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Synthetic Virus Systems for Systemic Gene Therapy

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I. INTRODUCTION

In the early 1980s the concept of treating disease through correction of aberrant gene function rose to the attention of both the scientific community and the general public. At the time techniques for gene transfer were underdeveloped, but in the following years efficient methods for gene transfer were established and preclinical studies demonstrated the feasibility of correcting disease phenotypes both in vitro and in vivo. These early proof of principal experiments successfully capped the first, "conceptual phase" (1) of the development of gene therapy and helped to ensure a position for the field in both the medical and popular lexicon. It is generally agreed that the second, translational phase of the development of gene therapy has been considerably less successful. The first human gene marking study performed at the National Institutes of Health (NIH) in 1989 was followed within a year by the first sanctioned therapeutic study (2). In the following decade hundreds of gene therapy clinical trials were initiated worldwide. Today each new advance in medicine is, almost without exception, accompanied by proposals for genetic intervention in associated disease states. In spite of this enthusiasm, gene therapy has been a clinical disappointment. Although numerous reports describe successful gene transfer and prolonged expression of therapeutic transgenes, most clinical trials have failed to demonstrate a therapeutic benefit to treating patients with gene therapy. This was officially recognized in 1995 when the director of the NIH, Harold Varmus, struck two independent committees to review both the scientific and the regulatory aspects of the field. The outcome of this analysis was the consensus that most of the failures of gene therapy could be attributed to the "discordant combination of overinterpreted clinical concepts and immature technology, including poor vector design" (3). This conclusion resulted in a shift in emphasis and expectations for the field, culminating in a recent refocusing on the design and implementation of vector systems for gene therapy as well as a reevaluation of appropriate disease targets. Once regarded as an experimental treatment for inherited genetic disorders, gene therapy is now considered a candidate for front-line therapy of acquired diseases such as cancer. Because the future success of gene therapy will be measured by the ability to compete with the clinical utility of conventional therapeutics, the areas of medicine thought to be most suited to gene therapy are those that have failed to respond to existing treatments (4). The advantage of gene therapy over conventional therapy, and hence its promise, is the ability to regulate gene function and achieve prolonged and specific activity.

In oncology, the holy grail is systemic administration of gene therapy to impact metastatic disease, the primary determinant of survival in most cancers. A defining attribute of metastatic disease is its disseminated nature. As such, treatment with local or regional therapeutics is unlikely to affect clinical outcome or overall disease progression. In the case of intracavitary administration (intrapleural or intraperitoneal), the surface of the tumor mass is coated by the vector, but intratumoral delivery is not achieved. It is increasingly apparent that relevant gene-delivery systems for oncology applications must be able to access distal dis-

ease sites following systemic administration. For this reason a number of investigators have chosen to focus on the development of gene-transfer systems that will have utility in this context (5-8).

Gene-transfer systems are based on one of two predominant platforms-viral or nonviral. Although recognized as being very efficient gene-transfer agents, viral vectors are limited by their inability to access disease sites upon systemic administration. Until significant progress has been made in improving the pharmacology and toxicology of viral vectors, their utility will be limited to local or regional administration in clinical application. Nonviral gene-transfer systems offer specific clinical and commercial advantages as potential therapeutics. Because nonviral systems use synthetic or highly purified components, they are chemically defined and free of adventitious agents. Nonviral systems do not contain adjuvants or undescribed molecular sequences inherent in many viral gene-transfer systems. Nonviral systems can be manufactured under controlled conditions which are not constrained by the biological considerations as cell growth and viability that define viral scale-up processes. In spite of these advantages, progress in the development of systemic nonviral vectors has been slow.

In this chapter we will discuss recent progress in nonviral gene therapy with an emphasis on developments that specifically attempt to address the limitations of current vector systems and their inability to overcome the first barrier to systemic gene delivery-delivery to the disease site and the target cell. Other important barriers to transfection include cytoplasmic delivery, endosomal release, and nuclear delivery. It is unlikely that one single approach will overcome all of these barriers. In fact, properties that are required for systemic disease site targeting have been shown to inhibit intracellular delivery and nuclear uptake. This supports the concept of a modular solution for systemic gene therapy, a vector with individual components fulfilling different functions in the transfection process. An ideal artificial virus would incorporate the most beneficial attributes from both viral and nonviral gene systems. Refinement of gene therapies can be viewed as the convergence of "top-down" deconstruction of viral systems and "bottom-up" engineering of nonviral systems. A number of investigators have chosen to address the limitations of viral systems by removing viral sequences from viral vectors in an attempt to reduce their undesirable characteristics. In this chapter we describe the status of the development of lipid-based systems with virus-like qualities and the potential to rationally incorporate other virus functions.

II. DESIRED PROPERTIES OF A SYNTHETIC GENE-DELIVERY SYSTEM FOR THE TREATMENT OF SYSTEMIC DISEASE

A. Definition of an Appropriate Vector

An objective inherent in all pharmaceutical development is to minimize the risks associated with treatment while maximizing the benefit to patient health. The most important risk to patients is the toxicity associated with the administration of poorly tolerated compounds or cytotoxic agents. Toxicity is often exacerbated by attempts to increase efficacy by escalating the administered dose. The differential between the minimum dose at which a therapeutic benefit is obtained and the maximum dose that can be safely administered to the patient is known as the "therapeutic window." Any reduction in toxicity or increase in potency results in a concomitant increase and improvement in the therapeutic window of a given drug. Toxicity is often the result of preferential accumulation of therapeutic agents in nontarget tissue. One strategy for reducing toxicity involves optimization of drug delivery to maximize delivery to the target site. Therapeutics designed for systemic administration must be capable of bypassing numerous obstacles to effective drug delivery. Drug distribution will be determined by physical and biochemical properties including stability, size, charge, hydrophobicity, interaction with serum proteins, and interaction with nontarget cell surfaces. In the context of an oncology application, effective therapeutics must be able to overcome obstacles associated with heterogeneous cell populations that are often proliferating rapidly at different stages of the cell cycle and do not conform to the patterns of organization established during the development of normal tissue. In particular, tumor growth is associated with changes in vascular organization and permeability that have the potential to affect drug delivery. Normal vascular endothelium is characterized by intact intracellular junctions, which permit only the passage of small molecules. However, the capillaries in tumors develop in a less organized fashion, leaving fenestrae or gaps in the endothelial layer ranging in size from 30 to 500 nm, (9). A few normal tissues, most importantly liver and spleen, have similar fenestrations in the vascular epithelium, which directly expose the underlying endothelial cells to material in the circulation. Importantly, these tissues are often the sites of drug accumulation upon systemic administration.

In conventional pharmacology in vivo studies are initiated once a drug formulation has been developed that exhibits the properties required to ensure effective drug delivery and pharmaceutical viability. Progress in gene therapy has been driven primarily by the pursuit of vectors that are

Table 1 Target Attributes of Carrier Systems for Systemic Gene Therapy

Attribute	Target value	Effect
Toxicity	Low	Facilitates dose escalation
Stability	> 6 months	Facilitates QC
Size	<100 nm	Disease site targeting and extravasation and endocytosis
Surface charge	Neutral or shielded	Disease site targeting and extravasation
Serum half-life	> 3 hours	Disease site targeting
Immunogenicity	Moderate	Multiple dosing
Manufacturability	Reproducible and scalable	Clinical utility

effective in vitro transfection reagents. Acknowledgment of the properties required for effective systemic gene transfer and pharmaceutical viability may require a strategic reevaluation of systemic vector development (Table 1). We propose the following definition of an ideal carrier for systemic gene therapy. The ideal synthetic vector for systemic gene therapy will have the following properties:

1. It must be safe and well tolerated upon systemic administration.
2. It must have appropriate pharmacokinetic attributes to ensure delivery to disease sites.
3. It must deliver intact DNA to target tissue and mediate transfection of that tissue.
4. It must be nonimmunogenic.
5. It must be stable upon manufacture so that large batches can be prepared with uniform reproducible specifications.

