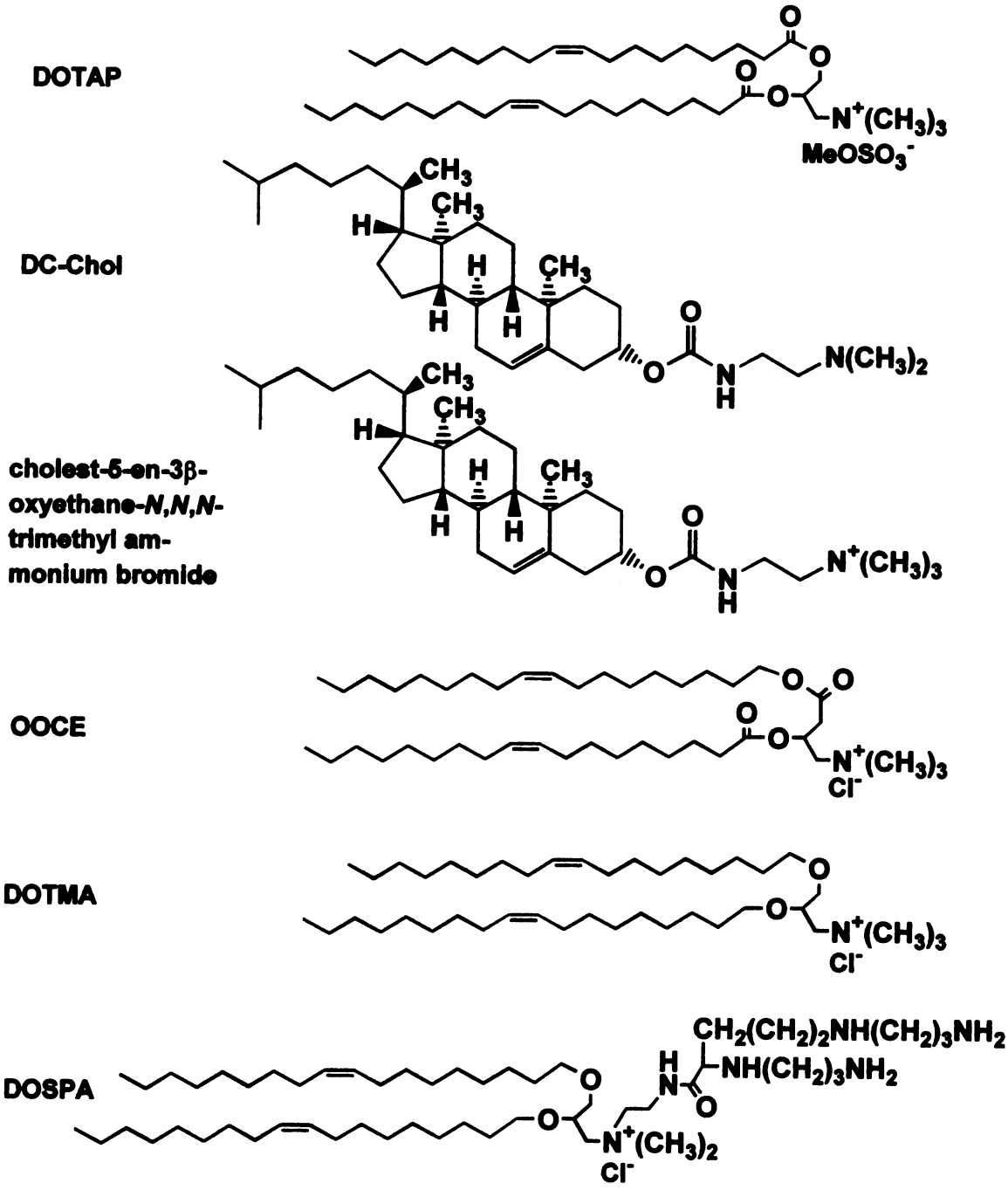
4.2 Introduction

Gene therapy has the potential to become an important therapeutic modality in the 21st century. This form of therapy introduces exogenous DNA sequences (genes) into cells to correct their gene defect or to generate a therapeutic gene product(119). Polymers(120), lipids(14,121) and peptides(122) have been utilized as synthetic delivery systems to transfer DNA into cells both in culture and *in vivo*. Cationic lipids have been the most common synthetic vectors used *in vivo* and a number of cationic liposome-DNA complexes (lipoplexes) have been investigated in clinical trials(123-125). Representative cationic lipids are shown in Figure 4-1.

However, after about 15 years of research, the efficiency of delivery remains the factor that limits the use of synthetic gene vectors in the clinic(126). One of the key barriers of gene delivery is that most of the cationic lipoplexes are trapped in the endosomes following cellular uptake and finally processed to lysosomes, where they are degraded(122,127). In fact, viruses which infect their host cells *via* the endocytic pathway rely on specific proteins to destabilize endosome membranes so as to translocate the viral genomic DNA into the cytoplasm.(127)

A number of endosome-disrupting agents have been incorporated into synthetic gene delivery systems. The endosotropic agents so far reported are triggered by the decrease of pH in the endosomal compartments(5,128) and exhibit a large diversity in structure, including synthetic peptides(122), polymers(51,129) and pH-titratable lipids(130). Although these reagents significantly enhanced gene transfection in cell culture, their success in animals is limited. Recently, Thompson and coworkers(56)

Figure 4-1: Structures of Selected Cationic Lipids for Gene Delivery



reported the synthesis of a pH-sensitive cationic lipid containing a spermidine headgroup and acid-labile diplasmenyl hydrophobic side chains, but the gene transfection activity of this lipid has not yet been published.

Ortho esters attract considerable interest in our lab, due to their exceptional sensitivity towards small drops in pH based on the work on their polymeric derivatives(11,131,132). In Chapters 2 and 3, it is shown that an ortho ester conjugate of PEG and distearoyl glycerol (POD) remains stable at neutral pH but is hydrolyzed to greater than 90% in 1 hour when the pH is decreased to 5. Liposomes composed of POD and PE were stable for more than 10 hours at pH 7.4, 37 °C but released most of their encapsulated contents within 30 minutes when the pH was decreased to 5.5. It is thus reasoned that, an ortho ester conjugate of a cationic headgroup and a cone-shaped lipidic alcohol could be used for the generation of cationic lipoplexes with an improved endosome-disrupting effect. At neutral pH, liposomes composed of the cationic conjugate and a fusogenic helper lipid should condense spontaneously with DNA into lipoplexes; upon endocytosis and acidification of the endosomes, the conjugate would be hydrolyzed to expose the conical lipidic alcohol and the fusogenic helper lipid. These lipids would in turn disrupt endosomal membranes and the previously complexed DNA would be released into the cytoplasm.

In this chapter, I describe the design, synthesis, pH-sensitivity and transfection acitivities of two categories of ortho ester-based cationic lipids and discuss their potential use for gene delivery.

4.3 Materials and Methods

4.3.1 *General Procedures and Materials*

The diketene acetal, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane (**2**) was received as a generous gift from Dr. Jorge Heller at Advanced Polymer Systems (Melon Park, CA). Triethylamine was purchased from Aldrich and re-distilled under Ar before use. Lipids (DOPE, DOTAP and DC-Chol) were purchased from Avanti Polar Lipids (Birmingham, AL). MiliQ water, which had a pH about 8 when freshly deionized, was used to prepare all aqueous buffers. All other chemical reagents and solvents were purchased from Aldrich or Fisher. Ratios of components in chromatography solvent systems are in volume unless stated otherwise. Ratios of lipid components in liposomes are in mole. CV-1 cells (a monkey fibroblast cell line) were provided by the UCSF cell culture facility. Female CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). All animals were handled in accordance with protocols described by the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and with the approval of the Committee of Animal Research at the University of California, San Franccisco. Animals were sacrificed at the stated times with a sodium pentabarbital overdose. Luciferase plasmid DNA (pLC0888.143) was a gift from Valentis, Inc. (Woodlands, TX). ^{1}H-NMR spectra were recorded on an Oxford AS 400 NMR spectrometer. Electrospray mass spectra (ESIMS) were recorded on a Sciex (PE, Forster City, CA) at Mass Spectrometry Facilities, University of California at San Francisco.

