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Liposomes as a gene delivery system

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Abstract

Gene therapy is an active field that has progressed rapidly into clinical trials in a relatively short time. The key to success for any gene therapy strategy is to design a vector able to serve as a safe and efficient gene delivery vehicle. This has encouraged the development of nonviral DNA-mediated gene transfer techniques such as liposomes. Many liposome-based DNA delivery systems have been described, including molecular components for targeting given cell surface receptors or for escaping from the lysosomal compartment. Another recent technology using cationic lipids has been evaluated and has generated substantial interest in this approach to gene transfer.

Key words
- Gene
- pH-sensitive liposome
- Cationic lipid
- Transfection

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Introduction

The introduction of genes into cells of various origins has been a major technique in cell biology research for more than a decade. In addition to being a powerful research tool, gene transfer is a new concept for gene therapy, a molecular therapeutic approach for curing inherited and many other diseases (1,2). Acquired diseases which have a genetic component can theoretically be a target of genetic correction based on the addition of needed genes. Among the genetic diseases that have been studied are cystic fibrosis, muscular distrophy, and familial hypercholesteremia. For cancer, most are not inherited but result from acquired mutations caused by external factors, and therefore correcting or compensating for these mutations could be one of the challenges of gene therapy.

Gene transfer may also be a potential weapon as a vaccine. The injection of a DNA fragment coding for a foreign antigen could trigger a potent immune response to this antigen. This therapeutic design could be applied to infectious diseases (viruses, parasites) or cancer (3,4). If the conceptual part of the gene therapy revolution has occurred, the central challenge remains, i.e., perfecting methods for delivering therapeutic genes to cells.

From gene transfer to gene therapy. A gene cannot enter a cell by itself for two reasons: it is a large piece of DNA and is surrounded by various anionic charges. For *in vitro* gene transfer, a variety of artificial techniques such as direct DNA microinjection, membrane perturbation by chemicals (organic solvents, detergents), physical means (mechanical or osmotic means, electric shocks) and liposomes have been used.

However, *in vivo*, the interactions between DNA and cells may be more complex. Wolff et al. (5) have reported that naked DNA injected into the muscle of animals was expressed as protein. Despite the electrical repulsion between cell surfaces and DNA, it appeared that a few cells were able to assimilate the molecule. This suggests that a small amount of tissue damage or increased pressure at the injection site could play a role. *In vivo* interactions of DNA with proteins could also modify the interactions

between cells and DNA. So, theoretically, it should be possible to inject naked DNA intramuscularly but the production of high local protein would be insufficient to be effective after its dilution in the bloodstream.

Therefore, to improve gene delivery, DNA compaction with polycations, DNA encapsulation into recombinant retroviruses (6), adenoviruses (7), or liposomes (8,9) have been evaluated. As far as retroviruses and adenoviruses are concerned, the possible production of recombination and the oncogenic effects of random insertion into the host genome have limited their use (10).

Since gene therapy has progressed rapidly into clinical trials in a relatively short time, the methods of gene introduction must be compatible with therapy. This encouraged the development of nonviral DNA-mediated gene transfer techniques such as liposomes. Only pH-sensitive liposomes and cationic lipids will be discussed in the present article.

Prerequisites for transfection *in vitro*

What is the most significant factor or rate-limiting step controlling the DNA transfer process? To answer this question, it is important to dissect the overall cell uptake process into individual steps.

In fact different studies have indicated that successful gene transfer *in vitro* involves: 1) the packaging of DNA, 2) the adhesion of packaged DNA to the cell surface, 3) internalization of DNA, 4) escape of DNA from endosomes if endocytosis is involved, 5) DNA expression in cell nuclei.

To perform all of the above steps, liposomes have been explored as a delivery system for DNA as early as in 1979 (11). The encapsulation of plasmid DNA into liposomes (12) and the introduction of poliovirus RNA and SV40 DNA into cells via liposomes (13,14) were reported between 1979 and 1980.

We shall also discuss the more recent transfection technique using cationic lipids.

pH-sensitive liposome strategy

Liposomes of various compositions can extensively bind to cell surfaces. For gene transfer, it was established that dioleylphosphatidylethanolamine (DOPE) is by far the most efficient lipid for *in vitro* gene transfection for pH-sensitive liposomes or as lipid helper in cationic liposomes (15-18). It has been assumed that the function of phosphatidylethanolamine (PE) is that of a membrane fusion promoter, since in fact this lipid undergoes changes upon acidification (for a review see Ref. 19). Cholesterol is often essential to achieve sufficient stability of these liposomes.