B. Barriers to Transfection

DNA is generally limited by poor pharmacokinetic attributes, which limit delivery to disease sites upon systemic administration. Once at the target cell, DNA is again limited in its ability to traverse the numerous biological barriers to transfection. Nucleic acids clearly require pharmaceutical enablement in the form of appropriate carriers, which are able to confer protection from degradation and facilitate delivery and uptake at the disease site. In order to achieve delivery to a disease site, an appropriate systemic carrier must overcome a number of pharmacological barriers. When in the blood compartment an effective gene-delivery system must be able to confer stability to the nucleic acid payload in spite of the presence of serum nucleases and other enzymatic activities that have the potential to degrade carrier components. Indiscriminate interaction with lipoproteins or serum proteins can cause aggregation before a carrier reaches the disease site. Systemic carriers encounter several nontarget cells in the blood compartment, such as blood cells and vascular endothelium, which may be only moderately differentiated from target cells. In addition, systemic delivery systems have a greater potential for inducing toxicity through interaction with complement and coagulation pathways. This is especially true for systems that contain large polyanionic molecules such as plasmid DNA. Other barriers to gene delivery include the microcapillary beds of so-called first-pass organs (lung and liver), the phagocytic cells of the reticuloendothelial system, and filtration by kidney glomemli. The critical parameters that must be measured and optimized to ensure the performance of systemic gene delivery systems are stability, circulation lifetime, biodistribution, and toxicity profile.

Accessing target cell populations requires extravasation from the blood compartment to the disease site. Carriers may leave the blood compartment by passing between endothelial cells or in some cases by transcytosis through specialized endothelial cell systems. Carriers of appropriate size pass through the fenestrated epithelium of tumor neovasculature. Delivery from the blood compartment is followed by diffusion through tissue. In the case of solid tumors, this is often an inefficient process. Although the disorganized nature of tumor growth would intuitively imply accessibility in practice, this is not always true. Tumor growth is often characterized by an underdeveloped lymphatic drainage, which leads to a build-up of hydrostatic pressure unfavorable to extravasation. Other physical barriers to extravasation include large areas of fibrosis and necrosis, which must be bypassed to access actively dividing target cell populations.

Although a prerequisite, delivery of a gene therapy vector to a target cell in no way guarantees transfection. Once at the cell surface, gene therapy vectors are confronted with a number of physical and biochemical barriers, each of which must be overcome in order to effect transfection and transgene expression. The first physical barrier to transfection is the plasma membrane. The plasma membrane is a

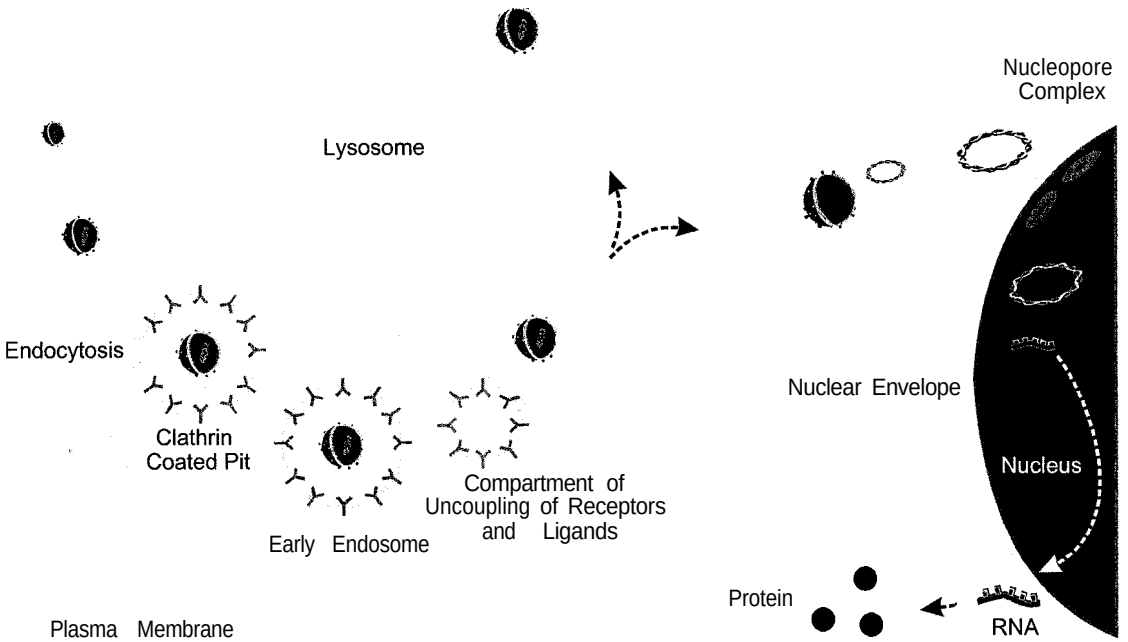


Figure 1 Physical and biochemical barriers to transfection. Most intracellular delivery is thought to occur through endocytosis via cell surface clathrin-coated pits. Endocytic vesicles undergo a series of morphological changes, which define the various stages of intracellular processing. Internalized vesicles or primary endosomes are differentiated from tubulovesicular early endosomes, multivesicular late endosomes, and lysosomes. Late endosomes have a significantly lower luminal pH than early endosomes and contain multivesicular bodies, carrier vesicles, and the prelysosomal compartment. Lysosomal maturation is accompanied by a further decrease in internal pH, a destabilization of the lysosomal membrane, and an increase in fusogenicity. Untargetednonviral vectors rely on diffusion to facilitate interaction with the nuclear envelope, the final physical barrier to transfection.

discontinuous phospholipid bilayer containing intercalated amphipathic membrane proteins (10). The external surface of the plasma membrane is protected by a carbohydrate coating, or glycocalyx, formed by the posttranslational glycosylation of transmembrane proteins. This carbohydrate layer may be up to 100 nm thick (11). The luminal side of the plasma membrane is supported by an actin-rich cytoskeletal matrix of microfilaments and microtubules. Although early models of lipid-mediated transfection invoked a putative fusion event between the plasma membrane and the membrane of the lipid vesicle, it is now generally agreed that the majority of intracellular delivery occurs through endocytosis.

Endocytosis is a complex process by which cells take up extracellular material by translocation across the cell surface membrane (12). This process is known to occur through the activity of cell surface clathrin-coated pits, invaginations in the plasma membrane which are subsequently pinched off into the cytoplasm (Fig. 1). When this occurs, internalized material remains on the exoplasmic side of the internalized vesicle, without direct access to the cytoplasm. The contents of the internalized vesicles are enriched in ligands such as low-density lipoproteins and transferrin (12). In contrast, the fluid content of the vesicle has the same composition as the extracellular medium. The nonspecific uptake of extracellular material through the internalization of coated vesicles is referred to as bulk uptake or fluid phase endocytosis.

Endocytic vesicles undergo a series of morphological changes, which define the various stages of intracellular processing. Internalized vesicles or primary endosomes are differentiated from tubulovesicular early endosomes, multivesicular late endosomes, and lysosomes. According to the maturation model of endocytosis, these organelles are transient entities which undergo successive remodeling to form the organelles associated with each subsequent stage of the pathway (13). This occurs by progressive addition or subtraction of individual organelle components at a rate that maintains the appearance of a homeostatic complement of organelles at each stage of maturation. The first

such change occurs within 5 minutes of uptake as internalized vesicles undergo a series of changes and form the tubulovesicular early endosome or compartment of uncoupling of receptor and ligand (CURL) (14). In this compartment sorting of receptors and ligands occurs. Early endosomes are transiently fusogenic (15). Late endosomes are differentiated from early endosomes both morphologically and biochemically. Late endosomes have a significantly lower luminal pH than early endosomes and contain multivesicular bodies, carrier vesicles, and the prelysosomal compartment. Lysosomal maturation is accompanied by a further decrease in internal pH, a destabilization of the lysosomal membrane and an increase in fusogenicity. Although the process of endocytosis has been well characterized, the processing and release of internalized nonviral vectors and/or their payload DNA is not well understood.