4.3.2 *Attempted Synthesis of 3,9-Diethyl-3-(cholest-5-en-3β-oxy)-9-(N,N,N-trimethylaminoethoxy)-2,4,8,10-tetraoxaspiro[5.5]undecane, Chloride Salt (4)*

Sodium heptansulfonate (606 mg, 3 mmol) and choline chloride (590 mg, 4.2 mmol) were dispersed in 80 mL $CHCl_3$ and stirred at room temperature for 4 hours. The mixture was filtered and the filtrate evaporated under reduced pressure. The residue of the evaporation was dried in high vacuum to obtain choline heptansulfonate salt (956 mg, ~3 mmol). The choline heptansulfonate salt was dissolved in 80 mL anhydrous $CHCl_3$ under Ar at 45 °C together with cholesterol (1.31 g, 3.4 mmol). A heat gun was used to melt **2** and 650 μL (715 mg, 3.4 mmol, d = 1.1) of the melted compound was taken by a dry syringe and injected into the $CHCl_3$ solution. One drop (~40μL) of 0.6 mg/mL *p*-toluenesulfonic acid in anhydrous THF was added and the reaction mixture was stirred at 45 °C under Ar for 2 hours. The reaction was stopped by adding 2 mL triethylamine. The reaction mixture was then filtered and the filtrate evaporated at 30 °C under reduced pressure. The residue of the evaporation was dissolved with minimum volume of $CHCl_3$/MeOH/(30 wt% NH_3 in H_2O) (50:50:1) and passed through an anion exchange column (AG-2 x 4, 20 g, chloride form, Biorad). However, the exchange of the heptansulfonate anion to the chloride anion was incomplete and significant degradation occurred as indicated by TLC. The reaction products were unstable, presumably due to the hydrolysis of the ortho ester functional groups. This prohibited further purification and characterization of the possible ortho ester conjugates.

4.3.3 *Synthesis of 3,9-Diethyl-3-(2,3-distearoyloxypropyloxy)-9-(N,N,N-trimethylaminoethoxy)-2,4,8,10-tetraoxaspiro[5.5]undecane, p-Toluenesulfonate and Chloride Salts (5)*

Silver *p*-toluenesulfonate (279 mg, 1 mmol) and choline chloride (140 mg, 1 mmol) were dispersed in 80 mL $CHCl_3$ and stirred in dark at room temperature for 4 hours. The mixture was filtered and the filtrate evaporated under reduced pressure. The residue of the evaporation was dried in high vacuum to obtain the choline *p*-toluenesulfonate salt (276 mg, 1 mmol). The choline *p*-toluenesulfonate salt was dissolved in 80 mL anhydrous $CHCl_3$ under Ar at 45 °C together with distearoyl glycerol (625 mg, 1 mmol). A heat gun was used to melt **2** and 200 µL (212 mg, 1 mmol, d = 1.1) of the melted compound was taken by a dry syringe and injected into the $CHCl_3$ solution. One drop (~40µL) of 0.6 mg/mL *p*-toluenesulfonic acid in anhydrous THF was added and the reaction mixture was stirred at 45 °C under Ar for 3 hours. The reaction was stopped by adding a basic ion exchange resin which was prepared as follows. The Dowex ion exchange resin (3 g, BioRad, Cat No. AG 2-X4) was washed with EtOH, dispersed in HEPES buffer and the pH adjusted to pH 9.45 by 6M NaOH. The mixture was stirred at 4 °C for 2 hours and filtered. The filtrate was sequentially mixed with triethylamine (500 µL) and silica gel (3 g). The mixture was evaporated under reduced pressure and the residue was poured on top on a silica gel column (40 g) equilibrated with $CHCl_3$/triethylamine (100:1). The column was eluted first with 100 mL $CHCl_3$/triethylamine (100/1) and then with $CHCl_3$/MeOH/triethylamine/(30 wt% NH_3 in H_2O) (100/15/1/1.15) to afford 142 mg (15% crude yield) product as a mixture of *p*-

toluenesulfonate and chloride salts. TLC R_f 0.58 in $CHCl_3$/MeOH/triethylamine/(30 wt% NH_3 in H_2O) (100/25/1/1). ^1H NMR (400 MHz, $CDCl_3$, chemical shifts relative to TMS signal) exhibited the following peaks corresponding to the functional groups of the conjugate: δ 7.75 (0.6H, d, J = 8 Hz, *p*-toluenesulfonate 3'H and 5'H), 7.15 (0.6H, d, J = 8 Hz, *p*-toluenesulfonate 2'H and 6'H), 5.22 (1H, m, glycerol methine), 3.2-4.4 (m, glycerol methylenes, $N^+CH_2CH_2O$ and N^+CH_2), 3.48 (s, OCH_2 on the spiro ring), 3.0-3.2 (m, $N^+(CH_3)_3$), 2.2-2.4 (m, CH_2COO), 1.7-1.8 (m, CH_2CH_2COO), 1.5-1.7 (m, CH_2CH_3 on spiro rings), 1.2-1.4 (56H, m, $CH_3(CH_2)_{14}CH_2CH_2COO$), 0.82-0.97 (12H, m, CH_2CH_3). However, other peaks due to impurities were also observed. The major peaks associated with the impurities were located at: 3.0-3.2 (m, $HN^+(CH_2CH_3)_3$), 1.4-1.5 (~20H, q, J = 7.2 Hz, $HN^+(CH_2CH_3)_3$). The impurity peaks interfered with the integration of some of the product peaks. ESIMS, calcd for $[M]^+$ excluding the negative counter ion: $C_{55}H_{106}O_{10}N$ 940.8, found 940.7. The compound was not stable at room temperature or at –20 °C even after extensive drying in high vacuum. The instability of the compound prohibited further purification and biological evaluation.

4.3.4 *Synthesis of N,N-Dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (DOC, 6)*

In a THF (anhydrous, 50 mL) solution of cholest-5-en-3β-oxyethan-2-ol (400 mg, 0.926 mmol) and *N,N*-dimethylethanolamine (630 mg, 0.71 mL, 7 mmol) under Ar was added sodium hydride (352 mg, 60% dispersion in mineral oil, 8.8 mmol) and the reaction mixture was stirred at room temperature for 30 minutes. α,α–Dichloromethyl methyl ether (**7**, 460 mg, 0.362 mL, 4 mmol) was injected in one bolus while the reaction mixture was vigorously stirred. The reaction mixture was then stirred at 45 °C under Ar for 10 h. Triethylamine (1 mL) was added and the reaction mixture was filtered. The precipitate was extracted with 1% of triethylamine in $CHCl_3$ and the extracts were pooled with the filtrate and evaporated under reduced pressure. The residue of the evaporation was dissolved with minimum volume of $CHCl_3$/MeOH/triethylamine (100/5/0.5, vol/vol/vol). A silica gel column (40 g) was equilibrated with $CHCl_3$/MeOH (100/5) and then washed with 20 mL of $CHCl_3$/MeOH/triethylamine (100/5/0.5, vol/vol/vol). The solution of the evaporation residue was loaded on top of the column and eluted with $CHCl_3$/MeOH/triethylamine (100/5/0.5) to yield 264 mg (51% purified yield) of the product. R_f 0.44, silica gel TLC, $CHCl_3$/MeOH/triethylamine (100/2/1). ^{1}H NMR (400 MHz, $CDCl_3$, chemical shifts relative to TMS signal) δ 5.34 (1H, d, J = 5.2 Hz); 5.20 (1H, s, ortho ester C*H*); 3.62-3.72 (6H, m, OCH_2); 3.35 (3H, s, OCH_3); 3.15-3.25 (1H, m); 2.57 (2H, t, J = 6 Hz, NCH_2); 2.33-2.39 (1H, m); 2.30 (6H, s, N(CH_3)$_2$); 2.15-2.25 (2H, m); 1.78-2.04 (6H, m); 0.85-1.60 (33H, m); 0.68 (3H, s). ESIMS $C_{35}H_{61}NO_4$: Calcd, 562.46, found, 562.53.

4.3.5 *Synthesis of N,N,N-Trimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amonium Iodide (8)*

N,N-dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (**6**, 180 mg, 0.32 mmol), iodomethane (100 μL, 1.6 mmol, d = 2.28) and sodium carbonate (200 mg, 1.9 mmol) were suspended in anhydrous THF (5 mL) under Ar at room temperature for 8 hours. TLC showed the disappearance of the spot of **6** at R_f 0.44 (silica gel TLC, $CHCl_3$/MeOH/triethylamine = 100/2/1) and the generation of a new more hydrophilic spot of the methylation product at R_f 0.06. The quaternary ammonium product showed an R_f value of 0.47 on silica gel TLC using the solvent system $CHCl_3$/MeOH/(30 wt% NH_3 in H_2O) (4/1/0.05). ESIMS, calcd for $[M]^+$ excluding the negative counter ions: $C_{36}H_{64}O_4N$ 576.48, found 576.52. However, upon exposure to the air, the compound quickly degraded, prohibiting further spectrometric and elemental analyses.