The composition of liposomes may play an important role in their interactions with cells. The size of liposomes and the type of cells are fundamental for an efficient capture by cells. Generally, liposomes are taken up by various endocytosic processes. Professional phagocytes such as macrophages and neutrophils can take up liposomes of various size and charge through active phagocytosis.

The vesicular pathway for cellular uptake. After binding to the cell surface, liposomes are internalized into endosomes where they encounter a more acidic pH than in the external medium. Early endosomes generally have an internal pH of 6.50 (20,21). Their contents are then transferred to a more acidic environment by maturation or vesicular fusion. The last endosome environment, with an internal pH of 5.5-6.0, is reached 10-15 min after uptake. The last endocytotic compartment, the lysosome, is further acidified to pH values of 5.0 or lower and is reached 20 min or more after uptake. The lysosomes are the main degrading compartment in the endocytotic pathway. Conventional, pH-insensitive liposomes and their content are delivered to lysosomes and degraded. The last requirement for plasmid liposomes after cell penetration is to avoid

accumulation in particular cell compartments such as lysosomes. In order to prevent this degradation, pH-sensitive liposomes have been proposed (15,16).

pH-sensitive liposomes were designed based on the concept of viruses that fuse with the endosomal membrane by means of a protein at pH 5-6, delivering their genetic material to the cytosol before reaching the lysosomes (Figure 1) (22,23).

Generally, the lipid used to design pH-sensitive liposomes is PE. PE represents a class of lipids which, when dispersed in pure form, assemble into nonbilayer structures in an inverted hexagonal phase (for a review see Ref. 19). To stabilize PE in the lamellar phase in liposomes a series of stabilizers possessing titratable acid headgroup such as oleic acid (OA), palmitoylhomocysteine (PHC) and cholesterolhemisuccinate (CHEMS) were used.

Liposomes composed of DOPE/OA/chol are capable of transfecting mouse Ltk-cells (cells lacking thymidine kinase (TK)) with an exogenous TK gene (9). In this study, pH-sensitive liposomes were 8-fold more efficient in gene delivery than pH-insensitive liposomes. Interestingly, the same investigators also demonstrated that plasmid DNA adsorbed to preformed empty pH-sensitive liposomes can transfect murine Ltk-cells *in vitro*. In contrast, negligible transfection by free plasmid DNA was observed.

In a study by Zhou et al. (24), pUCSV2 CAT DNA was used to prepare liposomes composed of DOPE/dioleylsuccinylglycerol (DOSG) (pH-sensitive formulation) or of dioleylphosphatidylcholine/DOSG (pH-insensitive formulation). The data showed that the acid sensitivity was directly related to the transfection activity. DOPE/DOSG liposome, which was the most sensitive to pH, transfected cells with the highest efficiency.

Legendre and Szoka (25) compared the transfection efficiency mediated by pH-sensitive, pH-insensitive and cationic (DOPE/dioleyloxypropyl-trimethylammonium bromide (DOTMA) liposomes using two different genes and five different cell lines. For all cell types investigated, cationic liposomes mediated the highest transfection level. While pH-sensitive liposomes mediated gene transfer, their efficiency was 1-30% of that obtained with DOPE/DOTMA and pH-insensitive liposomes did not induce transfection. It is important to emphasize the fact that separation of nonencapsulated (adsorbed or free) DNA was performed by pH-sensitive but not by cationic liposomes. This fact itself may interfere with the performance of the two types of liposomes.