Following endosomal release, plasmid DNA spends an indeterminate residency time in the cytoplasm prior to gaining entry to the nucleus. Unlike viral systems, which have evolved specific mechanisms to traverse this barrier, untargeted nonviral vectors rely on diffusion to facilitate interaction with the nuclear envelope. However, the cytoplasm is a highly organized space containing networks of cytoskeletal elements and membrane-bound organelles, which have the potential to interact with and accumulate vector systems that arrive at the cytosol intact. In particular, mitochondria have been shown to preferentially accumulate polycationic compounds. Until recently the cytoplasmic degradation of plasmid DNA has not been thought to be limiting transfection. However, recent results suggest that this assumption may be incorrect. The turnover of plasmid DNA in the cytoplasm can be measured by quantitative single-cell video analysis (16). When plasmid DNA is delivered by direct microinjection into the cytosol of mammalian cells, it is rapidly degraded by divalent-cation-dependent cytosolic nucleases. This finding is a partial explanation for the strikingly low efficiency of the nuclear translocation process and may have implications for vector design. Vector systems which either protect the DNA payload from degradation following endosome release or effectively minimize the cytoplasmic residency time would be expected to yield improved transfection efficiencies.

The nuclear envelope presents the final physical barrier to transfection. Evolution has led to the development of the nuclear envelope as a means of organizing and maintaining the integrity of the large genome of eukaryotic cells. The nuclear envelope also effectively isolates and protects genetic material from any adventitious elements, such as viruses or transposons, which may enter the cytoplasm of the cell. Appropriately designed gene therapy vectors must overcome this barrier. For this reason an understanding of the systems that mediate the nuclear import of plasmid DNA is essential. The importance of the nuclear envelope in the transfection process is underscored by the finding that nonviral transfection occurs more readily in mitotic cells (17,l8). This implies that the nuclear uptake of DNA is limited by the presence of an intact nuclear envelope, which is destabilized during mitosis. Strategies to overcome this barrier can take one of two forms, either targeting transfection reagents to cell populations with a high degree of mitotic activity, such as tumor tissue, or enhancing the low level of transfection that occurs in the absence of nuclear envelope breakdown.

III. PROPERTIES OF CURRENTLY AVAILABLE GENE-DELIVERY SYSTEMS

A. Viral Vectors

Although viral vectors have shown considerable promise in local and regional applications, the utility of viral vectors for systemic gene therapy remains limited (Table 2). This limitation is primarily due to the inability of viral vectors to overcome the first barrier to transfection-delivery to the target cell. Upon systemic administration most viral vectors are rapidly cleared by the organs of the reticuloendothelial system. The resulting gene expression is usually confined to the liver, a significant disadvantage unless the liver is the target organ (19). An additional disadvantage to the use of viral vectors in systemic gene therapy is the strong host immune response elicited by viral components.

Table 2 Relevant Properties of Viral Vectors for Systemic Gene Therapy

Virus	Size (nm)	Integration competent?	Mitosis requirement?	Intravenous transfection	Ref.
Adenovirns	70-90	No	No	Liver, lung	120
Adeno-associated virus	18-26	Yes (in replicating cells)	Yes (S phase)	Liver	121,122
Retrovirns	80-100	Yes	Yes	Liver	123
Vaccinia virus	200-400	No	No	Liver	124
Herpes simplex virus	120-200	No	No	Liver	124

Intravenous administration in particular elicits a powerful immune response (20). This immune response serves to both eliminate the vector and decrease expression of the transduced gene. For this reason many of successful preclinical studies performed in immunodeficient hosts have failed to translate to successful clinical protocols. Strategies to overcome this limitation include the transient suppression of host immunity (21) or modification of viral vectors in an effort to decrease their immunogenicity (22). Although recent results indicate the potential for development of viral vectors that are relatively nonimmunogenic, the most serious limitation of viral vector systems is their inability to access distal disease sites upon systemic administration. Until significant progress has been made in improving the pharmacokinetics and biodistribution of viral vectors, their utility is likely to be limited to local or regional applications.

B. Nonviral Vectors

The majority of nonviral vectors can be distinguished on the basis of their composition as belonging to one of three main classes: lipoplex, polyplex, or lipopolyplex. Polyplex vectors are defined as cationic polymer-nucleic acid complexes formed by the addition of nucleic acid to cationic polymer, lipoplex vectors are cationic lipid-nucleic acid complexes formed by the addition of nucleic acid to preformed liposomes, and lipopolyplex vectors are complexes that contain both polycationic polymers and cationic lipids (23).

Polyplex-mediated transfection has become an established approach since polylysine-DNA complexes were first shown to be capable of transfecting mammalian cells (24). In spite of early in vitro success, systemic utility has been elusive because of rapid clearance by the reticuloendothelial system and dose-limiting pulmonary and hepatic toxicities. In an attempt to address these limitations, some investigators have chosen to focus on the development of polyplex systems that are either less toxic or are capable of condensing plasmid DNA into smaller, more stable particles than are currently available or incorporation of targeting ligands in an attempt to redirect the distribution of these systems. (These systems are discussed in more detail in Chapter 7.)

Although lipid-mediated systemic gene delivery and expression was reported by Zhu et al. in 1993 (7), progress in developing lipoplex systems capable of delivering plasmid DNA to distal disease sites has also been slow. Early studies on the biodistribution and pharmacokinetics of DC-Chol:DOPE lipoplex (25,26) determined that cationic liposomes were cleared from the circulation within minutes of intravenous administration. The majority of lipid label accumulates immediately in the lung, with the remainder being distributed in the spleen, heart, and liver. Lipoplex vectors are cleared rapidly because the lungs, the first major organ encountered upon intravenous administration, have a large capillary bed with internal diameters < 10 um. Large cationic lipoplexes interact electrostatically with the pulmonary epithelium, and deposition presumably ensues. Attempts to overcome the inappropriate pharmacokinetics of lipoplex systems by escalating the delivered dose typically lead to dose-limiting toxicities (27).

Despite alterations in plasmid:liposome charge ratios or the nature of the cationic lipid or co-lipids in lipoplex systems, their pharmacokinetic limitations have not been overcome. This may be the result of strategies that have relied too heavily on empirical derivation of "systemically active" formulations in the absence of appropriate pharmacological analysis or an understanding of the effect of these modifications on the ability of the delivery system to overcome the biological barriers to transfection. Although several factors contribute to clearance, size and surface charge are thought to be most critical. The sizes of nonviral gene-transfer systems are determined by the physical chemistry of the self-assembly process. Lipoplex formation occurs spontaneously upon addition of plasmid DNA to cationic liposomes. Although theoretically it should be possible to standardize this process, in practice the process generates a heterogeneous mixture of complexes of lipid and plasmid DNA that range in size from 100 to 1000 nm in diameter, which often tends to aggregate over time. In such cases, this necessitates the preparation of lipoplex systems immediately before use, precluding the manufacture of large-scale batches or effective quality control over the final product. Due to the heterogeneous and unstable nature of lipoplex, it is difficult to determine which fraction represents the active form responsible for mediating transfection. The physical characteristics of the formulation change further upon intravenous administration and exposure to blood components by dissociation, aggregation, and/or fusion with blood cells (28-31).

