Chemical Approaches to Triggerable Lipid Vesicles for Drug and Gene Delivery

XIN GUO AND FRANCIS C. SZOKA JR.*
Departments of Pharmaceutical Chemistry and Biopharmaceutical Sciences, University of California at San Francisco, San Francisco, California 94143-0446

Received August 8, 2002

ABSTRACT

Effective drug delivery requires the precise spatial and temporal delivery of therapeutic agents to the target site. To this end, a variety of chemical and physicochemical approaches have been devised to create lipid vesicles (liposomes) that can be triggered to release their contents in a controlled fashion. The triggers include changes in pH, redox potential, temperature, or the level of specific enzymes. We review the chemistries that have recently been applied to exploit the pH and redox potential triggers so as to release vesicle contents in the appropriate biological location.

1. Introduction

Since the discovery of liposomes by Bangham and coworkers[1] 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). Despite this plethora of information, liposomes still have not attained their full potential as drug and gene carriers. There are three scientific 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 cellular barriers to reach the target site. The second is to synthesize ligands that are selective for particular cell types. The third is to devise mechanisms for the liposomes to transfer the encapsulated drug or gene into the target.

Ideally, a drug-loaded liposome should remain stable until it reaches the target site.[2] Upon accumulation of the liposome at the target, the drug must be released at a high enough level to mediate 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 effective targeted delivery systems.[2] The following sections review the 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.

2. Requirements for Targeted in Vivo Delivery Using Liposomes

2.1. Challenges of Drug Delivery after Intravenous Administration. The most common 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.[3] Premature leakage of the drug from the liposome will not only decrease the amount of drug that reaches the target site, but it will also result in systemic toxicity. Therefore, the liposome must tolerate insults from plasma proteins in the vascular system. Second, liposomes must remain in circulation long enough to have time to accumulate in the target cells. This requires the liposomes 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. 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. One important exception to this size limitation is in solid tumors, where the vasculature is more porous and allows the permeation and retention of large particles.[4] The Doxil sterically stabilized liposome formulation utilizes this phenomenon to passively target doxorubicin to solid tumors.

2.2. Additional Barriers for Gene Delivery. For therapeutic agents such as oligonucleotides, proteins, RNA, and DNA, additional mechanisms must be exploited to transfer these macromolecules across subcellular barriers to reach their intracellular target site.[2] Among these macromolecules, the delivery of plasmid DNA for gene therapy is the most formidable task. After reaching the target cell, DNA needs to transfer across the plasma membrane, either by fusion or by endocytosis.[5,6] Subsequently, the DNA must be released from the endosome into the cytoplasm[5] to avoid degradation in the lysosomes. Finally, the DNA must relocate from the cytoplasm into the nucleus to direct the expression of the gene products.[7] Clearly the delivery of high molecular weight, hydrophilic

Xin Guo (born in Shanghai, P. R. China, 1971) received his M.S. (Medicinal Chemistry) from Duquesne University and his Ph.D. (Pharmaceutical Chemistry) from University of California, San Francisco. His major research interests include designing novel molecules to be applied in drug and gene delivery systems and developing high-throughput methods to characterize protein folding, protein−protein interaction, and drug−protein interaction.

Francis Szoka (born in New York, 1943) received his M.S. (Microbiology) from the University of Maryland and his Ph.D. (Biochemistry) from State University of New York. His major research interests include applying chemical, biochemical, and biophysical approaches to the study of membrane fusion/destabilization and developing drug/gene delivery systems based upon defined physicochemical mechanisms of membrane destabilization and intracellular trafficking.

* To whom correspondence should be addressed. Fax: (415) 476-0688. Phone: (415) 476-3895. E-mail: szoka@cgl.ucsf.edu.

10.1021/ar9703241 CCC: $25.00
Published on Web 03/08/2003

molecules across biomembranes is one of the more challenging problems facing the pharmaceutical industry.

2.3. Current Status of Liposomal Drug Delivery Suggests the Need of Triggered Release. Over the past 20 years, numerous new chemical methodologies have been developed to address issues related to drug encapsulation, retention and stability of liposomes in circulation.[8] 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 location and time of drug release. First, the stimulus to trigger the drug release must be specific to the target site; second, the liposome must be sensitive enough to the trigger to yield effective release; third, the triggered release mechanism must be compatible with its preexisting properties such as drug retention, long circulation time, and deposition at the target site. Recent literature reviews have provided persuasive examples, data, and 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.[2,9,10]

3. Strategies of Engineering Liposomes for Triggered Release

The strategies developed to induce liposomal leakage in response to an environmental stimulus include (1) formation of defects and channels in the bilayer, (2) lamellar-micellar phase transition, (3) lamellar-hexagonal phase transition, (4) lipid phase separation, and (5) liposome fusion. These topics have been the subjects of several excellent reviews.[3,11−16]

The stimuli to induce release 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, enzymatic cleavage,[11] 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), cleavable lipids, functional ionizable polymers and peptides, ionizable detergents, cis−trans isomerization, and free-radical-generating compounds as photosensors.

