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Recent advances in liposome technologies and their applications for systemic gene delivery

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Abstract

The recent clinical successes experienced by liposomal drug delivery systems stem from the ability to produce well-defined liposomes that can be composed of a wide variety of lipids, have high drug-trapping efficiencies and have a narrow size distribution, averaging less than 100 nm in diameter. Agents that prolong the circulation lifetime of liposomes, enhance the delivery of liposomal drugs to specific target cells, or enhance the ability of liposomes to deliver drugs intracellularly can be incorporated to further increase the therapeutic activity. The physical and chemical requirements for optimum liposome drug delivery systems will likely apply to lipid-based gene delivery systems. As a result, the development of liposomal delivery systems for systemic gene delivery should follow similar strategies. © 1998 Elsevier Science B.V.

Keywords: Liposomal gene delivery systems; Cationic lipids; DNA encapsulation; Prolonged circulation lifetimes; Targeting; Fusogenic liposomes; Intracellular delivery; Transfection

Contents

1. Introduction

Recent advances in liposome technologies for conventional drug delivery have resulted in liposomal drugs with proven clinical utility [1,2]. Notable examples are liposome formulations of doxorubicin [3,4], all-*trans* retinoic acid [5], amphotericin B [6], daunorubicin [7] and vincristine [8,9]. These advances have led to the production of well-defined, relatively small liposomal systems that have the ability to entrap drugs with high efficiencies, reside

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in the circulation for extended periods, and accumulate at regional sites of disease, such as inflammation and tumor. The next generation of liposomal drug delivery systems will include drug-loaded liposomes with surface-associated targeting information that will increase drug accumulation in specific cells, as well as fusogenic liposomes that will enable more efficient intracellular drug delivery.

It is anticipated that these advances in liposome technologies will be directly applicable to the design of liposomal systems for systemic gene delivery. In many ways, the challenges facing the development of liposomal gene delivery systems are not unlike those that have faced liposomal drug delivery systems. The therapeutic index of the conventional or gene-based (plasmid DNA or RNA transcripts) drug will be enhanced by delivering more biologically active drug to target cells/tissues and less to non-target cells/tissues, to avoid drug-related toxicities. With gene-based drugs, however, delivery into appropriate cells represents only part of the problem; a number of intracellular barriers exist that can inhibit the biologic activity of gene-based drugs [10,11]. It is not clear what role, if any, liposomes will play in overcoming these intracellular barriers.

The potential of liposomes to systemically deliver DNA was recognized as early as the late 1970s (extensively reviewed in [12]), however, gene-based drugs have presented interesting challenges for systemic delivery systems. First, gene-based drugs are highly susceptible to degradation by the nucleases present in plasma. Although liposomes have the potential to encapsulate gene-based drugs and prevent inactivation by nucleases, procedures to efficiently encapsulate plasmid DNA in well defined, small liposomes or lipidic DNA particles have only recently been realized. Second, the efficacy of gene-based drugs is completely dependent on gaining entry into the target cell cytosol in an intact form. Therefore, for liposomes to be effective, they must incorporate agents that promote intracellular delivery. With few exceptions (i.e. skeletal muscle [13,14] and hepatocytes [15,16]), naked plasmid DNA alone is not taken up very efficiently by most cell types in vivo. Third, for certain gene therapy approaches, such as those involving the delivery of suicide genes, systemic gene delivery systems must have the potential to selectively deliver gene-based drugs to specific target cells. This review will highlight several of the advances made in liposome technologies and discuss how these advances may be applied to resolve the challenges facing the development of liposomes for the controllable and reproducible delivery of gene-based drugs.

2. Production of liposomes for conventional drug delivery

The major advances in liposome technology in the past decade arise from the ability to produce well-defined liposomes composed of a wide variety of lipids with different physical and chemical properties, having high drug-trapping efficiencies and having narrow size distributions, averaging less than 100 nm in diameter. These physical and chemical properties have been shown to significantly affect the stability and pharmacokinetics of liposomes [17]. A number of procedures have been established to produce well-defined liposomes (extensively reviewed in [18,19]). These include extrusion, where the liposomes are forced through filters with well-defined pore sizes under moderate pressures, reversed-phase evaporation, sonication and detergent-based procedures.