In an effort to address some of these issues, investigators have pursued a strategy that involves precondensation of plasmid DNA with protamine sulfate or other polycationic agents prior to the addition of cationic liposomes (8,3 1,32). Plasmid condensation results in the formation of a DNA core that presumably becomes coated with a lipid shell. The resulting lipopolyplex systems are complexes that contain both polycationic polymers and cationic lipids. Precondensing DNA with protamine sulfate results in an increase in protection from serum nucleases in vitro and improved gene expression following systemic administration, however, due to the large size and charged nature of these

particles the majority are rapidly cleared by the lung where the bulk of the gene expression is observed (32).

In summary, nucleic acids clearly require pharmaceutical enablement in the form of appropriate carriers, which are able to deliver intact DNA to disease sites without causing undue toxicity. Viral and nonviral gene-transfer systems suffer from common pharmacological issues, which limit their utility as systemic delivery agents. Attempts to address these issues through manipulation of the virology or molecular biology of these systems have met with limited success. The issues for nucleic acid drugs are similar to those faced by many low molecular weight chemotherapeutic drugs. The pharmacology of these toxic agents has been successfully enhanced by encapsulation within liposomes. Application of this approach to DNA should overcome a number of the limitations of first-generation delivery systems. In the following sections we will describe the benefits of encapsulation for the chemotherapeutic vincristine and discuss progress in the development of formulations that completely encapsulate plasmid DNA.

IV. CURRENT STATUS OF LIPID-BASED DRUG-DELIVERY SYSTEMS

As an example of the successful implementation of liposome carrier technology in an oncology application we will elaborate on the use of a liposomal carrier for vincristine. Vincristine is an plant alkaloid that effects growth arrest of mitotic cells by binding to and inhibiting tubulin polymerization. It is approved for use against lymphoblastic leukemia, lymphoma, and a number of childhood sarcomas and is often used in combination with other drugs. Evidence suggests that there is a correlation between antitumor efficacy and the duration of exposure to vincristine. One of the attractive features of vincristine is the relative lack of bone marrow toxicity common among many antineoplastic agents. The dose-limiting toxicity of vincristine is neurological, primarily peripheral and autonomic neuropathy (33,34). These qualities combine to make vincristine an attractive target for liposomal encapsulation.

Vincristine has been encapsulated in liposomes through a pH-dependent remote loading procedure. Briefly, liposomes are formed by rehydration of lipid films in low-pH buffer. Vincristine is then added to the preformed liposomes. Finally the pH of the liposome-drug mixture is raised. The pH differential between the external compartment and the interior aqueous space effectively draws the cationic vincristine molecule into the liposome. Entrapment efficiencies of greater than 98% can be achieved (35). Vincrisitine-containing liposomes composed of sphingomyelin and cholesterol (SM/Chol) were found to have mean diameters of 120-130 nm as measured by quasielastic light scattering (36). When the lipid and drug pharmacokinetics of vincristine-SM/Chol liposomes were evaluated in mice, the half-life for removal of vincristine-loaded liposomes from the circulation was 18.9 hours. Vincristine leakage from SM/Chol liposomes in vivo was also slow with 25% of the trapped drug remaining in the liposomes after 72 hours in the circulation. The half-life of vincristine derived from vincristine-loaded SM/Chol liposomes was 12.1 hours (Fig. 2a). This compares favorably with the behavior of free vincristine, which is rapidly removed from the circulation. To determine if the pharmacokinetic attributes of liposomal vincristine affect the ability of vincristine to accumulate in tumor tissue, SCID mice bearing solid A431 tumors were injected intravenously with either free or liposomal vincristine. Improved circulation half-life correlates with an increase in the accumulation of vincristine in distal subcutaneous A43 1 tumors (Fig. 2b). Free vincristine levels peak at 0.5 hour after injection at 0.856 μg/g tumor and decreased to 0.32 μg/g tumor at 24 hours after injection,' while liposomal delivery resulted in 3.2 and 2.8 μg/g tumor at 24 and 48 hours, respectively (Fig. 2b). This represents a lo-fold increase in drug concentration at later time points.

The antitumor efficacy of vincristine correlates with vincristine accumulation at the tumor site (Fig. 2~). A431 tumor-bearing mice that received no treatment required termination 10 days after tumor cell inoculation when the tumor volume reached 10% of the total body weight. Treatment with free vincristine results in a slight inhibition in tumor growth and increase in short-term survival (Fig. 2c). Liposomal formulation results in a significant improvement in antitumor efficacy. Mice treated with liposomal vincristine demonstrate complete tumor regression and an improved survival, a significant improvement over the performance of free vincristine.

The clinical utility of liposomal vincristine has been evaluated in a phase I clinical trial (37). Twenty-five patients with confirmed malignancies were treated with multiple doses of liposomal vincristine. Pharmacokinetic data were collected that suggest that liposomal encapsulation confers dramatically improved pharmacokinetics and serum half-life, supporting the preclinical data collected in animal studies. Based on the successful phase I study, a phase II clinical trial for pancreatic cancer and a separate phase II clinical trial for lymphoma have been initiated.

This example shows that lipid formulation can affect the pharmacology of small molecule drugs in a number of ways. Drugs that have limited serum half-lives can be enhanced by formulation in liposome carriers (38-41). Likewise, drugs that degrade rapidly in the presence of serum components can often be stabilized by liposomal formulation. Drugs that are specific to one phase of the