This review will discuss triggered release by stimuli inherent in the biology: the decrease of pH and the change of redox potential. The advantage of biological triggers, compared with externally applied stimuli such as heat and light, is that they do not require complicated medical engineering to apply the stimulus after the delivery system has distributed in the body.

4. pH-Sensitive Liposomes

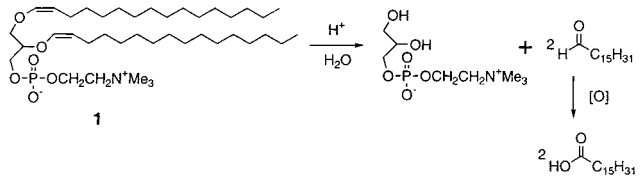
4.1. Overview of the Mechanism. The decrease of pH is implicated in many physiological and pathological processes such as endosome trafficking, tumor growth, inflammation, and myocardial ischemia.[12,13] Therefore, numerous pH-sensitive liposomes have been designed and intensively studied over the past two decades.[12,14−16] The mechanisms of pH-triggered liposome destabilization include (1) neutralization of negative lipids in the bilayer via protonation, leading to lamellar to hexagonal phase transition, (2) protonation of negative polymers or peptides, which in turn absorb to the bilayer 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, and (4) ionization of neutral surfactants into their positive and surface-active conjugate acids.

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 endosome in cells occurs in about 10−30 min with pH in the range of 5−6 before the endosomal contents traffick into the lysosome where extensive degradation takes place.[17] 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. At inflammatory tissues[18] and solid tumors,[19] the pH is only 0.4−0.8 units more acidic than that of the circulation, suggesting that liposomes designed for these purposes need to respond to a small stimulus and release enough drug for a therapeutic effect. It should also be noted that the requirements may vary depending on the delivered agent (see sections 2.1 and 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 bilayer and host cell membranes appears necessary to transfer DNA into the cytoplasm of most cell types.

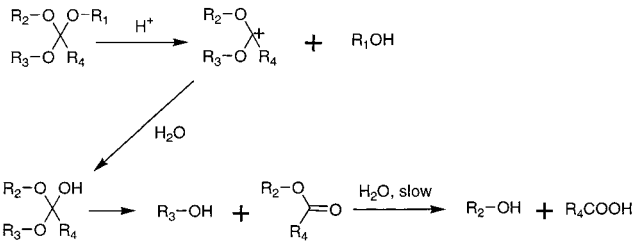
4.3. Neutralization of Bilayer Surfactants. The first pH-sensitive liposome system was introduced by Yatvin and colleagues.[20] This was composed of phosphatidylcholine and N-palmitoyl homocysteine. Since then, a variety of pH-sensitive liposomes that feature a surfactant with a pH-titratable carboxylate group and a fusogenic, conical-shaped lipid such as DOPE have been described.[14] The decrease of pH results in the neutralization of the excess negative charges of the carboxylate groups. This reduces the surface area of the headgroup and triggers the collapse of the PE-rich lamellae into a hexagonal phase with concomitant release of the encapsulated contents. These tritratable lipid systems have been extensively reviewed[12−14] and hence will not be covered here. The drawback of these surfactants, however, is that at neutral pH, the excess negative charges of the carboxylate groups on the liposome surface induce undesired interactions with plasma proteins and fixed macrophages, leading to rapid elimination of the liposomes from circulation[2].

4.4. Neutralization of Polyelectrolytes. In a similar vein to the titratable lipids, functional polymers and peptides with titratable acidic groups can be used to destabilize

Scheme 1. Hydrolysis of Diplasmenyl Phosphocholine



Scheme 2. General Mechanism of Acid-Catalyzed Hydrolysis of Ortho Esters



the liposomes at low pH. One category that was developed in our group, the GALA family of peptides, is particularly potent, and the mechanism has been reviewed.[21] There are excellent reviews on the various functional polyelectrolytes used for this purpose,[12,16] so they will not be covered here.

4.5. Surfactant Hydrolysis. One attractive approach to circumvent the problem of a negatively charged surface of pH-sensitive liposome is to design cleavable surfactants with noncharged functional groups whose hydrolysis is catalyzed by acidic conditions. The chemical principles behind this approach have been described in an outstanding review published almost three decades ago by Cordes and Bull.[22] Given these principles, chemists have considerable flexibility in tailoring lipid structures for specific application. Different headgroups, lipid chains, linker groups, and linkage configurations can be introduced to generate unique surfactants of desired properties.