A key question remains concerning the mechanism of pH-sensitive liposomes: do they react as originally intended? Ropert et

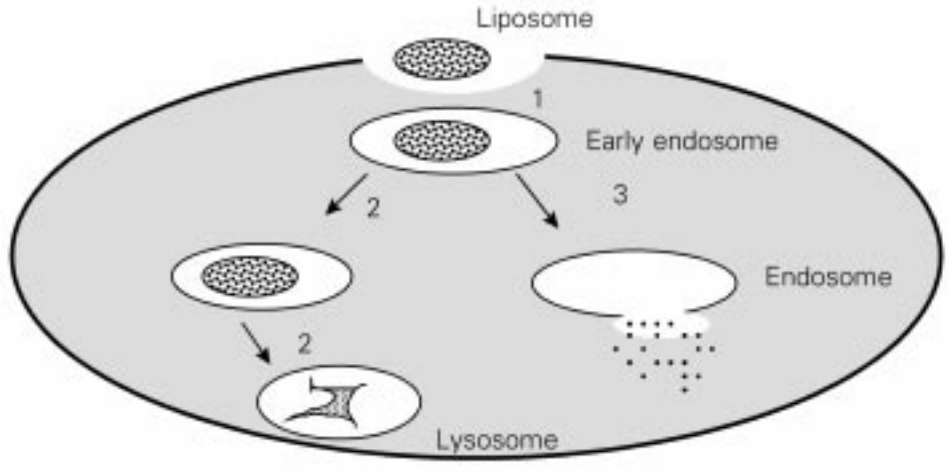

Figure 1 - Uptake and delivery by pH-sensitive and non-pH-sensitive liposomes. 1, Endocytosis process: liposome in an early endosome. 2, Degradation of a non-pH-sensitive liposome and its content in a lysosome. 3, Destabilization of a pH-sensitive liposome and liberation of its content into the cytosol.

al. (26,27) encapsulated antisense oligonucleotides into pH-sensitive liposomes, a short length of DNA directed against the *env* gene of the murine Friend retrovirus, to inhibit virus proliferation. They suggested that the greater activity of oligonucleotides encapsulated into pH-sensitive liposomes was not due to a destabilization of the DOPE liposome bilayer but to an increased association between pH-sensitive liposomes and cells. They reported that the efficiency of the viral inhibition obtained with oligonucleotides encapsulated into pH-sensitive liposomes was only twice that of oligonucleotides encapsulated into non-pH-sensitive liposomes. And a two-fold increase in cell association was also observed when pH-sensitive liposomes were compared to pH-insensitive liposomes. In fact, pH-sensitive liposomes are taken up more efficiently by cells than pH-insensitive liposomes, a fact probably leading to a better activity (22).

Cationic lipid strategy

The encapsulation of DNA into conventional liposomes could be a technical problem due to the plasmid size, representing a poor transfection system. On this basis, an alternative technology based on cationic lipids and PE was developed in the late 1980s (17). The idea was to neutralize the negative charge of plasmids with positively charged lipids to capture plasmids more efficiently and to deliver DNA into the cells. Generally, this is a simple procedure requiring mixing the cationic lipids with the DNA and adding them to the cells. This results in the formation of aggregates composed of DNA and cationic lipids.

The cationic lipid DOTMA was first synthesized and described by Felgner et al. (17). This lipid, either alone or in combination with other neutral lipids, spontaneously forms multilamellar vesicles (MLV) which may be sonicated to form small unilamellar vesicles (SUV). DNA interacts spontaneously with DOTMA to form DNA complexes with 100% of the DNA becoming associated. It is presumed that complex formation simply results from ionic interactions between the positively charged headgroup of DOTMA and the negatively charged phosphate groups of DNA. DOTMA is commercialized (Lipofectin™, Gibco-BRL, Gaithersburg, MD) as a one to one mixture with DOPE and has been widely used to transfect a wide variety of cells (28-31). In an effort to reduce the cytotoxicity of DOTMA, a series of metabolizable quaternary ammonium salts have been developed whose efficiency is comparable to that of Lipofectin when dispersed with DOPE (32).

As stated in the list of requirements, one important step for transfection is DNA compaction to improve cell penetration. Cationic amphiphiles able to compact genomic DNA, namely lipopolyamines, have been studied. Among them, DOSG (Transfectam™) has been shown to transfect many animal cells in a highly efficient manner (33-35). These amphiphiles have been shown to stably condense DNA into particles.

Common detergents of diverse structures (cetyl-trimethylammonium bromide (CTAB), dodecyl-trimethylammonium bromide (DDTAB)) have been compared for use in combination with DOPE. DDTAB seemed to be the most promising one and the DDTAB/DOPE formulation was patented (TransfectACE™). As reported by Farhood et al. (36), the role of DOPE in cationic liposome-mediated gene transfer seemed to be critical, and the compound has been extensively used. Since it has been postulated that the mechanism of DNA/cationic lipids uptake by cells is related to endocytosis, DOPE may favor the liberation of DNA into the cytosol as in pH-sensitive formulations. Electron microscopy observations have shown the endosome destabilizing effect of DOPE-containing cationic liposomes.