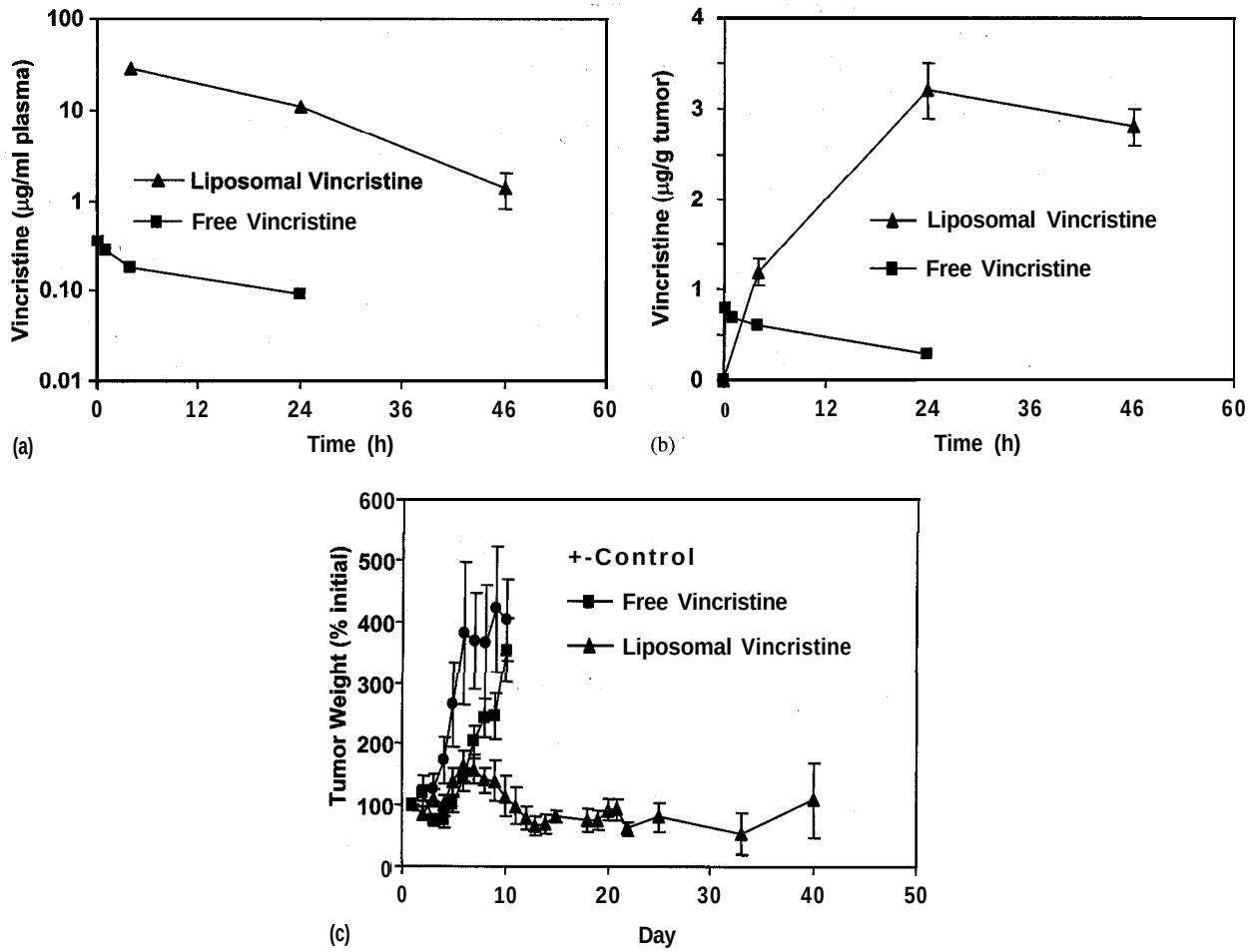


Figure 2 Pharmacology of liposomal vincristine. (a) Pharmacokinetics of liposomal vincristine. Female SCID mice were injected subcutaneously in the hind flank with 2 $\times 10^6$ A431 cells. Fourteen days after tumor seeding, 2.0 mg/kg free or liposomal vincristine was administered through the tail vein. Blood was collected at the indicated time points by cardiac puncture. Plasma was isolated from whole blood and analyzed for ^{3}H-vincristine by liquid scintillation. (b) Accumulation of vincristine in distal tumors following systemic administration. Tumors and other organs were removed and frozen at – 70°C prior to homogenization and analysis for ^{3}H-vincristine by liquid scintillation. (c) Efficacy of liposomal vincristine: inhibition of tumor growth. Tumors were seeded as in (a). Fourteen days after tumor seeding, a single dose corresponding to 2.0 mg/kg free or liposomal vincristine was administered through the tail vein. Animals received no treatment (circle), free vincristine (square), or sphingomyelin:cholesterol formulated vincristine (triangle). Error bars indicate SEM for six tumors.

cell cycle can be modified with liposomal formulation such that they are bioavailable through the entire cell cycle (41). An additional benefit of liposomal encapsulation of conventional drugs is the reduction in acute toxicity that often accompanies the shift in biodistribution associated with lipid formulation. Many chemotherapeutics suffer from toxicity resulting from the pooling of systemically administered drugs in organs such as the kidney, liver, or spleen. Liposomal formulations can be engineered to bypass the reticuloendothelial system and thereby avoid the pooling in nontarget organs, which is often the cause of the acute toxicity associated with systemic drug administration. The redirected biodistribution of liposomal drugs can be extended further. Appropriately designed liposomal carriers are able to take advantage of the phenomenon of "disease site targeting." The changes in vascular permeability associated with tumor growth and inflammatory disorders favor the local accumulation of liposomes with small size and long circulation lifetimes. Delivery systems that aim to take advantage of this unique physiology should have circula-

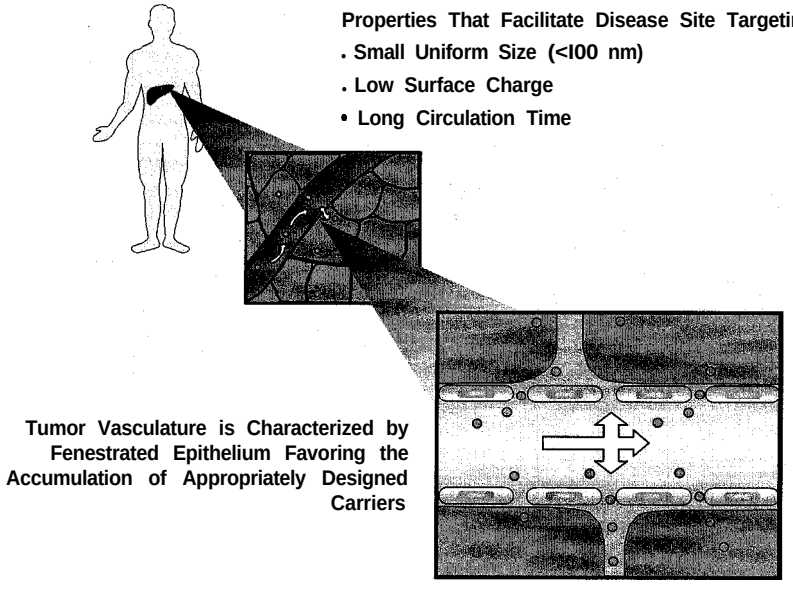


Figure 3 Systemic delivery and disease-site targeting. Accessing target cell populations requires delivery from the blood compartment to the disease site. Delivery from the blood compartment is followed by extravasation through tissue. The changes in vascular permeability associated with tumor growth and inflammatory disorders favor the local accumulation of liposomes with small size and long circulation lifetimes. Delivery systems with circulation times of hours or longer with homogeneous small size (≤100 nm) can take advantage of this unique physiology. This is small enough to exit the fenestrated epithelia of tumor neovasculature and well within the size limit for receptor-mediated endocytosis and intracellular delivery.

tion times of hours or longer in murine models and exhibit a homogeneous small size with a mean diameter of ≤ 100 nm (Fig. 3). This is small enough to exit the fenestrated epithelia of tumor neovasculature and well within the size limit for receptor-mediated endocytosis and intracellular delivery. The benefits of disease site targeting can be profound. In the case of delivery of chemotherapeutic drugs to distal tumor sites, for example, encapsulation in small long-circulating liposomes results in 50- to l00-fold enhancements in the amount of drug delivered to the disease site (36,40,41).

This approach has also been applied to other small-molecule drugs which are either undergoing clinical trial or have been approved for use (Table 3). One of these is the antineoplastic anthracycline doxorubicin (42). Doxorubicin is indicated for a variety of nonhematological tumors. The clinical utility of doxorubicin is limited by cumulative myocardial toxicity, which can result in congestive heart failure during or even years after treatment with the drug. Preclinical and clinical studies show that liposomal doxorubicin has a reduced myocardial toxicity, enhanced antitumor activity, and an improved therapeutic index (38, 39,42). In a similar manner, the toxicity of another anthracycline, daunorubicin, has been partially ameliorated through liposomal encapsulation (43). Liposomal formulations of both daunorubicin and doxorubicin are currently approved for use against AIDS-related Kaposi's sarcoma (Table 3). *cis*-Platinum (cisplatin) is a heavy metal-containing DNA cross-linking agent used against a wide variety of malignancies including ovarian, lung, testicular, bladder, and cervical cancers. Cisplatin is subject to a dose-limiting renal toxicity (44), which is significantly reduced upon liposomal encapsulation (45,46). Other examples of liposomal drugs currently under investigation are summarized in Table 3.