Thompson and associates reported a number of mono- and diplasmenyl lipids with an acid-sensitive vinyl ether linkage between the headgroup and one or both of the hydrocarbon side chains.[23,24] Upon exposure to low pH or photooxidation, the vinyl ether side chains are cleaved from the lipids (**1** in Scheme 1), leading to structure defects in the bilayer 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 min to release 50% of their contents at pH 2.3 and about 500 min for 50% release at pH 5.3.[25] The incorporation of dihydrocholesterol into the bilayer to improve the serum stability greatly reduces the pH sensitivity, and at pH 2.3, over 70 min is needed to effect 50% content release in 6:4 PlsPamCho/DHC liposomes. Liposomes composed of diplasmenyl phosphocholine (DPPlsC) possess better pH sensitivity and released 50% of encapsulated calcein in 230 min at pH 5.3.[26] 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 h. 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 the principle that the introduction of a pH-triggering mechanism into targeted liposomes can significantly increase the efficacy of the encapsulated therapeutic agents.

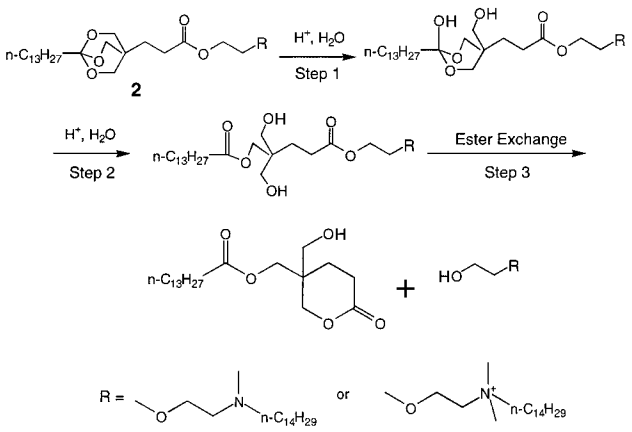
Boomer and Thompson[23,27] reported the synthesis of three novel diplasmenyl lipids based on a chiral 1,2-di-*O*-(1*Z*′,9*Z*′-octadecadienyl)-*sn*-glycerol platform. The lipids displayed three different headgroups: a neutral phosphocholine group, a sterically stabilizing poly(ethyleneoxide) group, and a cationic *O*-carbamoyl-*N*-diethylenetriamine group. The diplasmenyl PEG lipid, (*R*)-1,2-di-*O*-(1*Z*′,9*Z*′-octadecadienyl)-*sn*-glyceryl-3-(ω-methoxy-poly(ethyleneglycolate) (BVEP), was formulated with the hexagonal-phase-favoring lipid, DOPE, to generate unilamellar liposomes. BVEP:DOPE liposomes at 3:97 molar ratio offered the best pH-triggered leakage profile, releasing 50% of encapsulated calcein in 4 h at pH 4.5 and 15% at pH 7.4. Lipid mixing assays and transmission electron microscopy experiments suggested that the acid-triggered hydrolysis of BVEP primarily induced contents leakage, with membrane fusion and vesicle collapse occurring on a slower time scale. The PEG headgroup of BVEP shows considerable promise for in vivo applications of the diplasmenyl lipid. However, the leakage rate of the BVEP/DOPE liposomes at mildly acidic pH (5−6) may need to be increased to yield an optimal drug release system.

Surfactants containing the acid-labile ketal and methylene−hydrazine linkages have also been synthesized.[28−30] Song and Hollingsworth[30] reported a glycolipid conjugate of glucose and two palmitoyl side chains via an acid-labile acetal moiety. The glycol lipid self-assembles into lamellar structures in aqueous solution and 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 1−20% 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.

Compared with vinyl ethers and acetals, ortho esters are expected to hydrolyze more quickly in response to pH decrease, due to the stable dialkoxy cation intermediate as shown in Scheme 2.[22] Indeed, ortho esters are one of the most acid-sensitive functional groups in the literature[31] and polyortho esters have been extensively studied by Heller and co-workers for controlled drug release.[32]

Zhu and co-workers[33] incorporated an ortho ester functional group into cationic lipids. Two cationic lipids (**2** in Scheme 3) containing an ortho ester linker based on the structure of 3,5,8-trioxabicyclo[2.2.2]octane were synthesized and characterized. However, 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

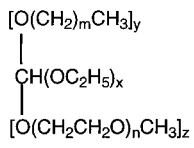
Scheme 3. Hydrolysis of Ortho Esters Derived from 3,5,8-Trioxabicyclo[2.2.2]octane



single-chain compounds. Such a hydrolysis pattern complicates the kinetics of bilayer destabilization by these surfactants and perhaps makes them too slow for effective use for delivery of DNA from the early endosome.

Hellberg et al.[34] prepared mixtures of ortho ester surfactants (**3**, Figure 1) by heating 1 equiv triethyl orthoformate with 1.5 equiv of methoxyPEG and 1.5 equiv of *n*-octanol or *n*-decanol. Without further separation, the hydrolysis and the loss of emulsification capacity of these mixtures were evaluated using NMR. The ortho esters degraded in an acid- and temperature-sensitive manner, with significant hydrolysis (>30%) at pH 6 within 5 h. These surfactants have not yet been purified or incorporated into lipid vesicles.