4.3.6 *Preparation of Cationic Liposomes and Lipoplexes*

A chloroform solution of a cationic lipid alone or in combination with other helper lipids was placed in a rotary evaporator and the solvent removed at room temperature under reduced pressure. The resultant lipidic film was dried for 1 hour under a high vacuum at room temperature. The lipidic film was hydrated by 10% glucose with 10 mM HEPES (pH 7.4) into a 9 mM suspension followed by vortexing for 5 min. The hydrated lipids were extruded through 200 nm filter 5 times to form a liposome

preparation. The stock liposome preparation was diluted to batches (200 μL each) of different concentrations in conical tubes. Luciferase plasmid DNA (200 μL, 600 μg/mL) in water was then added to each liposome batch followed by brief agitation of the tube to form lipoplexes of appropriate N/P ratios (nitrogens from the cationic lipid headgroups divided by DNA phosphates). The lipoplexes were then diluted appropriately to transfect CV-1 cells or CD-1 mice in different doses.

4.3.7 *Particle Diameter Measurements*

Photon Correlation Spectra of the samples was obtained with a Malvern apparatus (Zeta 1000, Malvern Instruments Ltd., Southborough, MA, USA) and used to determine the size distribution of the liposomes and lipoplexes. Typically, a small aliquot of the sample was diluted in 2 mL 10 mM HEPES buffer (pH 7.4) to reach a scattering intensity of 150-200 kilocounts per second. Three measurements of 3-5 min were made for each sample, using the automatic algorithm mode for data analysis. The average of three cumulant results of each sample that agreed within 10% is reported.

4.3.8 *Zeta Potential Measurements*

The Zeta potential of the liposomes were determined in 10 mM HEPES buffer (pH 7.4) by a laser electrophoretic mobility instrument, (Zeta Sizer 4, Malvern Instruments Ltd., Southborough, MA, USA) according to the manufacturer's specifications. In the beginning of each experiment, the instrument was calibrated with a latex sphere standard, STD0050 as recommended by the manufacturer. The average of three measurements was reported for each sample. The standard error of the measurements for each sample is within 5 mV.

4.3.9 *CV-1 Cell Transfection Protocol*

CV-1 cells (a monkey fibroblast cell line) were seeded onto a 24-well microtiter plate at 20,000 cells/well in DME-H21 media (1 mL/well) containing 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 0.1 mg/mL streptomycin). The cells were grown overnight at 37 °C in a humidified atmosphere containing 5% CO_2. The lipoplexes previously formed in conical tubes were left at room temperature for 20-30 min before use in transfection. The cells were transfected by replacing the growth media with the diluted lipoplexes in 1 mL DME-H21 media free of serum and antibiotics. The cells were incubated at 37 °C for 5 h, and the complex-containing media was replaced by 1 mL per well of DME-H21 containing 10% fetal bovine serum and antibiotics. The cells were cultured for an additional 24 h at 37 °C. Then, the media were aspirated and the

cells were lysed by adding 200 μL of 1 x Reporter Lysis Buffer (5 fold dilution of Reporter Lysis Buffer, 5x, Promega, Madison, WI) followed by 2 cycles of freezing at -20°C and thawing at room temperature. After completion of cell lysis, 10 μL of the lysate was used to measure luciferase activity according to the recommendations of the manufacturer (Promega; Luciferase Assay System, no. E1501) using an Optocomp I luminometer (MGM Inc., Hamden, CT). Protein concentration of the cell lysates was determined using the Bio-Rad protein assay (Richmond, CA) in a 96-well plate format according to the manufacturer's specifications.

4.3.10 *Animals*

Female CD-1 mice were purchased from Simonsen (Gilroy, CA, USA). All animals were studied in accordance with guidelines established by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, and with the approval of the Committee on Animal Research at the University of California, San Francisco. The studies utilized female 4-5-week-old CD-1 mice 25 g to 30 g in weight.

4.3.11 *Intratracheal Gene Transfection to CD-1 Mice*

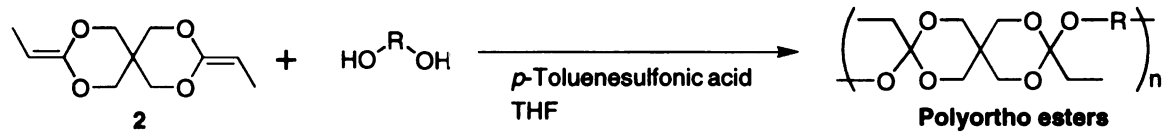
The intratracheal gene delivery protocol is based on the procedure reported by Meyer and coworkers(133). Animals were anesthetized with an intraperitoneal (i.p.) injection of 100 μL anesthetics cocktail (44 mg/kg ketamine, 2.5 mg/kg xylazine and

0.75 mg/kg acepromazine) followed by inhalation with isoflurane. The cationic lipoplexes were delivered to the airways by intratracheal catheter delivery. The trachea was exposed with an anterior midline incision to visualize the insertion of a blunted 18-gauge needle into the trachea. Polyethylene PE-10 catheter tubing (Becton Dickinson, Parsippany, NJ, USA) was threaded through and extended 1-2 mm beyond the tip of the blunted needle. The lipoplex preparations (5 μg plasmid DNA per animal in 100 μL) were then dispensed through a syringe connected to the catheter tubing. An empty syringe connected to the blunted needle was then used to deliver 0.5 mL of air to disperse the dose throughout the lung. The incision was then sutured and recovery of the mouse from anesthesia followed within 30 min. After 24 h, the animals were anesthetized with an intraperitoneal (i.p.) injection of 100 μL anesthetics cocktail (44 mg/kg ketamine, 2.5 mg/kg xylazine and 0.75 mg/kg acepromazine) followed by inhalation with isoflurane. Approximately 1 mL of blood was removed by cardiac puncture, and the lung vasculature flushed by injecting 1 mL of PBS into the cardiac right ventricle. The heart, lung, and about 300 mg of liver of each mouse was excised and transferred into separate tubes with 1.2 mL of lysis buffer (Promega) and 1 g of zirconium beads. The tissues were then homogenized in a Mini-Beadbeater (Biospec Products, Inc., Bartlesville, OK) for 20 s. The homogenate was clarified by centrifugation in a microcentrifuge at 12,000 rpm, 4°C for 5 min. The supernatant (10 μL) was used to measure luciferase according to the recommendations of the manufacturer (Promega; Luciferase Assay System, no. E1501) using a Optocomp I luminometer (MGM Inc., Hamden, CT). Protein concentration of the cell lysates was determined using the Bio-Rad protein assay (Richmond, CA) in a 96-well plate format according to the manufacturer's specifications.

4.4 Results

4.4.1 *Chemistry*

The initial strategy to prepare the ortho ester-based lipidic conjugates is based on the work of Heller and associates, who showed that, in the presence of an organic acid catalyst, 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane (2) smoothly reacts with a series of diols to generate acid-labile linear polymers(131,134)(Scheme 4-1). In Chapter 2, it is shown that, under similar reaction conditions (40°C, THF as the solvent), the monomethyl ether of PEG can be conjugated to distearoyl glycerol *via* the spiro-linker molecule in moderate yield. It is thus anticipated that an ortho ester conjugate of appropriate cationic alcohols and lipidic alcohols could be synthesized in a similar manner.

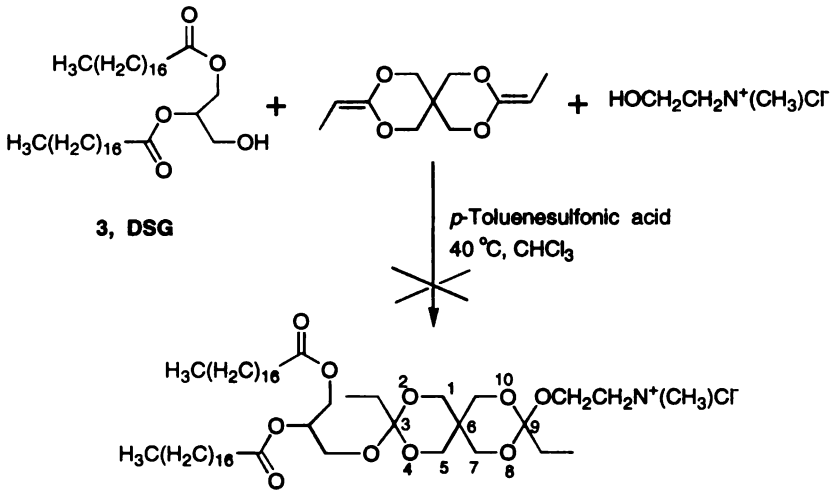


2 Polyortho esters

Scheme 4-1: Synthesis of polyortho esters using 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane and diols in the presence of an organic acid catalyst.