Another significant advance has come from the ability to entrap drugs in liposomes with high efficiencies while maintaining the integrity of the liposome structure. Drug loading can be achieved either passively (i.e. the drug is encapsulated during liposome formation) or actively (i.e. after liposome formation). Hydrophobic drugs can be directly incorporated into liposomes during vesicle formation, and the extent of uptake and retention is governed by drug–lipid interactions. Trapping efficiencies of 100% are often achievable, but this is dependent on the solubility of the drug in the liposome membrane. Passive encapsulation of water-soluble drugs relies on the ability of liposomes to trap aqueous buffer containing a dissolved drug during vesicle formation. Trapping efficiencies (generally less than 30%) are limited by the trapped volume contained in the liposomes and drug solubility. Another approach to enhance the passive encapsulation of water-soluble drugs is to impart an amphipathic nature to the drugs by conjugating or complexing the drugs to lipids

[20,21]. Alternatively, water-soluble drugs that have ionizable amine functions can be actively entrapped by employing pH gradients [22], which can result in trapping efficiencies approaching 100%.

3. Production of liposomes for gene delivery

Based on our experience with liposomal drug delivery systems, it is envisioned that the ideal liposomes for systemic gene delivery will encapsulate plasmid DNA with high efficiencies, will protect the DNA from degradation by plasma nucleases, will have a narrow size distribution, averaging 100 nm or less in diameter, in order that the liposomes can access extravascular regions, and will have the potential to incorporate a wide range of lipids, especially lipids that promote fusion with cellular membranes and/or enhance liposome stability in the circulation. The feasibility of passively encapsulating DNA in liposomes was demonstrated in the late 1970s using a number of the methods indicated above. For example, high molecular weight DNA is entrapped in egg phosphatidylcholine liposomes by hydrating the lipid film in the presence of DNA [23]. In a similar manner, metaphase chromosomes are passively entrapped in, or tightly associated with, egg phosphatidylcholine–cholesterol (7:2, mol/mol) liposomes [24]. Alternatively, DNA can be encapsulated in cochleate lipid cylinders that are formed from the calcium-induced fusion of phosphatidylserine liposomes [25]. Reversed-phase evaporation procedures have also been employed to encapsulate plasmid DNAs with good but variable encapsulation efficiencies [26,27]. More recently, freeze drying methods have yielded DNA-containing multilamellar vesicles with encapsulation efficiencies of 50–60% [28]. For the most part, however, these procedures yield relatively large multilamellar vesicles with low DNA encapsulating efficiencies and generally low gene transfer capabilities. Extrusion of the DNA-containing multilamellar vesicles to reduce the particle size have resulted in poor recoveries of DNA-containing liposomes.

In the late 1980s, it was shown that cationic lipids, when incorporated in dioleoylphosphatidylethanolamine (DOPE)-containing liposomes, could enhance the efficiency of gene delivery to cultured cells in vitro [29] by (1) increasing the association of plasmid DNA with liposomes and (2) increasing the binding of cationic liposome–plasmid DNA complexes to cells. This has prompted many researchers to synthesize different cationic lipids that exhibit improved gene transfer and cell tolerability properties [30–32], as well as to develop novel procedures to efficiently encapsulate plasmid DNA within lipid-based carriers. The addition of plasmid DNA to preformed cationic liposomes often results in the formation of a heterogeneous mixture of unstable complexes of cationic lipids and plasmid DNA [33–35]. This heterogeneity and instability are undoubtedly responsible for the poor reproducibility in the transfection activity observed in vivo when these complexes are administered intravenously [36–39].