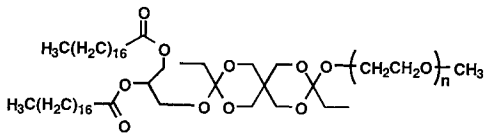
Despite this huge diversity of pH-sensitive liposomes in the literature, very limited success has been reported in the use of these systems for in vivo drug or gene delivery. Such a disappointing history prompted us to reconsider the physical and pathological scenarios where the decrease of pH takes place. We designed and synthesized a lipid conjugate (**4**, Figure 2) of poly(ethylene glycol) (PEG2000) and distearoyl glycerol via an acid labile diortho ester linker.[35] We chose PEG 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.[8] The 3,9-diethyl-2,4,8,10-tetraoxaspiro[5,5]undecane



3

x = 0-3, y = 0-3, z = 0-3; x + y + z = 3; m = 7, 9; n_{mean} = 7.8 -16.7

FIGURE 1. Structure of triethyl orthoformate-derived ortho esters.



4

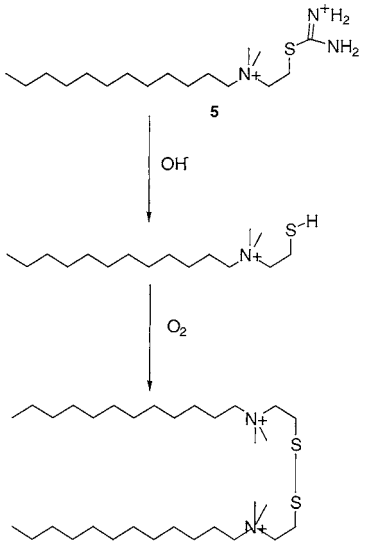
FIGURE 2. Structure of **4**, a diortho ester conjugate of PEG and distearoyl glycerol.

moiety was chosen as the diortho ester linker, on the basis of previous research by Heller and co-workers,[36] who characterized its pH sensitivity and biocompatibility in polymeric drug delivery systems. The first step of the hydrolysis of either of the two ortho ester groups would lead to the immediate cleavage of the PEG headgroup from the conjugate. A variety of hydrophobic anchors could be attached via the ortho ester to the PEG. In **4**, the distearoyl glycerol moiety, with two long and saturated hydrocarbon side chains, was chosen to serve as a stable anchor to incorporate the conjugate into lipid bilayer. When the conjugate is formulated into liposomes of unsaturated phosphatidylethanolamine, distearoyl glycerol will be regenerated in the bilayer upon hydrolysis and would favor the formation of hexagonal phases due to its conical structure.

The PEG2000−ortho ester−distearoyl glycerol conjugate (POD) was relatively stable in pH 7.4 buffer at 37 °C but degraded completely in 1 h when the pH was decreased to 5. POD/DOPE liposomes (1/9 in molar ratio) are as stable in serum as the sterically stabilized and pH-insensitive control liposomes (DSPE-PEG2000/DOPE = 1/9, mol/mol) for up to 12 h. 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 min, a rate consistent with endosome trafficking at this pH.[17] Contents release was due to liposome collapse after the PEG was cleaved.[37] DNA encapsulated in liposomes stabilized by POD transfected cells in culture.[38] Upon i.v. injection into mice, liposomes composed of 10% POD and 90% DOPE were eliminated from circulation with a half-life of 200 min, an elimination half-life that is comparable to the sterically stabilized but pH-insensitive liposomes (DSPE-PEG2000/DOPE = 1/9, mol/mol). The fast degradation kinetics of POD at low pH and its ability to stabilize liposomes in blood circulation may provide the conjugate with considerable advantages for triggered drug and gene delivery in mildly acidic bioenvironments such as endosomes, solid tumors, and inflammatory tissues.

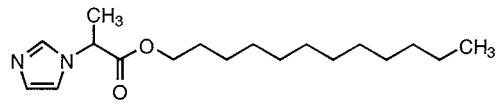
Lipid structures that are labile to mildly alkaline conditions have also been devised, not for pH-triggered release rather for pH-triggered assembly of DNA delivery systems. Ouyang, Remy, and Szoka[39] synthesized a series of novel cationic detergents that contain a quarternary amine group and a cleavable hydrophilic isothiuronium headgroup (**5** in Scheme 4). These detergents were used to control the assembly of plasmid DNA into small stable particles with high DNA concentrations. The detergents 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. After the detergents have accumulated on a DNA template, the unmasked sulfhydryl groups can then dimerize to form disulfide-linked cationic lipids containing two alkyl chains. Using the C12 detergent, a ~6 KB plasmid DNA was compacted into a small particle with an average diameter of around 40 nm and a

Scheme 4. Base-Triggered Cleavage and Dimerization of Cationic Isothiuronium Detergents

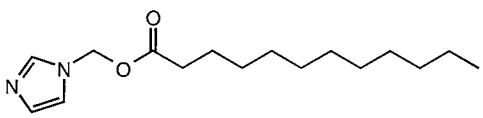


$\sim$ d$-$13 mV zeta potential at high DNA concentrations (up to 0.3 mg/mL). Under appropriate conditions, the small particle retained transfection activity.