V. METHODS OF ENCAPSULATING PLASMID DNA

In order to capitalize on the pharmacology and disease-site targeting demonstrated by liposomal drug carriers, it is necessary to completely entrap plasmid DNA within the contents of a liposome. Unlike small-molecule drugs, plasmid DNA cannot be "loaded" into preformed liposomes using pH gradients or other similar strategies. A number of investigators have evaluated alternative approaches to entrapping plasmid DNA. Lipid encapsulation of high molecular weight DNA was first demonstrated in

Table 3 Liposomal Drugs in Clinical Development

Drug	Indication	Investigator	Status	Ref.
Amikacin (Mikasome)	Urinary tract infection	NeXstar	Phase II	
Amphotericin B (AmBisome)	Antifungal	NeXstar	Approved	125
Annamycin	Breast cancer	Aronex Pharmaceuticals Inc.	Phase II	
Cisplatin (SPI-77)	Ovarian cancer	Sequus Pharmaceuticals Inc.	Phase II	126
Cyclophosphamide	Non-Hodgkin's lymphoma	Case Western Reserve University	Phase II	
Daunorubicin (DaunoXome)	Kaposi's sarcoma	NeXstar Pharmaceuticals Inc.	Approved	43
Doxorubicin (Doxil)	AIDS-related Kaposi's sarcoma	Sequus Pharmaceuticals Inc.	Approved	127,
	Leukemia and non-Hodgkin's lymphoma		Phase I/II	128
	Cancers of gynecological origin and carcinoma of the liver or bile ducts		Phase III	
Doxorubicin (Evacet)	Breast cancer	The Liposome Company	NDA	
Doxorubicin (LED)	Solid tumors	Neopharm	Phase I/II	
Lurtotecan (NX-211)	Ovarian cancer, solid tumors	NeXstar	Phase I	
Mitoxantrone	Breast cancer	University Hospital of Zurich	Phase II	129
Nystatin (Nyotran)	Antifungal	Aronex Pharmaceuticals Inc.	NDA	130
Paclitaxel (LEP)	Solid tumors	Neopharm	Phase I	
Trans-retinoic Acid (Atragen)	Leukemia	Aronex Pharmaceuticals Inc.	NDA	
	Non-Hodgkin's lymphoma		Phase II	
	Renal cell and bladder cancer		Phase I/II	
Tretinoin (Atragen, L-ATRA)	Acute promyelocytic leukemia	Aronex Pharmaceuticals Inc.	Phase II	131
Vincristine (ONCO-TCS)	Lymphoma	Inex Pharmaceuticals Corp.	Phase II	37

the late 1970s prior to the development of cationic lipid-containing lipoplex (47-49). Although previous attempts to encapsulate plasmid DNA yielded mostly large multilamellar vesicles with poor transfection efficiency (50-52), recent improvements in formulation technology have resulted in the production of cationic lipid-containing particles with much greater transfection potential. Many of these methods are summarized in Table 4. Plasmid DNA has been encapsulated by reverse-phase evaporation (53-56), either injection (57,58), detergent dialysis in the absence of PEG stabilization (55,58), lipid hydration-dehydration techniques (52,59,60), sonication (61-63), and others (64,65). The two approaches that have shown the most promise differ conceptually in that one relies on a spontaneous internalization of plasmid DNA in preformed liposomes (6), while the other, detergent dialysis, is a process in which unilamellar vesicles containing plasmid DNA are formed upon removal of detergent from a DNA:lipid solution (5). We will briefly describe the invaginated liposome approach (covered in detail in Chapter 12) and then describe in detail the detergent dialysis approach.

Templeton et al. have described a novel formulation of stable complexes composed of DOTAP, cholesterol, and plasmid DNA (6). DOTAP:cholesterol liposomes (1:NOl) were formed by hydration of a lipid film, sonication, and extrusion through a series of filters of decreasing size. Particular attention was paid to optimization of conditions in order to maximize the colloidal properties of the resulting delivery system. Extrusion of the large unilamellar liposomes with excess surface area results in the formation of unique vase-like structures. Upon addition of plasmid DNA, plasmid is condensed in a space formed by invagination or complete circling and fusion of two lipid bilayers. Systemic administration of these particles results in high levels of reporter gene expression in the lung and other organs, a portion of which can be redirected to the liver by the incorporation of the targeting ligand, succinylated asialofetuin. This work clearly demonstrates the advantages of protective encapsulation of the plasmid DNA payload and reduction in surface charge upon incorporation of a targeting ligand, two concepts that support the concept of an artificial virus for systemic gene delivery.

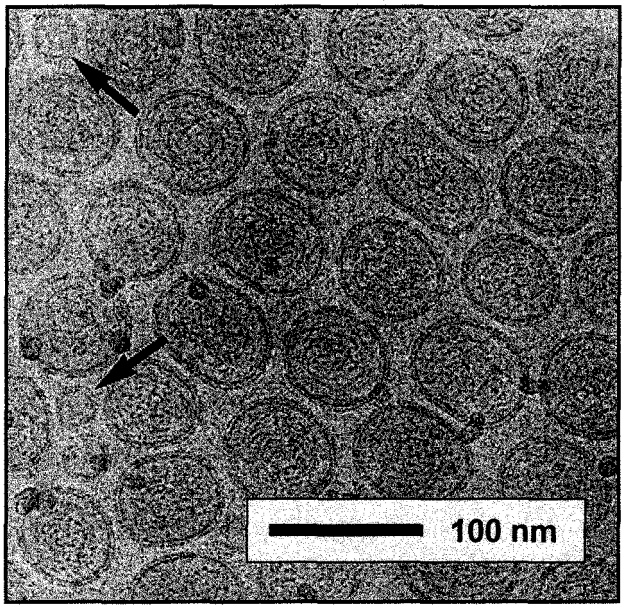
Novel encapsulation methods have been developed in an effort to improve the clearance properties of encapsulated plasmid systems containing high ratios of cationic lipid and associated surface charge. It is of interest to extend these procedures to generate plasmid-containing cationic liposomes stabilized in a manner analogous to liposomal drug formulations, which have been shown to facilitate disease-site targeting (66). In particular, lipo-