In the past couple of years, there have been significant advances made in the formulation of plasmid DNA into relatively small, stable plasmid DNA-containing lipidic particles or liposomes that protect plasmid DNA from degradation by nucleases. For example, Gao and Huang [40] describe a procedure where the addition of polylysine or other polycationic polymers to plasmid DNA, prior to or during the addition of cationic liposomes, results in particles with membranous structures of less than 100 nm in diameter. The plasmid DNA in the presence of polylysine alone or polylysine and cationic liposomes appears to be resistant to nuclease attack, remaining supercoiled when incubated with 5 μl of fetal bovine serum at 37°C for 1 h. As characterized by negative stain electron microscopy, however, the plasmid DNA does not appear to be fully encapsulated by a lipid membrane. Moreover, the particles formed are heterogeneous in nature, having varying plasmid DNA–polylysine–lipid ratios that affect the transfection activity in Chinese hamster ovary (CHO) cells in vitro. The transfection active particle can be isolated by sucrose density gradient ultracentrifugation and the purified particle is reported to be stable for up to three months at 4°C, with no increase in particle size. Similar particles can be generated by the addition of DOPE, cholesteryl hemisuccinate and folate–poly(ethylene glycol)–phosphatidylethanolamine conjugates (6:4:0.01, mol/mol/mol) to plasmid DNA–polylysine complexes [41]. These particles were shown to be highly active

in transfecting KB cells in vitro, being 20–30 times more active than 3-β-[*N*-(*N′*,*N′*-dimethylethane)carbamoyl]cholesterol–DOPE, (6:4, mol/mol) cationic liposome–plasmid DNA complexes. The stability and pharmacokinetics of these particles upon intravenous administration, or the ability of these particles to transfect cells in vivo, have not been reported.

Recently, Hofland et al. [42] described a detergent-based procedure to form stable plasmid DNA–lipidic particles by the addition of various amounts of cationic lipids [2,3-dioleoyloxy-*N*-(2(sperminecarboxamido)-ethyl)-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate] and DOPE (1.5:1, mol/mol) solubilized in buffered 1% octylglucoside to plasmid DNA, followed by removal of the detergent by dialysis. The particles can be stored frozen or as a suspension at 4°C for 90 days with no loss in transfection activity in NIH 3T3 cells in vitro. The physical properties of the active particles have not been defined. However, the active particles can be pelleted by centrifugation at 3000 *g* for 15 min, indicating that they are relatively large particles. Moreover, in vitro transfection efficiency is affected by the presence of serum, with a 70% reduction in transfection activity in the presence of as little as 1% fetal bovine serum in the culture medium. Another detergent-based method that has yielded active particles has recently been described by Liu et al. [43,44]. In this procedure, stable emulsions of cationic lipids and plasmid DNA are produced by the addition of non-ionic surfactants. These particles are not well defined, but are relatively large in size. The average diameter of lipid particles for emulsions containing various surfactants range from 170 to 250 nm. Upon mixing with plasmid DNA, the particle size increases five- to fourteen-fold in diameter, depending on the type of non-ionic surfactant used for preparing the emulsions. The use of detergents containing branched polyoxyethylene chains as the hydrophilic head group are more effective in preventing the formation of large DNA–emulsion complexes. The stability and biodistribution of these particles upon intravenous administration, or the ability of these particles to transfect cells in vivo have not yet been described in the literature.

An alternative approach that has recently been developed takes advantage of the hydrophobic plasmid DNA–cationic lipid complex formed by the addition of cationic lipids, added in monomer or micellar form, to plasmid DNA [45,46]. This complex can serve as a well-defined intermediate in the preparation of plasmid DNA-containing liposomes with good properties for systemic gene delivery applications (unpublished results). For example, the addition of excess neutral lipids, such as dioleoylphosphatidylcholine or DOPE, to these intermediates results in the formation of plasmid DNA-containing liposomes that have a narrow size distribution, averaging 70–100 nm in diameter (Fig. 1). Typically, plasmid DNA encapsulating efficiencies of 70% are obtained using this procedure. A wide variety of lipids that alter the biodistribution of the liposomes can be readily incorporated into these liposomes. For example, the incorporation of at least 10 mol% poly(ethylene glycol) conjugated to phos-