4.6. Ionization of Bilayer Surfactants. In an effort to improve the gene transfection efficiency of cationic liposomes, Liang and Hughes[40,41] reported a number of biodegradable, pH-sensitive surfactants (BPS) as potential endosomotropic agents. The surfactants possess a single 12-carbon side chain and a pH-titratable imidazole group that becomes cationic at acidic pH. Liposomes composed of PC and BPS underwent fusion and content leakage that are dependent on pH and the molar ratio of BPS to membrane lipids. Among the three reported BPS, dodecyl 2-(1′-imidazolyl)propionate (DIP, **6** in Figure 3) 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 (SKn-SH), compared with the control formulation of DOTAP/DOPE in 1:1 molar ratio. The use of the BPS for in vivo applications might be hampered by the propensity of single chain surfactant to readily transfer from liposomes into biological membranes such as red blood cell membranes.



6



7

FIGURE 3. Structure of imidazole-derived pH-sensitive surfactants.

For instance, Asokan and Cho[13] recently reported a series of acyloxyalkyl imidazole lipids which induce hemolysis in a pH-dependent manner. Among the reported surfactants, myristoyloxymethylimidazole (**7** in Figure 3) completely disrupts human erythrocytes within 10 min at pH 5.5, where as at pH 7.0 and above, 50 min is needed for complete hemolysis. Interestingly, the kinetics of the hemolysis consists a lag phase and a burst phase, suggesting that, along with the protonation of the imidazole headgroup, there may be some acid-triggered chemical degradation of **7**[35] which contributes to the hemolysis.

5. Redox-Triggered Release Systems

Reduction of disulfides is a tactic exploited in the biosphere by various proteins as a trigger to alter function. Savvy investigators have synthesized lipids that contain a disulfide bond and used them to prepare liposomes that are susceptible to a thiolysis trigger mechanism. The rationale behind this approach is that the cytoplasm has a lower redox potential and substantially more molecules with free sulfhydryl groups such as glutathione than does plasma. Kirpotin and colleagues[42] prepared a PEG headgroup conjugated to a DSPE via a dithiopropionyl linkage (mPEG$-$DTP$-$DSPE, **8** in Figure 4). Liposomes composed of 3 mol % mPEG$-$DTP$-$DSPE and 97 mol % fusogenic DOPE were stable in plasma for over a day but released most of their contents within 1 h of incubation with 10 mM dithiotreitol. They also synthesized a disulfide lipid, mPEG$-$DTB$-$DSPE[43] (**9** in Figure 4), which was reduced at submillimolar concentrations of the amino acid cysteine, a milder thiolytic reagent than DTT.

Disulfide containing cationic lipids[44$-$49] have been devised as improved reagents for use for gene transfection. The concept is that the cationic functionality would be stable in serum but released from the lipid anchor in the presence of glutathione levels found in the cytoplasm. The reduction of the disulfide would alter the multivalent binding between the cationic liposomes and the attached DNA so that the DNA could migrate into the cytoplasm.

Tang and Hughes[44] synthesized a 1,2-dioleoyl-*sn*-glycero-3-succinyl-2-hydroxyethyl disulfide ornithine conjugate (DOGSDSO, **10** in Figure **4**) and combined it with DOPE to prepare liposomes. Reduction of the disulfide bond increased the release of pDNA. The amount of transgene produced using the DOGSDSO/DOPE was up to 50 times greater than that of its nondisulfide analogue and modestly greater than a standard transfection system. In a subsequent study,[45] they used dithiodiglycolic acid to prepare a cholesterol hemidithiodiglycolyl tris(aminoethyl)amine (CHDTAEA, **11** in Figure **4**). Liposomes prepared from CHDTAEA had more than 2 orders of magnitude greater transfection activity than the standard cholesterol-based reagent in CHO cells and 7 times greater transfection activity in SKnSH cells. The reducible analogue was slightly less toxic to cells in culture than was the nonreducible analogue.

Balakirev and co-workers[46] reported a lipoic acid-derived cationic lipid that contains four sulfhydryl groups