Table 4 Procedures for Encapsulating Plasmid in Lipid-Based Systems

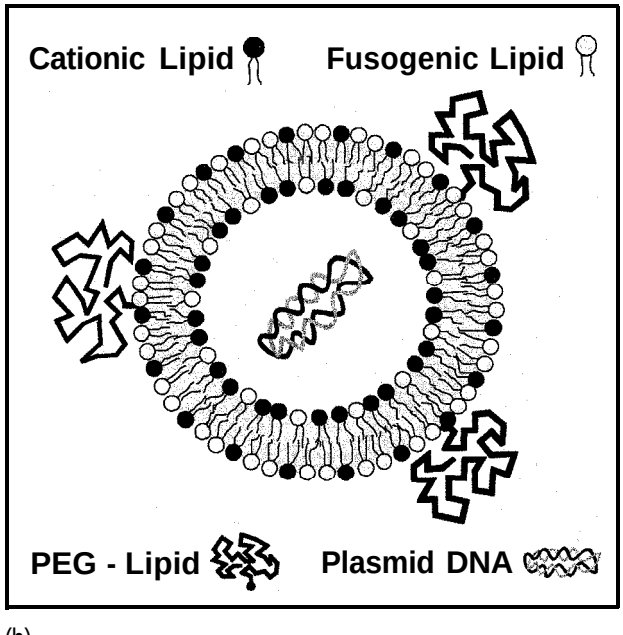
Procedure	Lipid composition	Length of DNA	Trapping efficiency[a] (%)	DNA-to-lipid ratio"	Diameter	Ref.
Reverse-phase evaporation	PS or PS:Chol (50 : 50)	SV40 DNA	30-50	<4.2 μg/μmol	400 nm	53
Reverse-phase evaporation	PC:PS:Chol (40: 10:50)	11.9 kb plasmid	13-16	0.23 μg/μmol	100 nm to 1 μm	54
Reverse-phase evaporation	PC:PS:Chol (50: 10:40)	8.3 kb, 14.2 kbp plasmid	10	0.97 pg/pmol	ND	55
Reverse-phase evaporation	EPC:PS:Chol (40: 10:50)	3.9 kb plasmid	12	0.38 ,ug/pmol	400 nm	56
Ether injection	EPC:EPG (91: 9)	3.9 kb plasmid	2-6	<1 μg/μmol	0.1-1.5 μm; Aug = 230 nm	58
Ether injection	PC:PS:Chol (40: 10:50) PC:PG:Chol (40: 10:50)	3.9 kb plasmid	15	15 pg/pmol	ND	57
Detergent dialysis	EPC:Chol:stearylamine (43.5:43.5: 13)	sonicated genomic DNA (approximately 250,000 MW)	11	0.26 μg/μmol	50 nm	97
Detergent dialysis, extrusion	DOPC:Chol:oleic acid or DOPE:Chol:oleic acid (40 : 40 : 20)	4.6 kb plasmid	14-17	2.25 ,ug/pmol	180 nm (DOPC) 290 nm (DOPE)	104
Lipid hydration	EPC:Chol (65 : 35) or EPC	3.9 kb, 13 kb plasmid	ND	ND	0.5-7.5 μm	59
Dehydration-rehydration, extrusion (400 or 200 nm filters)	Chol:EPC:PS (50:40: 10)	ND	ND	0.83 μg/μmol (200 nm) 1.97 μg/μmol (400 nm)	142.5 nm (200 nm filter) 54.6 nm (400 nm filter, ultracentrifugation)	60
Dehydration-rehydration	EPC	2.96 kb, 1.25 kb plasmid	35-40	2.65-3.0 μg/μmol	l-2 μm	52
Sonication (in the presence of lysozyme)	asolectin (soybean phospholipids)	1 .O kb linear DNA	50	0.08 μg/μmol	100-200 nm	61
Sonication	EPC:Chol:lysine-DPPE (55:30: 15)	6.3 kb ssDNA 1.0 kb dsRNA	60-95 ssDNA 80-90 dsRNA	13 μg/μmol ssDNA; 14 μg/μmol dsRNA	100-150 nm	62
Spermidine-condensed DNA, sonication, extrusion	EPC:Chol:PS (40:50: 10) EPC:Chol:EPA (40:50: 10) or EPC:Chol:CL (50:40: 10)	4.4 kb, 7.2 kb plasmid	46-52	2.53-2.87 μg/μmol	400-500 nm	63
Ca^{2+}-EDTA entrapment of DNA-protein complexes	PS:Chol (50: 50)	42.1 kbp bacteriophage	52-59	22 μg/μmol	ND	65
Freeze-thaw, extrusion	POPC:DDAB (99 : 1)	3.4 kb linear plasmid	17-50	ND	80-120 nm	64
SPLP	DOPE:PEG-Cer: DODAC (84 : 10 : 6)	4.4 to 10 kb plasmid	60-70	62.5 μg/μmol	75 nm (QELS); 65 nm (freeze-fracture)	5

ND = not determined.

a Some values calculated based on presented data.



(a)



(b)

Figure 4 Structural model of SPLP. (a) Cryo-electron microscopy of SPLP. SPLP were prepared from DOPE:DODAC:Peg-$CerC_{20}$ and pCMVLuc and purified employing DEAE column chromatography and density gradient centrifugation. Arrows indicate the presence of residual "empty" vesicles formed during the detergent dialysis but not removed by the density gradient centrifugation purification step. (b) Structural schematic of SPLP. SPLP are formed with neutral fusogenic lipid, cationic lipid, PEG-ceramide, and plasmid DNA. The process results in the formation of small (approximately 70 nm diameter) particles containing one plasmid surrounded by a lipid bilayer.

somes that incorporate PEG conjugates in the lipid bilayer have been shown to yield long circulation lifetimes (67-69). PEG conjugates are thought to sterically stabilize liposomes by forming a protective layer, which shields the hydrophobic lipid layer (70). This prevents the association of serum proteins and resulting uptake by the reticuloendothelial system (71,72). This approach has been investigated with a view towards improving the stability and pharmacokinetics of lipoplex (73). However, lipoplex incorporating PEG-phosphatidylethanolamine demonstrate an improved stability at the expense of transfection activity.

A method of encapsulating plasmid in small, PEG-coated lipid vesicles has recently been described (5). In this method hydrophobic and hydrophilic carrier components are simultaneously solubilized in a single detergent-containing phase. Particle formation occurs spontaneously upon removal of the detergent by dialysis. Under appropriate conditions thermodynamics favor the formation of small (approximately 70 nm in diameter) stabilized plasmid lipid particles (SPLP) containing one plasmid/vesicle in combination with plasmid-trapping efficiencies approaching 70% (Fig. 4). The SPLP protocol results in stable particles with low levels of cationic lipids, high levels of fusogenic lipids, and high DNA-to-lipid ratios. SPLP can be concentrated to achieve plasmid DNA concentrations > 1 mg/mL. These attributes compare favorably with the previously reported plasmid encapsulation processes summarized in Table 4. The SPLP method yields the highest plasmid DNA-to-lipid ratio (62.5 $\mu g/\mu mol$) of any of these methods and is remarkably stable when compared to other encapsulated systems. The stability of SPLP facilities a thorough characterization of their physical properties. In particular it is possible to monitor the efficiency of the encapsulation process and the degree to which plasmid DNA is protected from exogenous nuclease activity. Although the SPLP process results in the formation of empty lipid vesicles and unencapsulated DNA in addition to SPLP, free plasmid DNA can be removed by ion-exchange chromatography while density gradient centrifugation effectively removes empty vesicles. The plasmid-to-lipid ratio of pure SPLP, 65 $\mu g/\mu mol$, corresponds to one plasmid per SPLP (5). This is achieved without the use of extreme conditions, which have the potential to compromise the integrity of plasmid DNA.

Several parameters have been shown to be critical for SPLP formation (5,74). Ionic strength, cationic lipid, and PEG lipid content must be optimized to maximize plasmid entrapment and minimize aggregation (Fig. 5). Entrapment is a sensitive function of cationic lipid content (5). The first stage of dialysis is proposed to result in the formation of macromolecular intermediates, possibly lamellar lipid sheets or micelles. Plasmid DNA is recruited by electro-