Choline was chosen as the cationic alcohol because the trimethylated ammonium group is present in many reported cationic lipids that mediate gene transfection(14). However, it appeared difficult to find a reaction solvent for the conjugation reaction due to the large difference in the polarity of choline and lipidic alcohols. Lipidic alcohols

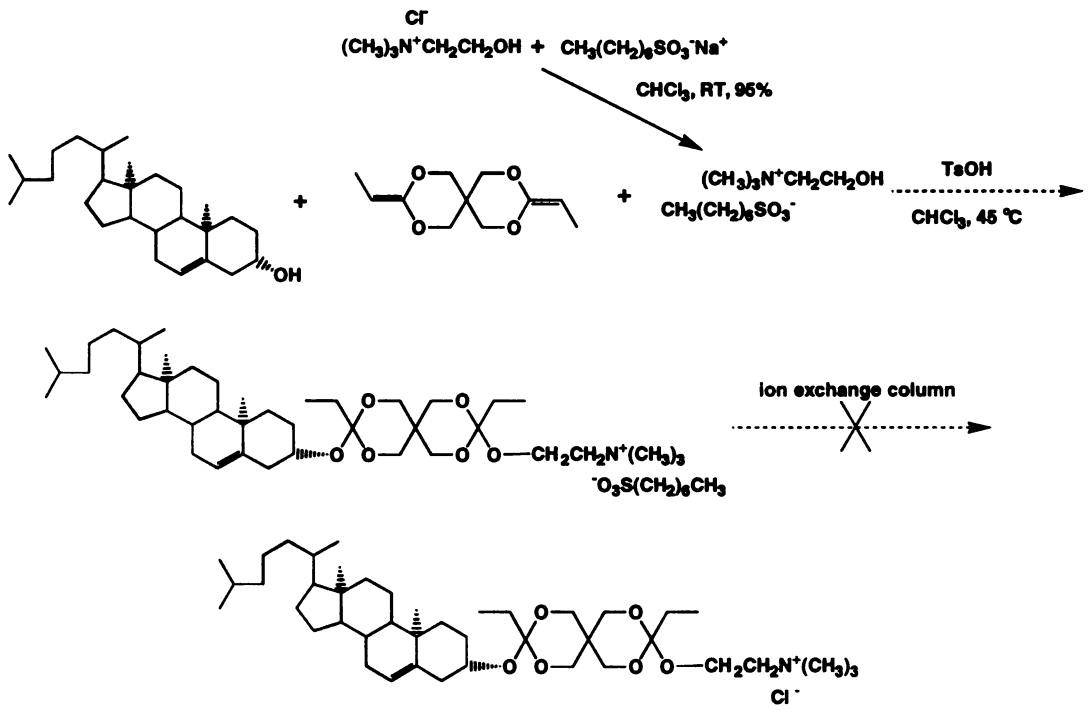
such as cholesterol dissolve in nonpolar solvents such as THF and $CHCl_3$, whereas choline chloride as a highly hydrophilic salt dissolves only in water and MeOH, which are not compatible with the conjugation reaction. An attempted reaction using a $CHCl_3$ suspension of choline chloride and a lipidic alcohol, distearoyl glycerol (**3**), yielded no product (Scheme 4-2).



Scheme 4-2: Attempted conjugation of DSG (3), 3,9-diethylidene-2,4,8,10-tetraoxa-spiro[5.5]undecane (2) and choline in $CHCl_3$ suspension.

In order to increase the solubility of the choline cation in aprotic solvents, the chloride counter ion was exchanged with a large hydrophobic anion, heptansulfonate (Scheme 4-3). After the conjugation reaction, the heptansulfonate counter ion could be changed back to the more biocompatible chloride anion by an anion exchange column. The heptansulfonate salt of choline was readily prepared by stirring choline chloride with sodium heptansulfonate in $CHCl_3$ followed by filtering the NaCl salt. Eighty milliliter of

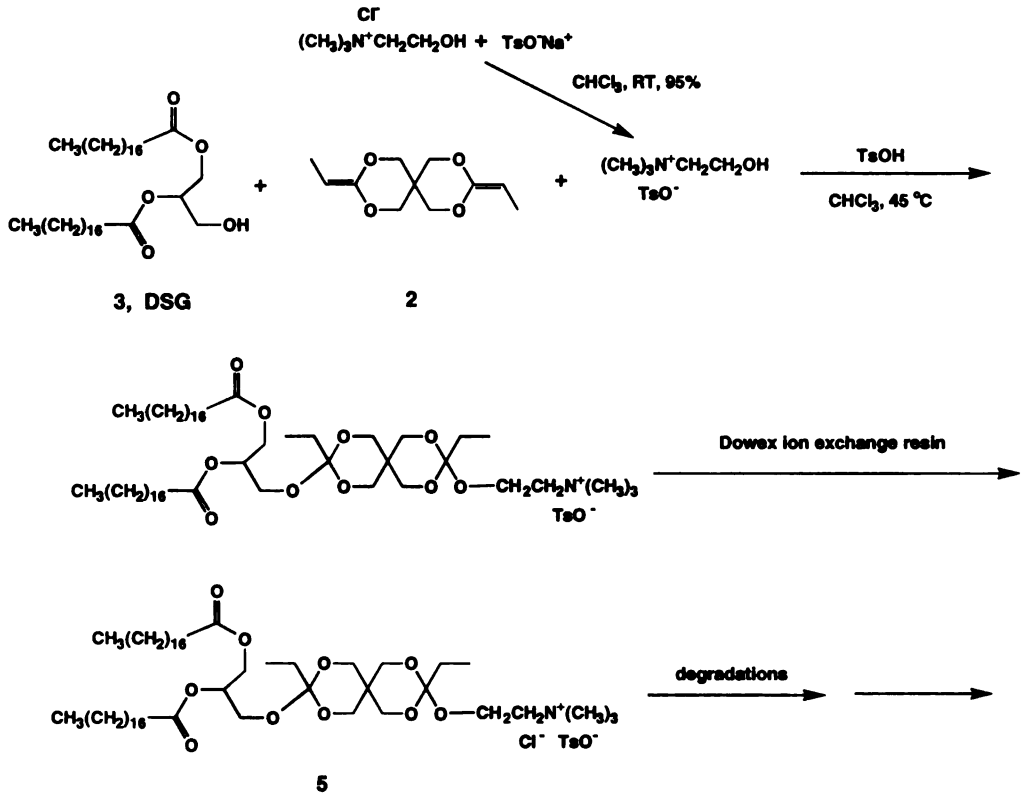
$CHCl_3$ dissolved 3 mmol of choline heptansulfonate (956 mg), which is sufficient for organic reactions in gram scale. Choline heptansulfonate was then dried in high vacuum with cholesterol, a lipidic alcohol, and then dissolved by anhydrous $CHCl_3$. The melted diketene acetal **2** was added with a dry syringe and trace amount of *p*-toluenesulfonic acid was used to initialize the reaction. After the reaction mixture was stirred for 2 hours under Ar, TLC showed the appearance of a new spot whose polarity is similar to that of DOTAP, suggesting the generation of the desired cationic lipid. In order to shorten the preparation procedure so as to minimize the degradation of the product, the reaction mixture was passed through a chloride anion exchange column without separation. Unfortunately, the reaction mixture underwent considerable degradation during the chromatography process, prohibiting the separation of possible ortho ester conjugates.



Scheme 4-3: Attempted synthesis of 3,9-diethyl-3-(cholest-5-en-3β-oxy)-9-(*N*,*N*,*N*-trimethylamino-ethoxy)-2,4,8,10-tetraoxaspiro[5.5]undecane, chloride salt (**4**).