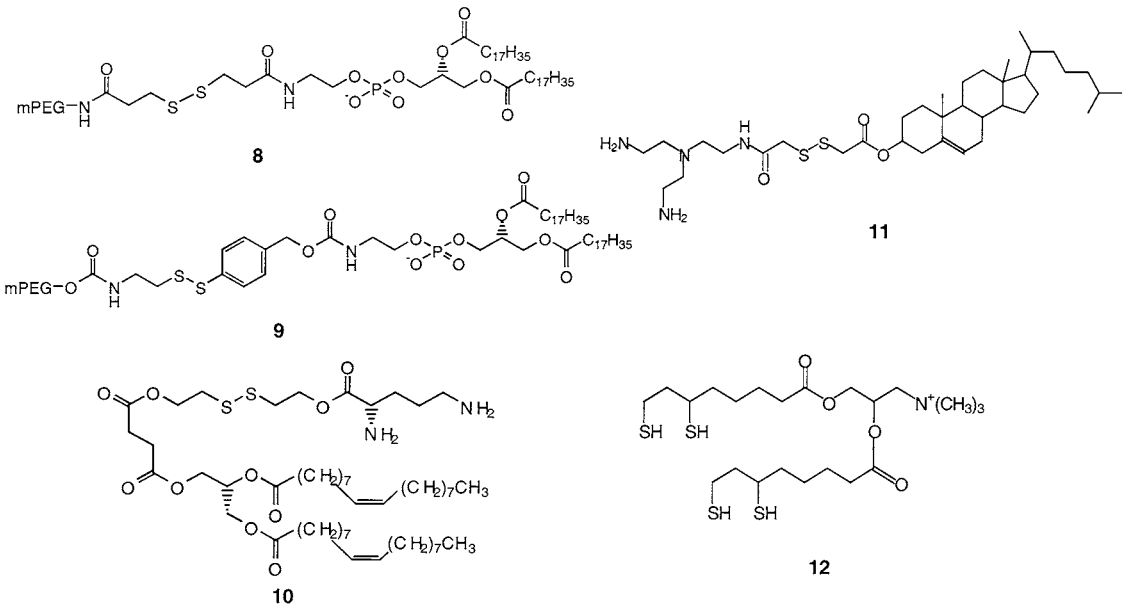


FIGURE 4. Structure of disulfide-derived lipids.

at the end of its hydrophobic chains (**12** in Figure **4**). In the oxidized state, the lipid polymerized and condensed DNA into spherical lipoplexes. Upon reduction, the lipoplexes swelled and released the DNA. Transfection with the lipoplexes in vitro showed a severalfold increase of transgene expression compared with DOTAP, an efficient cationic lipid for gene delivery.

Byk, Scherman, and co-workers[47,48] developed a series of lipopolyamines consisting a polyamine headgroup, fatty chains, a side chain entity, and a disulfide group at different positions within the structure. Compared with a nonreducible analogue, surfactants with the disulfide bridge between one of the fatty chains and the rest of the molecule yielded up to 1000-fold higher reporter-gene activity, whereas inserting the disulfide bond between the polyamine and the lipid chains resulted in a loss of transfection efficiency.

Despite the above promise of improved activity and lower toxicity from these cleavable disulfide containing lipids, 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 small molecules with free sulfhydryl groups, such as glutathione and cysteine, that exist predominantly inside the cells[50] are required for the triggered-release of the liposomes. 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. Moreover, disulfide reduction is not favored at low pH. Another possible mechanism involves membrane-bound reductive enzymes. Ryser[51] and associates have demonstrated that protein disulfide isomerase (PDI), a chaperon enzyme present in the ER and the plasma membrane of eukaryotic cells, plays a pivotal role in the thiolysis of macromolecules that do not diffuse through biomembranes. Inhibition of PDI eliminated the activity of disulfide-containing toxins such as diphtheria toxin.[52] Thus, it is conceivable that disulfide-containing liposomes could be triggered by attaching to the cellular surface followed by PDI-catalyzed thiolysis. This possibility could be evaluated by including protein disulfide isomerase inhibitors in cell based assays that evaluate the sensitivity of disulfide-containing liposomes toward redox triggering.

6. Summary

The attributes that are associated with effective liposome drug delivery are now well understood and the first generation systems have attained therapeutic and commercial success. Chemists can now exploit this information to design "smart" molecules that can be incorporated into liposomal formulations that are triggered by biological factors and would enable further improvements in drug and gene delivery.

We gratefully acknowledge support from NIH DK 46052 and GM 61851.

References

(1) Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **1965**, *13*, 238−252.
(2) Drummond, D. C.; Meyer, O.; Hong, K.; Kirpotin, D. B.; Papahadjopoulos, D. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev.* **1999**, *51*, 691−743.
(3) Needham, D. Materials engineering of lipid bilayers for drug carrier performance. *MRS Bull.* **1999**, *24*, 32−40.
(4) Maeda, H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv. Enzyme Regul.* **2001**, *41*, 189−207.
(5) Xu, Y.; Szoka, F. C. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **1996**, *35*, 5616−5623.
(6) Huang, L.; Hung, M.-C.; Wagner, E. *Nonviral Vectors for Gene Therapy*; Academic Press: San Diego, 1999; p 442.
(7) Labat-Moleur, F.; Steffan, A. M.; Brisson, C.; Perron, H.; Feugeas, O.; et al. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Ther.* **1996**, *3*, 1010−1017.
(8) Woodle, M. C.; Lasic, D. D. Sterically stabilized liposomes. *Biochim. Biophys. Act* **1992**, *1113*, 171−199.