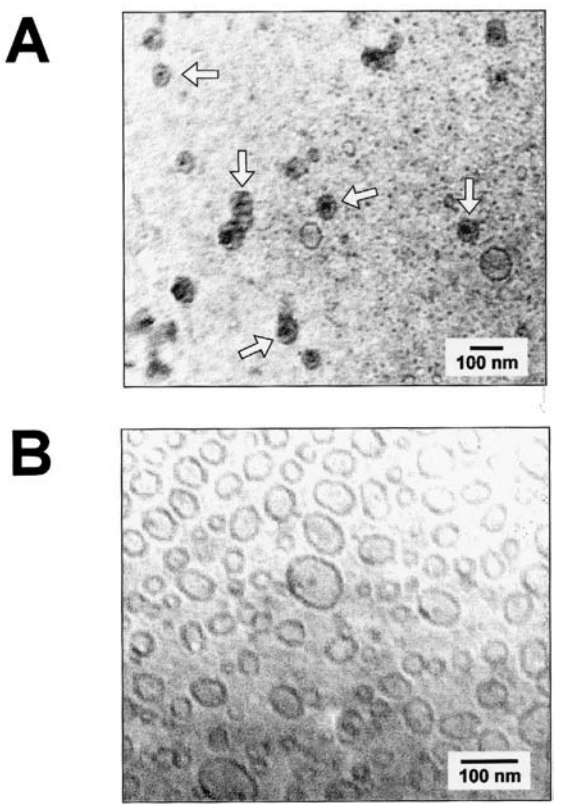

Fig. 1. Cryo-electron micrographs of plasmid DNA encapsulated in liposomes. Panel (A) represents vesicles formed in the presence of and (B) in the absence of plasmid DNA.

phatidylethanolamine results in plasmid DNA-containing liposomes that have a circulation half-life approaching 10–12 h in mice. Moreover, these DNA-containing liposomes appear to be stable in the circulation of mice, with the majority of the plasmid DNA extracted from the circulating liposomes at 24 h post-injection remaining intact.

4. Liposomes with prolonged circulation lifetimes

The use of liposomes for systemic drug delivery requires that the liposomes have the ability to avoid immediate uptake by phagocytic cells of the reticuloendothelial system (RES) and remain in circulation for extended periods of time in order to enhance the opportunity for the liposomal drugs to reach non-RES target tissues. A significant advance in the development of liposomal drugs has come with the use of specialized lipids, such as monosialoganglioside G_{M1} or poly(ethylene glycol) (PEG-)-modified phosphatidylethanolamine, that engender long circulation lifetimes when incorporated into liposomes [47–50]. It has been proposed that these PEG–lipid conjugates provide a 'steric stabilization' of the surface by virtue of the hydrophilic brush coat provided by the PEG polymer [51]. This coat has been shown to inhibit serum protein binding to the liposomal surface [52,53], which would otherwise promote uptake by the RES, complement activation and destabilization of the liposomal membranes. It has been demonstrated that increased circulation lifetimes enhance the opportunity for liposomes, administered systemically, to leave the vascular compartment and enter certain extravascular regions [54–56].

The ability to generate sustained circulating liposomal gene delivery systems using the PEG–lipid technology should prove useful for systemic gene delivery applications. For instance, the ability of long circulating liposomes to accumulate within tumors will be advantageous for cancer gene therapy applications involving tumor suppressor genes or suicide genes. Furthermore, the avoidance of RES uptake, especially by Kupffer cells, the resident macrophages of the liver, would enhance the opportunity for liposomes to deliver genes to hepatocytes, the target cell of several gene therapies for blood protein deficiencies.

The biodistribution of intravenously administered cationic liposome–plasmid DNA complexes is not appropriate for such systemic applications. For instance, it has recently been demonstrated that cationic liposome–plasmid DNA complexes, exhibiting strong positive zeta potentials, are cleared rapidly from the circulation [57,58]. These intravenously administered cationic liposome–plasmid DNA complexes [*N*-(2,3-bis(oleyloxy)propyl-*N*,*N*,*N*-trimethylammonium chloride or dimethyldioctadecylammonium bromide and DOPE-containing liposomes] are rapidly eliminated from the plasma, with 50–60% of the dose taken up by the liver within 5 min, and 20–30% of the dose taken up by the lung within 1 min, falling to 10% after 5 min [57]. The cationic liposome–plasmid DNA complexes are predominantly taken up by the Kupffer cells in the liver. Moreover, a recent study has shown that cationic lipid–DNA complexes, harboring excess positive surface charge, are potent activators of the complement system, potentially a barrier to the efficient delivery of genes when using high lipid doses [59]. Although there have been a few reports demonstrating the feasibility of using these complexes to deliver genes to a number of different tissues (such as the liver, lung, spleen, heart, skeletal muscle, kidney, uterus, bone marrow cells, peripheral blood and ovary) after intravenous administration [36–39,57,58], the observed levels of gene delivery are low and often are not reproducible. This may be a consequence of the rapid elimination of the majority of the injected dose of cationic liposome–plasmid DNA complexes by the RES.