The diketene acetal **2** polymerizes with primary and secondary diols, but tertiary diols do not react,(93) indicating that the formation of ortho esters is favored by alcohols with less steric hindrance. In the synthesis of cationic ortho esters, the minimization of steric hindrance may be even more critical since the attack of the cationic alcohol (e.g. choline) to the dialkoxy carbocation intermediate is already hampered by electric repulsion. Therefore, in the second attempt (Scheme 4-4) to synthesize cationic ortho ester lipids, a primary alcohol, distearoyl glycerol was selected in place of the secondary alcohol, cholesterol to facilitate the reaction. The expected product would also have less steric strain at the linker moiety, and hence improved stability. The *p*-toluenesulfonate anion was used instead of heptansulfonate for two reasons. First, the *p*-toluenesulfonate anion has a more hydrophilic structure than heptansulfonate, and hence would exchange more rapidly with the chloride anion in aqueous solution. Secondly, owing to its aromatic structure, the *p*-toluenesulfonate anion assumes distinctive spectrometric properties, such as a strong UV absorbance at 280 nm and low field peaks in ^{1}H-NMR. These properties facilitate the speedy analysis of the anion exchange process. The solubility of choline *p*-toluenesulfonate is lower than choline heptansulfonate in $CHCl_3$, but 80 mL was sufficient to dissolve 1 mmol of the *p*-toluenesulfonate salt for a small scale reaction. A basic Dowex resin was used to exchange the *p*-toluenesulfonate anions into chlorides after the conjugation reaction followed by separation of the product using silica gel chromatography. In search of a proper eluting solvent system for the chromatography separation, it was found that a small percentage of H_2O is needed together with $CHCl_3$ and MeOH to dissolve the cationic conjugate and to prevent streaking. Therefore, the optimized solvent system consists of $CHCl_3$, MeOH,

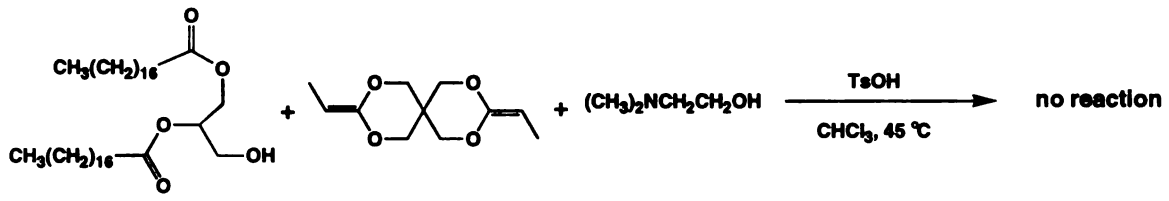
triethylamine as the basic stabilizer and a small percentage of H_2O in the form of a concentrated aqueous solution of ammonia (30 wt%). Fractions containing the desired product were pooled and evaporated. The Electrospray Mass Spectrometry of the residue gave a peak at m/z 940.7 (56%), as expected from the cationic conjugate (caculated m/z 940.8) without the counter ion. However, the 100% peak occured at m/z 375.3, corresponding to a cationic complex of one *p*-toluenesulfonate anion and two protonated triethylamine molecules. The presence of these impurities in the separated conjugate was confirmed by ^{1}H-NMR peaks at δ 7.75, 7.15, 3.0-3.2 and 1.4-1.5. The impurities indicated that: 1) the ion exchange of *p*-toluenesulfonate with chloride is not complete; 2) although the presence of water is necessary to dissolve the cationic conjugate, it protonated and hence entrapped the strong base, triethylamine during the separation process. The conjugate also underwent degradation during the ion exchange and the separation procedures, even though they were carried out at low temperature (4 °C) and under strongly basic conditions. At this point, the synthesis of cationic ortho ester lipids using **2** and the choline cation encountered a technical dilema. The charged conjugate needs water to dissolve into an organic solvent system for purification, but, in the presence of water, the degradation and contamination of the conjugate is difficult to prevent.



Scheme 4-4: Synthesis of 3,9-diethyl-3-(2,3-distearoyloxypropyloxy)-9-(*N*,*N*,*N*-trimethylamino-ethoxy)-2,4,8,10-tetraoxaspiro[5.5]undecane, p-toluenesulfonate and chloride salts (**5**).

One possible way to solve this problem is to design a clean reaction so that the chromatographic purification of the charged and water-labile product can be avoided. The methylation of tertiary amines into quaternary ammoniums using iodomethane(135) is an appealing approach since the reaction can be driven to completion at room temperature by excess iodomethane, which can be easily removed by evaporation during the workup. The reaction of *N*,*N*-dimethylethanolamine with distearoyl glycerol (**3**) and **2** was thus attempted (Scheme 4-5), in the expectation that the resultant conjugate could

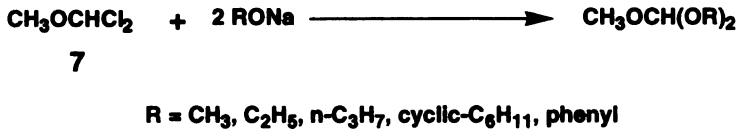
then be methylated at the dimethylamino headgroup. However, the conjugation reaction yielded no product, probably because the basic starting material, dimethylethanolamine quenched the catalytic activity of toluenesulfonic acid.



3, DSG 2

Scheme 4-5: Attempted conjugation of distearoyl glycerol (3), 3,9-diethylidene-2,4,8,10-tetraoxa-spiro[5.5]undecane (2) and dimethylethanolamine.

In search of a bi-functional linker molecule that allows the formation of an orthoster moiety in the presence of tertiary amines, the displacement reactions of a commercially available molecule, dichloromethyl methyl ether (7) was discovered in the literature. Gross and Rieche(136) reported that dichloromethyl methyl ether (7) readily reacts with deprotonated primary alcohols in THF to form ortho esters as dialkoxy-methoxy-methanes in structure (Scheme 4-6).

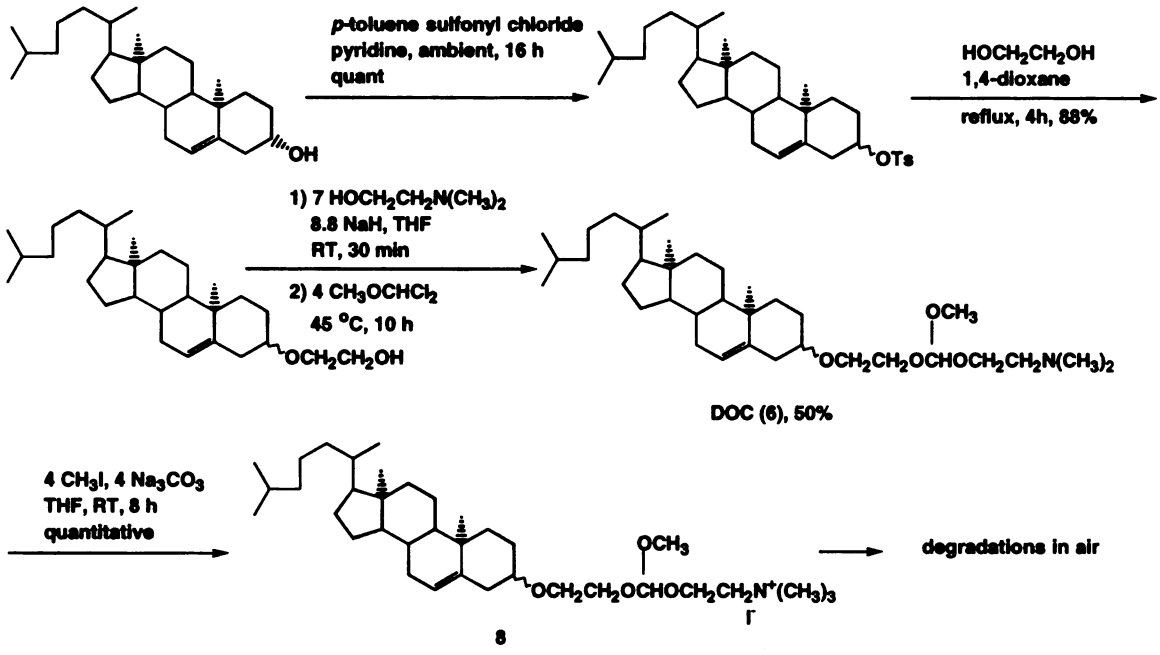


7

Scheme 4-6: Displacement of α,α–dichloromethyl methyl ether (7) with 2 equivalent of deprotonated alcohols.