(9) Bally, M. B.; Lim, H.; Cullis, P. R.; Mayer, L. D. Controlling the drug delivery attributes of lipid-based drug formulations. *J. Liposome Res.* **1998**, *8*, 299−335.

(10) Fenske, D. B.; MacLachlan, I.; Cullis, P. R. Long-circulating vectors for the systemic delivery of genes. *Curr. Opin. Mol. Ther.* **2001**, *3*, 153−158.

(11) Meers, P. Enzyme-activated targeting of liposomes. *Adv. Drug Deliv. Rev.* **2001**, *53*, 265−272.

(12) Drummond, D. C.; Zignani, M.; Leroux, J. Current status of pH-sensitive liposomes in drug delivery. *Prog. Lipid Res.* **2000**, *39*, 409−460.

(13) Asokan, A.; Cho, M. J. Exploitation of intracellular pH gradients in the cellular delivery of macromolecules. *J. Pharm. Sci.* **2002**, *91*, 903−913.

(14) Chu, C.-J.; Szoka, F. C. pH-Sensitive Liposomes. *J. Liposome Res.* **1994**, *4*, 361−395.

(15) Gerasimov, O. V.; Rui, Y. J.; Thompson, D. H. *Triggered Release from Liposomes Mediated by Physically and Chemically Induced Phase Transition. Vesicles*; Marcel Dekker: New York, 1996; pp 680−746.

(16) Thomas, J. L.; Tirrell, D. A. Polyelectrolyte-Sensitized Phospholipid Vesicles. *Acc. Chem. Res.* **1992**, *25*, 336−342.

(17) Schmid, S. L. Toward a biochemical definition of the endosomal compartment. Studies using free flow electrophoresis. *Sub-Cellular Biochem.* **1993**, *19*, 1−28.

(18) Gallin, J. I.; Goldstein, I. M.; Snyderman, R. *Inflammation-Basic Principles and Clinical Correlates*, 2 ed.; Raven Press: New York, 1992; pp 511−533.

(19) Gerweck, L. E. Tumor pH: implications for treatment and novel drug design. *Seminars Radiat. Oncol.* **1998**, *8*, 176−182.

(20) Yatvin, M. B.; Kreutz, W.; Horwitz, B. A.; Shinitzky, M. pH-sensitive liposomes: possible clinical implications. *Science* **1980**, *210*, 1253−1255.

(21) Nir, S.; Nicol, F.; Szoka, F. C. Surface aggregation and membrane penetration by peptides: relation to pore formation and fusion. *Mol. Membr. Biol.* **1999**, *16*, 95−101.

(22) Cordes, E. H.; Bull, H. G. Mechanism and catalysis for hydrolysis of acetals, ketals, and ortho esters. *Chem. Rev.* **1974**, *74*, 581−603.

(23) Boomer, J. A.; Thompson, D. H. Synthesis of acid-labile diplasmenyl lipids for drug and gene delivery applications. *Chem. Phys. Lipids* **1999**, *99*, 145−153.

(24) Thompson, D. H.; Gerasimov, O. V.; Wheeler, J. J.; Rui, Y.; Anderson, V. C. Triggerable plasmalogen liposomes: improvement of system efficiency. *Biochim. Biophys. Acta* **1996**, *1279*, 25−34.

(25) Gerasimov, O. V.; Schwan, A.; Thompson, D. H. Acid-catalyzed plasmenylcholine hydrolysis and its effect on bilayer permeability: A quantitative study. *Biochim. Biophys. Acta* **1997**, *1324*, 200−214.

(26) Rui, Y. J.; Wang, S.; Low, P. S.; Thompson, D. H. Diplasmenylcholine-folate liposomes: An efficient vehicle for intracellular drug delivery. *J. Am. Chem. Soc.* **1998**, *120*, 11213−11218.

(27) Kim, J. M.; Shin, J.; Shum, P.; Thompson, D. H. Acid- and oxidatively-labile vinyl ether surfactants: Synthesis and drug delivery applications. *J. Dispersion Sci. Technol.* **2001**, *22*, 399−407.

(28) Jaeger, D. A. Cleavable Surfactants. *Supramol. Chem.* **1995**, *5*, 27−30.

(29) Jaeger, D. A.; Li, B.; Clark, T. Cleavable Double-Chain Surfactants with One Cationic and One Anionic Head Group That Form Vesicles. *Langmuir* **1996**, *12*, 4314−4316.

(30) Song, J.; Hollingsworth, R. I. Synthesis, conformational analysis, and phase characterization of a versatile self-assembling monoglucosyl diacylglycerol analogue. *J. Am. Chem. Soc.* **1999**, *121*, 1851−1861.

(31) Atkins, M. P.; Golding, B. T.; Howes, D. A.; Sellars, P. J. Masking the carboxy group as a 2,6,7-trioxabicyclo[2.2.2]octane: application to the synthesis of alkylcobaloximes containing ester and carboxy groups. *J. Chem. Soc., Chem. Commun.* **1980**, *5*, 207−208.