Our recent findings show that DOPE-containing cationic liposomes can be stabilized in the circulation of mice by reducing the cationic lipid content of the liposomes and incorporating at least 2 mol% PEG–phosphatidylethanolamine derivatives [60]. In vitro, the addition of serum to cationic liposomes composed of dioleoyldimethylammonium chloride–DOPE (85:15, mol/mol) induces a rapid aggregation of the cationic liposomes, forming large fused aggregates (>1 μm in diameter) [60]. Amphipathic PEG–lipid conjugates can stabilize DOPE-containing liposomes by inhibiting the fusogenic activity of these liposomes [61,62].

However, the fusogenic activity is essential for efficient gene delivery [30,31,41,63,64] and, thus, an essential property of the amphipathic PEG–lipid conjugates is that they have the ability to dissociate from the carrier at some later time, restoring the fusogenic activity of the liposomes and allowing the liposomes to fuse with target cells. The feasibility of this approach has recently been demonstrated [61]. The rate at which fusogenic activity is recovered is shown to be controlled to a large extent by the same parameters that regulate spontaneous transfer of lipids between bilayers.

5. Targeted delivery to specific cells

In general, liposomes are effective delivery systems because they alter the pharmacokinetics of the free drug, leading to enhanced drug bioavailability to specific target cells that reside in the circulation or, more importantly, to extravascular disease regions. The ability to selectively deliver drugs to specific cells, such as tumor cells, within these regions will further enhance the therapeutic index of liposomal drugs. Targeted delivery and improved therapeutic activity of liposomal drugs in vivo has been achieved by coupling site-directive targeting ligands, such as monoclonal antibodies [65–68], to the surface of liposomes by either covalent or non-covalent methods [68,69]. A significant advance in this area has been the advent of novel PEG–phosphatidylethanolamine lipids that allow targeting ligands to be conjugated at the distal ends of the PEG spacer [70–73]. These conjugates increase target cell binding in vitro, as well as prolong circulation times. Furthermore, in addition to antibodies, glycolipids [74–77], proteins [78–80] and vitamins [41,71] have been used to selectively target specific cells via cell surface receptors.

For liposomal gene delivery systems, targeting ligands need to function not only to increase the binding of the liposomes to specific target cells, such as hepatocytes, but also to promote the cellular uptake of the liposomes via an endocytic pathway. Endocytosis is believed to play a major role in plasmid DNA delivery to cultured cells in vitro [10,63,81,82]. The feasibility of using targeting ligands to increase the cellular uptake of plasmid DNA-containing liposomes has recently been demonstrated in vitro by a number of investigators. For example, Lee and Huang [41] have shown that folate, conjugated to the distal end of PEG–phosphatidylethanolamine, enhances the plasmid DNA uptake and transfection efficiency of KB cells in vitro by employing plasmid DNA-containing pH-sensitive DOPE–cholesteryl hemisuccinate (6:4, mol/mol) liposomes, particularly when the liposomes carry an overall negative surface charge. This study clearly demonstrates that components which enhance the binding of liposomes to cells, mediated either by the use of targeting ligands or by a strong positive surface charge, are essential for efficient liposomal gene delivery systems. The addition of transferrin to cationic liposome–plasmid DNA complexes increases the amount of DNA taken up by human hepatoma HepG2 cells in vitro twofold, accompanied by a significant increase in the number of β-galactosidase-positive cells (98–100% in the presence of transferrin compared to 3–4% in the absence of transferrin) [83]. Transferrin presumably acts to further facilitate the uptake of cationic liposome–DNA complexes via a receptor-mediated process. Similarly, asialofetuin [79,80] and galactose-containing lipids [84] have been shown to increase the transfection efficiency of HepG2 cells in vitro. Kikuchi et al. [85] have shown that the addition of epidermal growth factor to cationic liposomes enhances the in vitro luciferase gene expression in epidermal growth factor receptor-overexpressing HEC-A cells and not in epidermal growth factor receptor-deficient HRA cells. The coupling of antibodies to pH-sensitive liposomes [86] or to cationic liposomes [87] has been shown to also enhance transfection activity in vitro compared to that found in non-targeted DNA-containing liposomes.