However, the displacement of **7** by two different alcohols, as desired in the synthesis of cationic ortho ester lipids, has not been published. Nonetheless, dichloromethyl methyl ether (**7**) is an attractive linker molecule, since the displacement reaction should, in theory, be compatible with tertiary amines. Thus, the bi-functional displacement of **7** was attempted to conjugate dimethylethanolamine with a lipidic alcohol (Scheme 4-7). Cholest-5-en-3β-ethan-2-ol(137) was selected as the lipidic alcohol because it is a primary alcohol with minimal steric hindrance and it does not have functional groups that are sensitive to strong bases. Furthermore, a number of cholesterol-based cationic lipids (Figure 4-1) such as DC-Chol(125,138) and cholest-5-en-3β-oxyethane-*N,N,N*-trimethyl ammonium bromide(139) significantly enhance gene transfection in cell culture and *in vivo*. Since cholest-5-en-3β-ethan-2-ol needs to be prepared in lab by two steps of organic synthesis, the conditions of the displacement reaction were optimized to maximize the use of this intermediate. One equivalent of cholest-5-en-3β-ethan-2-ol and 7 equivalents of dimethylethanolamine were treated with 8.8 equivalent of NaH at room temperature for 30 min(140) to ensure complete deprotonation of the alcohols. Four equivalents of dichloromethyl methyl ether (**7**) were then added to start the displacement reaction. The starting materials were set in such molar ratios that most of cholest-5-en-3β-ethan-2-ol would displace one of the chloride groups of **7**. The reaction was continued for 10 h at 45 °C, and the product, as a neutral tertiary amine analogue (DOC, **6**), was readily purified (51% yield) by silica gel chromatography at 4 °C, using a MeOH/$CHCl_3$-based solvent system with triethylamine as a stabilizing base. DOC (**6**) was found to be stable for more than 6 months if stored as a dried wax under Ar at -20 °C. DOC (**6**) was directly used for gene transfection because

the basic dimethylethanolamine headgroup becomes protonated in neutral buffers, which turns the compound into a cationic lipid. It was also used for synthesizing its trimethylated quaternary ammonium analogue **8**.



Scheme 4-7: Synthesis of pH-sensitive, cationic lipidic conjugates of tertiary/quarternary amines with cholesterol using α,α–dichloromethyl methyl ether (**7**).

Iodomethane was used to convert DOC (**6**) to its trimethylated analogue, **8** (Scheme 4-7). Excess equivalents of Iodomethane (100 μL, 228 mg, 1.6 mmol) were stirred with **6** in THF in the presence of sodium carbonate (200 mg, 1.9 mmol) at room temperature for 8 h. TLC showed the dissappearance of the spot of **6** at R_f 0.44 (silica gel, $CHCl_3$/MeOH/triethylamine = 100/2/1), indicating the complete conversion of the starting material. The quaternary ammonium product **8** appeared near the spotting origin on TLC using the same solvent system and at R_f 0.47 when a more polar solvent system

($CHCl_3$/MeOH/(30 wt% NH_3 in H_2O), 4/1/0.05) was used. The product gave a peak at m/z 576.5 in Electrospray Mass Spectrometry, as expected from the molecular weight of the cationic lipid without the negative counter ion. Unfortunately, once exposed to the air during workup, the quaternary ammonium analogue quickly decomposed. TLC showed the generation of hydrophobic impurities, presumably analogues of cholest-5-en-3β-ethan-2-ol from the hydrolysis of **8**. Mixing **8** with the base sodium carbonate was unable to prevent the degradation.

To summarize the efforts to synthesize the ortho ester-based cationic lipids, two reaction schemes were studied. The first one utilized 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane (**2**) as the key bi-functional linker molecule and carried out acid-catalyzed addition of alcohols on the two ethylidene groups of **2**. With the help of the *p*-toluenesulfonate counter ion, the choline cation was dissolved in $CHCl_3$ and conjugated with the lipidic alcohol, distearoyl glycerol (**3**) *via* the linker molecule **2**. However, the conjugation of the dimethylethanolamine with **2** yielded no product, probably because the basic dimethylethanolamine quenched the catalytic activity of toluenesulfonic acid. The second synthetic scheme involves the neucleophilic displacement of α,α–dichloromethyl methyl ether (**7**). Dimethylethanolamine was successfully conjugated with cholest-5-en-3β-ethan-2-ol by this method and the resultant lipid, DOC (**6**), can be readily purified and stored in its neutral form. The quaternary ammonium analogue (**8**) of **6** can be prepared by the methylation of DOC with Iodomethane. Unfortunately, the quaternary ammonium analogues prepared by both synthetic schemes are not stable, which prohibited their purification and the evaluation of their biological activities.

4.4.2 *Size and Zeta Potential of Cationic Liposomes and Lipoplexes*

Following their synthesis, one of the ortho ester-based cationic lipids, DOC (**6**) was used to prepare catioinic liposomes and lipoplexes. A lipid film composed of equal molar ratio of DOC and DOPE was readily hydrated and extruded through 200-nm membranes to form the liposomes. The liposomes were then mixed with the luciferase plasmid DNA to form cationic lipoplexes in different N/P ratios(141), where N is the nitrogen from the dimethylethanolamine headgroups of DOC and P is the phosphate from the plasmid DNA. In order to study the colloidal properties of these liposomes and lipoplexes, the size and the Zeta potential of the particles were measured. Table 4-1 shows the size of the liposomes and their corresponding lipoplexes (N/P = 5/1). The Zeta potential of the liposomes and lipoplexes of different N/P ratios are shown in Table 4-2. In comparison, the liposomes and lipoplexes of two other lipid compositions, DC-Chol/DOPE (1/1) and DOTAP/Chol (5/4) were prepared and characterized in the same manner.

Table 4-1

Size Distribution of Cationic Liposomes and Lipoplexes[a]

Composition (mole/mole)	Liposomes		Lipoplexes (N/P[b] = 5/1)	
	D[c] (nm)	P.I.[d]	D[c] (nm)	P.I.[d]
DOC/DOPE (1/1)	219±53.2	0.208	282.4±105.5	0.139
DC-Chol/DOPE (1/1)	177.9±61.8	0.120	530.0±330.9	0.388
DOTAP/Chol (5:4)	198.2±80.5	0.165	287.8±139.2	0.234

[a]Samples were measured in 10 mM HEPES buffer, pH 7.4. [b]Nitrogen to phosphate ratio: N stands for the mole of nitrogen from the dimethylethanolamine headgroup of DOC and P stands for the mole of phosphate from plasmid DNA. [c]Pariticle diameter reported as mean ± standard deviation from the cumulant result of Photon Correlation Spectrometry. [d]Polydispersity Index.

All the cationic liposomes were readily extruded through the polycarbonate membranes of 200 nm in pore diameter and have a diameter around 200 nm following the extrusion(Table 4-1). The lipoplexes are larger and more heterogeneous than their corresponding liposomes. The DOC/DOPE and DOTAP/Chol lipoplexes have a diameter around 300 nm and a polydispersity index below 0.25. The lipoplexes consisting of DC-Chol/DOPE are larger and more heterogeneous, with a mean diameter of 530 nm and a polydispersity index of 0.388.

The Zeta potential plays an important role in the interaction of lipid vesicles with biological membranes(130,142-144) and may affect the gene transfection mechanism and efficiency. This is because the cell surface carries a net negative charge and the positively charged lipoplexes interact with the cell surface *via* electrostatic interactions. The excess positive charges on the surface of lipoplexes, as reflected by a highly positive Zeta potential, also prevent the lipoplexes from aggregation and precipitation. The Zeta

potential (Table 4-2) of the DOC/DOPE liposomes is strongly positive as the other liposomes, confirming that DOC (**6**) becomes cationic *via* protonation in the neutral buffer. All the three types of lipoplexes are strongly positive when the cationic lipids are in large excess over the negative charges from DNA phosphates (N/P = 5/1, 3/1). However, when the cationic lipids were mixed with plasmid DNA containing equimolar of phosphates (N/P =1), the DOC/DOPE-DNA and DC-Chol/DOPE-DNA lipoplexes exhibited different properties from DOTAP/Chol-DNA complexes. The DOC/DOPE-DNA and DC-Chol/DOPE-DNA complexes have strongly negative Zeta potentails, whereas the DOTAP/Chol-DNA lipoplexes precipitated out of the solution. This result is due to the difference between the tertiary amine headgroups of DOC and DC-Chol, and the quaternary ammonium headgroup of DOTAP. At neutral pH, the headgroups of DOC and DC-Chol are only partially protonated, which reduces the number of positive charges. This in turn results in the excess negative charges on the lipoplexes when the N/P value is 1. In the case of DOTAP, each quaternary ammonium headgroup carries a positive charge regardless of the buffer pH and the N/P ratio is equivalent to the charge ratio. Therefore, at N/P ratio 1, the DOTAP/Chol-DNA lipoplexes are near charge neutrality, and hence cannot stay in the solution as stable colloids(145).