Although efforts to synthesize new cationic lipids led to the discovery of more efficient transfection agents, their efficiency does not correlate with their ability to deliver DNA after systemic administration to animals (37). The physicochemical properties of the DNA/lipid complex may determine its stability in plasma and its biodistribution or pharmacokinetics.

In an effort to determine the physicochemical properties of the complex, cationic lipids associated with DOPE and with various amounts of three different cationic surfactants have been investigated by cryo-transmission electron microscopy (TEM) (38). Cryo-TEM analysis suggests that an excess of lipids in terms of charge leads to entrapment of the DNA molecules between the lamellae in clusters of aggregated multilamellar structures. The choice of surfactant does not appear to affect the morphology of the DNA-lipid-complexes. Furthermore, the system containing DOPE results in more compact aggregates than similar formulations using egg lecithin. Templeton et al. (39) have proposed a model for the assembly of DNA-lipid (N-1(2,3-dioleyloxy) propyl, N,N,N-trimethyl ammonium methyl sulfate-DOTAP)-chol complexes in which DNA adsorbs onto the invaginated and tubular liposomes via electrostatic interactions. This generates closed structures in which DNA may be protected.

Farhood et al. (36) proposed the endocytosis as the major route for DNA-lipid complex uptake by cells during transfection. The surface-bound complex is internalized by endocytosis into endosomes and lysosomes in which a large part of the DNA would be degraded. According to Hui and Zhao (40), the most evident pathway for DNA entry into CHO cells is also endocytosis and not direct fusion of the complex with the plasma membrane. Once inside the cell, how and when DNA and lipids become separate remains in question.

Prerequisite for transfection *in vivo*

One important goal for gene therapy is to develop a delivery system that can be injected into the bloodstream and deliver the DNA sequence to the appropriate tissue. The use of conventional liposomes for gene therapy implies a preferential localization in the liver, spleen and lung. Numerous studies have shown that most intravenously injected liposomes are retained in these tissues (41). This phenomenon is primarily due to liposome uptake by the mononuclear phagocytic system (MPS), analogous to the behavior of other intravenously injected inert particulate materials or microorganisms. The propensity of the MPS to remove colloidal drug carriers from the systemic circulation has thus far limited the prospects of targeting liposomes to tissues other than liver, spleen and lung. To reduce the affinity of particles for MPS cells and hence to prolong the circulation time of intravenously injected drug carriers, polymers such as poly (ethylene glycol) and poloxamer have been proposed (42-44). They can either be adsorbed or covalently coupled to the particles, increasing the hydrophilia of the particle surface. The increase of liposome longevity could be a fundamental factor in the case of tumors. In fact, Gabizon et al. (45) have shown that long circulating liposomes offer the advantage of a selective tumor accumulation due to increased microvascular permeability.

Another alternative to specifically target cells and tissues is to use liposomes with attached surface immunoglobulins or other ligands. Such ligand-directed liposomes can be used to probe specific interactions with cell surface receptors and to target drugs and other macromolecules to specific cells and tissues (46,47). But this procedure was not efficient for *in vivo* targeting because antibody-conjugated conventional liposomes were removed nonspecifically by the phagocytic cells of the liver and the spleen. There-

fore, it has been demonstrated that long circulating liposomes with antibodies directed at cancer cells and endothelial cells can be achieved *in vivo* (48,49).

To improve the efficiency of gene expression it would be essential to design the plasmid expression vector as a function of the tissue, as shown by Thierry et al. (50). These investigators have shown that the cytomegalovirus promoter is capable of more efficient expression in spleen than in lung compared to the Rous sarcoma virus promoter. So, the promoter-driving expression of the plasmid may greatly influence the efficacy of the gene delivery system, possibly leading to a certain degree of tissue specificity. This could be one key to the success of gene therapy.

Furthermore, the association of the plasmid with an adequate DNA carrier seems to be fundamental to reach the appropriate cells and to affect a disease process.

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