6. Fusogenic liposomes for intracellular delivery

Fusogenic liposomes can potentially facilitate the intracellular delivery of encapsulated drugs by fusing with the target cell. A variety of approaches can be envisioned for constructing fusogenic liposomes. Examples include the inclusion of lipids that are able to form non-bilayer phases, such as DOPE, which

can promote destabilization of the bilayer, inducing fusion events [88,89]. Furthermore, alterations in the lipid composition can render liposomes pH sensitive, leading to enhanced fusogenic tendencies in low pH compartments such as endosomes [41,86,90]. Non-phospholipid fusogenic liposomes composed primarily of dioxyethylene acyl ethers and cholesterol have been shown to fuse with plasma membranes of erythrocytes and fibroblasts [91]. Alternatively, efficient fusogenic liposomes can be achieved by incorporating fusogenic proteins into the liposome membrane [92–94] or entrapped within liposomes [95]. The feasibility of this approach has been demonstrated for the delivery of the diphtheria toxin A subunit using liposomes produced from influenza virus envelopes [94]. Fusogenic peptides can be conjugated to the liposomes [96–98] and may also promote intracellular delivery. The encapsulation of a 30-amino acid fusogenic peptide has recently been shown to promote relatively efficient endosomal release of propidium iodide, with 20–25% of the encapsulated propidium iodide gaining access to KB cell chromosomal DNA after 48 h [99].

The effectiveness of liposomal gene-based drugs is dependent on their ability to access the cytosol of target cells. For optimum efficiency, therefore, lipid-based gene delivery systems should exhibit fusogenic activity. A number of studies illustrate that the above approaches to enhance the fusogenic activity of liposomes can be applied to enhance the efficiency of lipid-based gene delivery systems. For example, the addition of replication-deficient adenovirus, which enhance endosomal escape, to cationic liposome–plasmid DNA complexes results in an approximately fivefold increase in chloramphenicol acyl transferase activity detected in FAO hepatoma and 3T3-F442A adipocyte cells in vitro [100], and up to a 1000-fold increase in luciferase expression in human smooth muscle cells in vitro [101]. Similarly, the incorporation of the fusogenic protein from Sendai virus, by fusing Sendai virus with preformed DNA-containing liposomes, results in a liposome with improved gene delivery properties [102–104]. For the majority of cationic lipids, DOPE is required for efficient gene delivery to cells in vitro [30,31,63,64]. However, the observation that certain cationic lipids (such as dioctadecyldimethylammonium chloride or 1,2-dioleyloxypropyl-3-trimethylammonium bromide propane) can function in the absence of helper lipids [30,31] or in the presence of cholesterol (such as dioctadecylammonium bromide) [38] suggests that these cationic lipids may, by themselves, possess properties that promote endosomal release of plasmids via a mechanism other than a membrane fusion event. As previously mentioned, plasmid DNA-containing pH-sensitive liposomes are efficient gene delivery systems in vitro, provided that they have targeting ligands coupled to their surface [41,86].

7. Conclusions

The development of controllable and reproducible liposomal systems for systemic gene delivery necessitates the establishment of methods to efficiently encapsulate gene-based drugs in well-defined, relatively small liposomes. Traditional methods for encapsulating drugs in liposomes have proven to be ineffective for gene-based drugs. However, recently developed detergent-based procedures to produce stable plasmid DNA lipidic particles or plasmid DNA-containing liposomes appear promising. In vitro studies have shown that these systems are active in delivering plasmid DNA to a number of cultured established cell lines. The in vivo studies are certainly forthcoming. Although these are early stages for liposomal gene delivery systems, several of the advances made in liposomal drug delivery technologies can be directly applied to these systems. Noteworthy is the use of exchangeable PEG–lipid conjugates to stabilize the plasmid DNA-containing lipid-based carriers in the circulation. This should expedite the development of systemic liposomal gene delivery systems that exhibit targeted and enhanced intracellular delivery.

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