Table 4-2

Zeta Potential of Cationic Liposomes and Lipoplexes[a]

Composition (mole/mole)	Liposomes (mV[b])	Lipoplexes (mV[b])		
		5/1[c]	3/1[c]	1/1[c]
DOC/DOPE (1/1)	65.7	59.5	57.6	-40.1
DC-Chol/DOPE (1/1)	57.3	56.1	45.0	-46.7
DOTAP/Chol (5:4)	57.3	54.4	53.6	Ppted[d]

[a]Samples were measured in 10 mM HEPES buffer, pH 7.4. [b]Zeta potential in millivolts. The standard deviation is less than 5% of the reported mean value. [c]N/P ratio, where N stands for the mole of nitrogen from the dimethylethanolamine headgroup of DOC and P stands for the mole of phosphate from plasmid DNA. [d]Particles precipitated.

Table 4-3

Change of Zeta Potential of Cationic Lipoplexes Composed of

DOC and DOPE (1/1) and Luciferase Plasmid DNA

N/P ratio[a]	Temperature (°C)	pH[b]	Time	Zeta Potential[c] (mV)
5/1	RT[d]	7.4	0 min	59.5
5/1	37	5.5	10 min	60.9
5/1	37	5.5	60 min	61.4
5/1	37	5.5	24 h	39.5
3/1	37	4.5	0 min	60.6
3/1	37	4.5	60 min	61.9
3/1	37	4.5	24 h	Ppted[e]

[a]N/P ratio: N stands for the mole of nitrogen from the dimethylethanolamine headgroup of DOC and P stands for the mole of phosphate from plasmid DNA. [b]HEPES buffer (10 mM) was used for pH 7.4. For acidic pHs, 50 mM NaOAc/HOAc buffer was used. [c]Zeta potential in millivolts. After incubation of the lipoplexes at specified pH and temperature, a small aliquot of the sample is diluted in 10 mM HEPES buffer (pH 7.4) and analyzed. The mean of three measurements is reported. The standard deviation is less than 5% of the reported mean value. [d]Room temperature. [e]Particles precipitated.

In order to test the pH-sensitivity of the lipoplexes consisting of DOC, the DOC/DOPE-DNA lipoplexes were incubated at acidic pH and the change of their Zeta potentail was monitored (Table 4-3). When the lipoplexes with an N/P ratio of 5/1 were incubated at pH 5.5, 37 °C, the Zeta potential did not change for the first 1 h but decreased from about 60 mV to 40 mV after 24 h. The lipoplexes at N/P ratio of 3/1 remained stable after 1 h of incubation at pH 4.5, 37 °C, but precipitated when the incubation was continued for 24 hours, indicating that most of the excess positive charges of the lipoplexes were lost due to the hydrolysis of DOC.

4.4.3 *Gene Transfection in CV-1 Cell Culture Mediated by DOC/DOPE Liposomes*

CV-1 cells (a monkey kidney fibroblast cell line) in culture were treated with DOC/DOPE-DNA lipoplexes to test their ability to mediate gene transfection (Figure 4-2). DC-Chol/DOPE-DNA lipoplexes were tested as a control formulation since DC-Chol is structurally similar with DOC but is stable at mildly acidic pHs. At appropriate charge ratios and plasmid DNA doses, DOC/DOPE liposomes are significantly more efficient than the DC-Chol/DOPE liposomes in transfecting the CV-1 cells. For example, at charge ratio 5/1 and plasmid DNA dose of 3 μg per well, DOC/DOPE is about 10 fold more efficient than DC-Chol/DOPE (~5.6 x 10^8 RLU/mg cellular protein vs. ~0.56 x 10^8 RLU/mg cellular protein). At charge ratio 3/1 and plasmid DNA dose of 3 μg per well, DOC/DOPE is about 7-fold more efficient than DC-Chol/DOPE (~8.4 x 10^8 RLU/mg cellular protein vs. ~1.2 x 10^8 RLU/mg cellular protein). No transfection was observed for naked plasmid DNA at the dose of 3 μg per well.

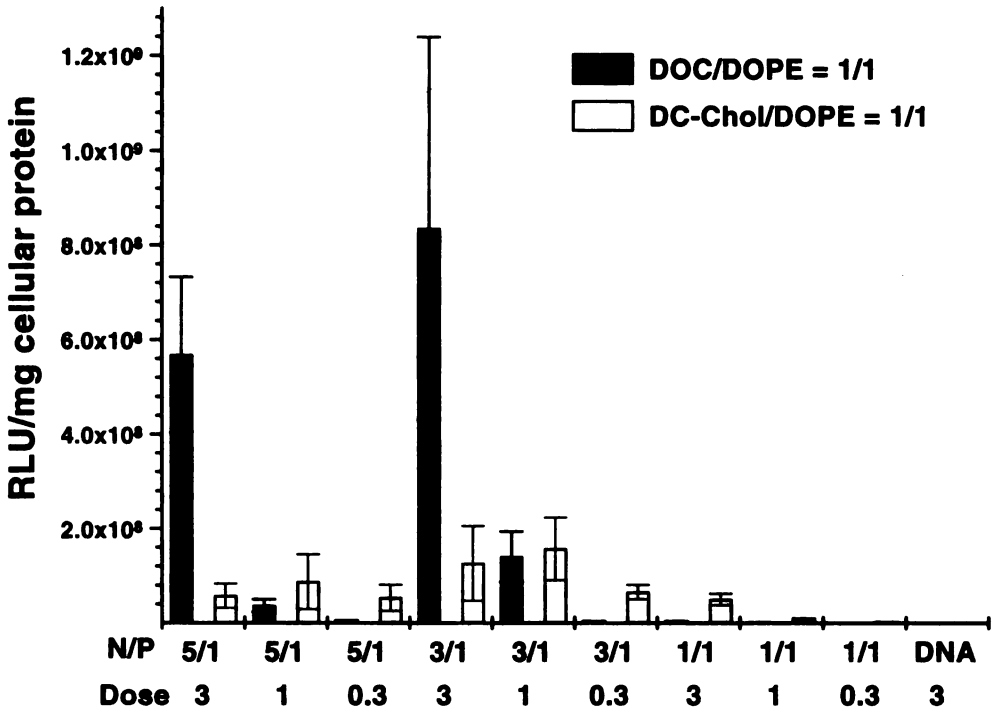


Figure 4-2: Transfection of Cationic Lipoplexes to CV-1 cells. The gene expression level is expressed as relative light units (RLU) per milligram cellular protein. N/P is the nitrogen to phosphate ratio of the lipoplexes. Dose of the lipoplexes are in μg plasmid DNA/well. DNA stands for the plasmid alone.

4.4.4 *Intratracheal Transfection in Female CD-1 Mice*

On the basis of the transfection results in CV-1 cell culture as well as reports in the literature(133), we evaluated the activity of DOC and DC-Chol to deliver the luciferase plasmid DNA into female CD-1 mice after intratracheal administration (Table 4-4). In the specified formulation (N/P ratio = 5:1, with equimolar DOPE as the helper lipid), DOC provided significantly better transfection activity. As expected after

intratracheal administration, most of the luciferase gene expression occurred in the lung for both formulations. DOC/DOPE-DNA lipoplexes mediated more than 5-fold as much gene expression in the lung compared to its pH-insensitive control, DC-Chol/DOPE-DNA (2.68×10^5 RLU/mg cellular protein vs. 4.81×10^4 RLU/mg cellular protein). Some residual gene expression was also observed in heart, liver and spleen, where DOC showed more activity than DC-Chol as well.

Table 4-4

Intratracheal Transfection[a] in Female CD-1 Mice

Tissue	DOC/DOPE (1/1)		DC-Chol/DOPE (1/1)	
	Mean	S.D.	Mean	S.D.
Heart	5.67×10^2	4.51×10^2	0	0
Lung	2.68×10^5	1.50×10^5	4.81×10^4	1.79×10^4
Liver	3.27×10^2	2.36×10^2	62.2	71.3
Spleen	5.46×10^3	9.34×10^2	0	0

[a]Five μg luciferase plasmid DNA in 100 μL buffer was administered to each animal and the gene expression level 24 h post administration is expressed as relative light units (RLU) per mg tissue protein. The mean and the standard deviation (S.D.) of at least four animals are reported.