(32) Heller, J.; Barr, J.; Ng, S. Y.; Shen, H. R.; Schwach-Abdellaoui, K.; et al. Poly(ortho esters) − their development and some recent applications [published erratum appears in *Eur. J. Pharm. Biopharm.* **2000**, *50* (2), 327]. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 121−128.

(33) Zhu, J.; Munn, R. J.; Nantz, M. H. Self-cleaving ortho ester lipids: A new class of pH-vulnerable amphiphiles. *J. Am. Chem. Soc.* **2000**, *122*, 2645−2646.

(34) Hellberg, P.-E., Bergstrom, K., Juberg, M. Nonionic cleavable ortho ester surfactants. *J. Surfact. Deterg.* **2000**, *3*, 369−379.

(35) Guo, X.; Szoka, F. C. Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG-diorthoester-lipid conjugate. *Bioconjugate Chem.* **2001**, *12*, 291−300.

(36) Heller, J. Controlled drug release from poly(ortho esters)-a surface eroding polymer. *J. Controlled Release* **1985**, *2*, 167−177.

(37) Guo, X.; MacKay, J. A.; Szoka, F. C. Mechanism of pH-triggered collapse of phosphatidyl ethanolamine liposomes stabilized by an ortho ester polyethylene glycol lipid. *Biophys. J.* **2003**, In press.

(38) Choi, J. S.; MacKay, J. A.; Szoka, F. C. J. Low pH-sensitive PEG-stabilized plasmid-lipid nanoparticles: preparation and characterization. *Bioconjugate Chem.*, In press.

(39) Ouyang, M.; Remy, J. S.; Szoka, F. C. Controlled template-assisted assembly of plasmid DNA into nanometric particles with high DNA concentration. *Bioconjugate Chem.* **2000**, *11*, 104−112.

(40) Liang, E.; Hughes, J. A. Membrane fusion and rupture in liposomes: effect of biodegradable pH-sensitive surfactants. *J. Membr. Biol.* **1998**, *166*, 37−49.

(41) Liang, E.; Hughes, J. Characterization of a pH-sensitive surfactant, dodecyl-2-(1′-imidazolyl) propionate (DIP), and preliminary studies in liposome mediated gene transfer. *Biochim. Biophys. Acta* **1998**, *1369*, 39−50.

(42) Kirpotin, D.; Hong, K.; Mullah, N.; Papahadjopoulos, D.; Zalipsky, S. Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). *FEBS Lett.* **1996**, *388*, 115−118.

(43) Zalipsky, S.; Qazen, M.; Walker, J. A.; Mullah, N.; Quinn, Y. P.; et al. New detachable poly(ethylene glycol) conjugates: cysteine-cleavable lipopolymers regenerating natural phospholipid, diacyl phosphatidylethanolamine. *Bioconjugate Chem.* **1999**, *10*, 703−707.

(44) Tang, F.; Hughes, J. A. Introduction of a disulfide bond into a cationic lipid enhances transgene expression of plasmid DNA. *Biochem. Biophys. Res. Commun.* **1998**, *242*, 141−145.

(45) Tang, F. X.; Hughes, J. A. Use of dithiodiglycolic acid as a tether for cationic lipids decreases the cytotoxicity and increases transgene expression of plasmid DNA in vitro. *Bioconjugate Chem.* **1999**, *10*, 791−796.

(46) Balakirev, M.; Schoehn, G.; Chroboczek, J. Lipoic acid-derived amphiphiles for redox-controlled DNA delivery. *Chem. Biol.* **2000**, *7*, 813−819.

(47) Byk, G.; Wetzer, B.; Frederic, M.; Dubertret, C.; Pitard, B. et al. Reduction-sensitive lipopolyamines as a novel nonviral gene delivery system for modulated release of DNA with improved transgene expression. *J. Med. Chem.* **2000**, *43*, 4377−4387.

(48) Wetzer, B.; Byk, G.; Frederic, M.; Airiau, M.; Blanche, F. et al. Reducible cationic lipids for gene transfer. *Biochem. J.* **2001**, *356*, 747−756.

(49) Ajmani, P. S.; Tang, F.; Krishnaswami, S.; Meyer, E. M.; Sumners, C. et al. Enhanced transgene expression in rat brain cell cultures with a disulfide-containing cationic lipid. *Neurosci. Lett.* **1999**, *277*, 141−144.

(50) Huxtable, R. J.; Lafranconi, W. M. Biochemistry of Sulfur; Plenum Press: New York, London, 1986; 445.

(51) Mandel, R.; Ryser, H. J.; Ghani, F.; Wu, M.; Peak, D. Inhibition of a reductive function of the plasma membrane by bacitracin and antibodies against protein disulfide-isomerase. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4112−4116.

(52) Ryser, H. J.; Mandel, R.; Ghani, F. Cell surface sulfhydryls are required for the cytotoxicity of diphtheria toxin but not of ricin in Chinese hamster ovary cells. *J. Biol. Chem.* **1991**, *266*, 18439−18442.

AR9703241