4.5 Discussions

In both the reaction schemes used to synthesize ortho ester-based cationic lipids, the quaternary ammonium derivatives have very poor stability, which prohibited their purification and biological evaluations. This is in contrast with the tertiary amine derivative DOC (**6**), as well as the PEG derivative, POD (**1**), which can be readily purified and stored for months as a dried solid at -20 °C. The reason for the poor stability of the quaternary ammonium-derived ortho esters is not fully understood, but may be attributed to the high degree of hydration at the ortho ester linker region due to the positive charge of the quaternary ammonium. In studies on the ortho ester-based polymers, it was found that the accessibility of water molecules to the ortho ester groups is a critical factor for the hydrolysis of the polymers(146). With a positive charge at the headgroup, the quaternary ammonium-based ortho ester conjugates are likely to absorb a considerable amount of moisture upon exposure to the air. The entrapped water molecules, predominantly around the charged headgroup, would in turn facilitate the hydrolysis of the adjacent ortho ester linker. In the cases of POD (**1**) and DOC (**6**), however, the headgroups are not ionic and hence would be less hydroscopic. The tertiary amine-based headgroup of DOC would further stabilize the conjugate by providing an alkaline condition close to the ortho ester linker.

The Zeta potential of the DOC/DOPE-DNA lipoplexes are very similar to DC-Chol/DOPE-DNA lipoplexes at different N/P ratios. For all three types of lipoplexes, the Zeta potential at N/P ratio 3/1 is highly positive and close to the Zeta potential at N/P ratio 5/1 (less than 10 mV different). This is consistent with the previous findings(145,147) that the Zeta potential of cationic lipoplexes reaches close to a plateau

when the charge ratio of the preparation (positive charges from the headgroup of the cationic lipid over negative charges from the DNA phosphate) is higher than 2/1. At N/P ratio of 1/1, both DOC/DOPE-DNA and DC-Chol/DOPE-DNA lipoplexes gave a highly negative Zeta potential around -40 mV, consistent with a partial protonation of the tertiary amine headgroups in both type of lipoplexes. The similar Zeta potential values of the two types of lipoplexes at different charge ratios also suggest that the headgroups of DOC and DC-Chol have similar pKa values, which is expected from the same headgroup structure as dimethyl-β-alkoxyethylamine. The similarity between the structures of DOC and DC-Chol (Figure 4-1) and the similar Zeta potentials of their lipoplexes, support the use of DC-Chol as a control lipid for the biological characterization of the ortho ester-based cationic lipid, DOC.

After incubation at pH 4.5 for 1 h, the Zeta potential of DOC/DOPE-DNA lipoplexes (N/P = 3/1) was unchanged. This observation indicates that the hydrolysis of DOC in its cationic lipoplexes is significantly slower than POD, which is completely hydrolyzed within 1 h at pH 5 (Figure 2-2). The slower hydrolysis of DOC may be attributed to the field effect(148,149) of the lipoplexes with a high positive charge density. It is well documented that the protonation of a basic nitrogen considerably decreases the apparent pKa of a nearby basic nitrogen(150) because the positive charge from the protonated nitrogen hinders the approach of another cationic proton *via* electric repulsion. Recently, Meidan and coworkers(151) studied the interaction between cationic liposomes and a fluorescent-labeled oligonucleotide. The spectrometric change of the fluorescent probe indicated that the local pH within the cationic complexes was elevated by up to 0.7 pH unit. Similarly, the pH within the cationic DOC/DOPE-DNA lipoplexes

could be significantly higher than the bulk solution, leading to the decreased rate of DOC hydrolysis.

The greater than 5 fold increase in the reporter gene expression mediated by DOC (**6**) over the control lipid, DC-Chol, strongly supports the concept that the incorporation of a pH-sensitive functionality into a cationic lipid facilitates the delivery of the complexed plasmid DNA. The decrease of the Zeta potential of DOC/DOPE-DNA lipoplexes (N/P = 5/1) at pH 5.5, as well as the aggregation of the lipoplexes (N/P = 3/1) at pH 4.5, conforms with the rationale that, after being taken up by the cells, the lipoplexes would be triggered by the acidification of the endocytic pathway(31,152) and would facilitate the escape of DNA from the endosomal/lysosomal compartments to the cytoplasm(27,28). The change of the Zeta potential of DOC/DOPE-DNA lipoplexes is relatively slow at pH 5.5 and 4.5, which may account for the modest increase in the transfection activity observed for this derivative. However, more biological and biophysical studies of the cellular processing of DOC-based lipoplexes are needed to confirm the proposed mechanism of the improved transfection efficiency.

During the process of my studies described in this Chapter, Zhu and coworkers(62) reported a number of acid-labile cationic lipids (see structures in Chapter 1, Scheme 1-5) containing an ortho ester linker based on the structure of 3,5,8-trioxabicyclo[2.2.2]octane. However, due to the particular configuration of the linker, the first two fast hydrolysis steps of the ortho ester functionality do *not* fragment the cationic lipid, but rather add two hydroxy groups near the cationic headgroup region. It is only after the final slower step of the hydrolysis, which is the cleavage of an ester group, that the lipids convert to two single-chain compounds. Since the authors postulated that it is

the single-chain degradation products that induce defects on membrane structures, such hydrolysis pattern may complicate the kinetics of bilayer destabilization by these cationic ortho esters. Since these ortho esters were reported in a communication article, the details of the chemical and biological characterizations of these compounds were not described in the article, but found in the supplementary materials of the paper. However, even in the supplementary pages, only a text description of the leakage assay and a leakage percentage at a single time point was reported (about 40% leakage after incubation at pH 3.5 for 10 minutes); no kinetic data of the destabilization of liposomes were shown. In NIH 3T3 cell culture, one of these derivatives mediated about 10-fold more luciferase gene expression compared to two control cationic lipids, DOTAP and DC-Chol. However, the overall transfection level, including the control lipids, was at least 100-fold lower than what is normally reported in the literature for the same cell line. Using 1 μg plasmid DNA per well in 24-well plates, the maximum transfection level was about 6×10^5 RLU per well in this study, whereas the usual transfection level of NIH 3T3 cells by cationic liposomes is 1×10^8 to 1×10^9 RLU/mg cellular protein(153,154), corresponding to about 5×10^7 RLU per well. Such discrepancy suggests that the improved gene transfection by one of the cationic ortho ester surfactants derived from 3,5,8-trioxabicyclo[2.2.2]octane may be attributed in part to the unoptimized cell culture assays.

The improved gene transfection by DOC-based lipoplexes opens considerable opportunities for the design of a new generation of cationic lipids, and hence for the development of more efficient gene delivery systems. Up to now, a large number of cationic lipids (Figure 4-1) have been reported(14,142,155) and their gene transfection

activities documented in the literature. Empirical rules on the structural features of the efficient cationic lipids have been established, which could be used to design cationic ortho ester-based lipids of superior transfection activity. For example, the dioleoyl side chains appear in many cationic lipids of high transfection activity (DOTAP(156), DOTMA(157), DOSPA(158), OOCE(147)), and hence could be used as the hydrophobic moiety of the cationic ortho ester conjugates. The spermine-like headgroups of DOSPA(158) and cholesterol polyamine carbamates(150) could also be used as the headgroup of the ortho esters.

4.6 Summary

Three cationic lipids with an acid-labile ortho ester linker between the headgroup and the hydrophobic tail were synthesized in an effort to design cationic liposomes that help the translocation of DNA from the endosome compartment to the cytoplasm. One of the prepared lipids, *N*,*N*-dimethyl-(4-methoxy-(cholest-5-en-3β-oxy)hept-3,5-dioxa-yl)amine (DOC, **2**) was combined with a fusogenic helper lipid, DOPE and used to prepare cationic liposomes. The cationic DOC/DOPE liposomes at N/P ratios of 5/1 and 3/1 spontaneously condense with plasmid DNA into cationic lipoplexes. Upon incubation at acidic pHs, the lipoplexes lost their positive charges as a result of DOC hydrolysis. Both in CV-1 cell culture and in CD-1 mice, lipoplexes containing DOC increased the luciferase gene expression more than 5 fold compared with the pH-insensitive control lipid, DC-Chol. These observations support the hypothesis that the incorporation of a pH-sensitive functionality into a cationic lipid enhances its ability to deliver DNA into the cytoplasm.

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