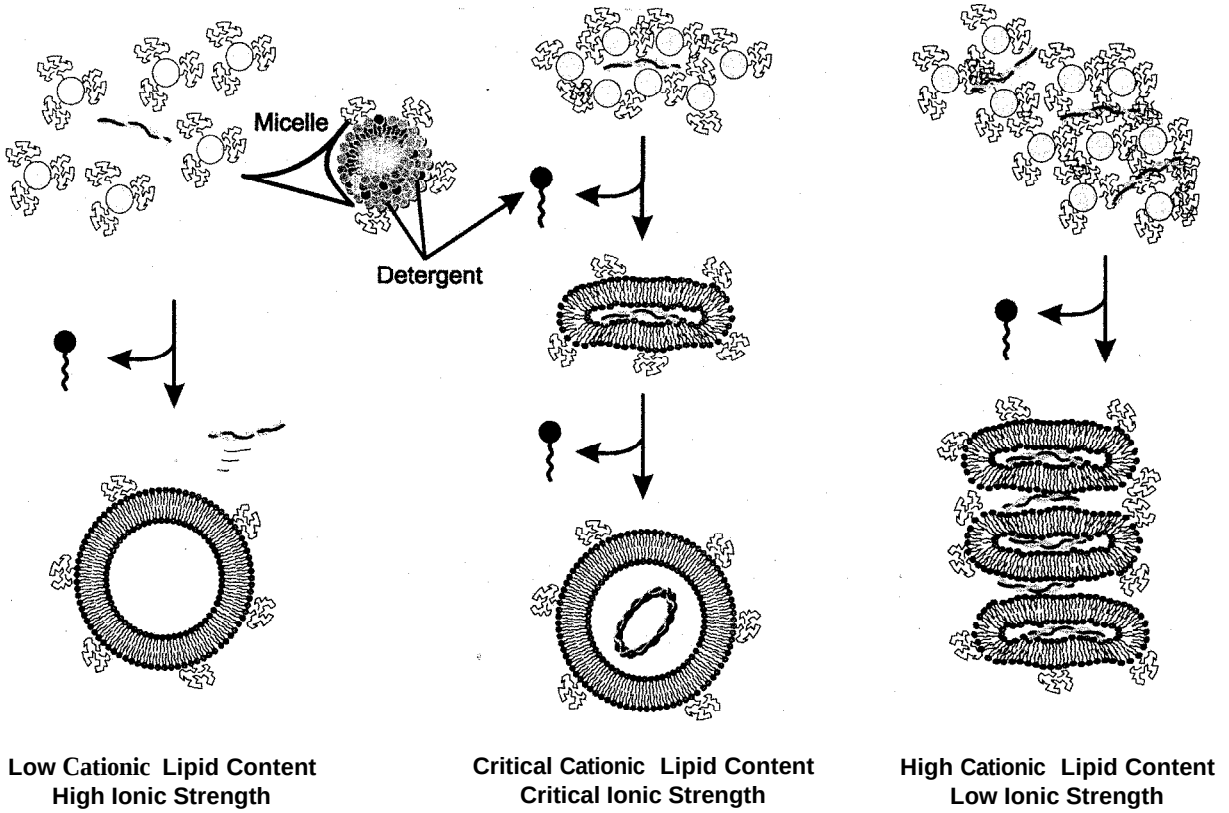


Figure 5 Critical parameters for SPLP formation. Dialysis is proposed to result in the formation of macromolecular intermediates, possibly lamellar lipid sheets or micelles. If the cationic lipid content is too low, plasmid fails to associate with these intermediates, favoring the formation of empty vesicles. If the cationic lipid concentration is too high, the surface charge on the lipid intermediate attracts excess plasmid DNA leading to the formation of polydisperse aggregates. At optimal cationic lipid concentrations, plasmid DNA is proposed to associate with the lipid intermediates in such a way as to reduce the net positive charge on the lipid surface. Association of further lipid leads to the formation of vesicles containing encapsulated plasmid. The electrostatic interaction between plasmid DNA and cationic lipids is also affected by the ionic strength of the dialysis buffer. However, at buffer concentrations above the optimal range, the positive charge on lipid intermediates is shielded to such an extent that formation of empty vesicles is favored. Likewise, if the ionic strength is suboptimal, large plasmid-lipid aggregates are formed.

static attraction. If the cationic lipid content is too low, plasmid fails to associate with these intermediates, favoring the formation of empty vesicles. If the cationic lipid concentration is too high, the surface charge on the lipid intermediate attracts excess plasmid DNA leading to the formation of polydisperse aggregates. At optimal cationic lipid concentrations plasmid DNA is proposed to associate with the lipid intermediates in such a way as to reduce the net positive charge on the lipid surface. Association of further lipid leads to the formation of vesicles containing encapsulated plasmid (5). Initial experimentation determined that the optimal cationic lipid content was 6-7% when formulation was attempted in HBS buffer. However, the electrostatic interaction between plasmid DNA and cationic lipids is also affected by the ionic strength of the dialysis buffer. Polyvalent buffers such as citrate can be used to shield the positive charge on cationic lipids to facilitate the formulation of SPLP containing higher concentrations of cationic lipid (74). However, at citrate concentrations above the optimal range, the positive charge on lipid intermediates is shielded to such an extent that formation of empty vesicles is favored. Likewise, if the ionic strength is suboptimal, large plasmid-lipid aggregates are formed. By careful manipulation of ionic strength SPLP can be formed containing up to 30 mol% cationic lipid (74). In a similar manner, selective replacement of individual lipid components can be achieved without sacrificing small uniform size and encapsulation efficiency.

VI. PHARMACOLOGY OF ENCAPSULATED PLASMID DNA

The properties of small size, serum stability, low levels of cationic lipid (approximately 6 mol%), and the presence of the PEG coating suggest that SPLP should exhibit extended circulation lifetimes and disease-site-targeting properties following intravenous administration. A direct test of the pharmacokinetic properties of SPLP particles is shown in Figure 6a (75). SPLP were formulated containing trace amounts of ^{3}H-cholesteryl hexadecyl ether (CHE), a non-exchangeable lipid marker routinely used to label liposomes or vesicle preparations (76). SPLP were administered intravenously to mice bearing distal hind-flank tumors. Blood was drawn at the indicated time points and subjected to analysis for ^{3}H-CHE lipid by liquid scintillation analysis. SPLP are cleared from serum gradually with a measured serum half-life of between 3 and 6 hours (75,77). The serum half-life of unprotected plasmid DNA is known to be less than 5 minutes (28,77). SPLP accumulate in distal tissue following intravenous administration. SPLP accumulate primarily in the tumor and liver, bypassing the capillaries of the lung. Twenty-four hours after tail vein injection, the tumor and liver have accumulated l-3 and 5-10% of the total injected lipid dose, respectively. The ability of SPLP to bypass the lung, unique among cationic lipid-containing gene-delivery systems, is thought to correlate with the properties required for disease-site targeting, small uniform size, and surface charge shielding. The stability of SPLP protects plasmid DNA and facilitates intratumoral delivery of intact plasmid following systemic administration (75). In contrast, intact DNA is not delivered to the tumor at any of the time points assayed following intravenous administration of naked plasmid DNA.

The observation that SPLP are able to transfect mammalian cells in vitro demonstrates that SPLP plasmid DNA retains biological activity through the formulation process. Furthermore, SPLP are capable of overcoming the barriers to transfection, intracellular delivery and nuclear uptake, when applied directly to cells. SPLP containing reporter gene constructs have been shown to be transfection competent in vivo when applied either regionally (74) or systemically (5). SPLP are capable of mediating reporter gene expression in distal tumors following systemic administration (77). This supports the concept of the artificial virus in that the properties required for disease-site targeting, stability and low surface charge, are not necessarily incompatible with those needed to facilitate intracellular delivery and transfection. This suggests that SPLP containing plasmid coding for an appropriate therapeutic transgene may be capable of mediating an antitumor effect following systemic administration. There are a number of reports of antitumor efficacy following systemic administration of lipoplex or lipopolyplex systems containing therapeutic plasmids, however, supporting pharmacokinetic and accumulation data have not been reported. Furthermore, it is increasingly apparent that some disease models are responsive to nonspecific effects associated with the delivery of vector components or plasmid DNA. For this reason the choice of therapeutic transgene and appropriate controls is critical to ensure the collection of meaningful data. One strategy that allows for partial differentiation between gene specific and nonspecific effects is the use of suicide genes. Transfection of mammalian cells with suicide genes sensitizes them to a nontoxic prodrug. Transfection in the absence of prodrug treatment has no specific therapeutic benefit. To test the therapeutic potential of SPLP, SPLP were manufactured containing plasmid DNA coding for the herpes simplex virus thymidine kinase (HSV-TK) and evaluated for antitumor efficacy upon systemic administration. Mice bearing MCA-207 tumors were treated with five tail vein injections of either TK-SPLP or a frameshift mutant control over the course of 10 days in combination with daily intraperitoneal administration of the prodrug ganciclovir. Those animals treated with TK-SPLP in combination with ganciclovir demonstrated the greatest antitumor response (Fig. 6c.). Four out of eight animals demonstrated complete tumor regression. Upon tumor rechallenge, three of these four animals failed to develop new MCA-207 tumors, a possible indication of adaptive tumor immunity. The long-term effects of SPLP-mediated systemic suicide gene therapy are even more pronounced than the results of short-term tumor growth-inhibition studies (Fig. 6d). In a larger study 60% of the animals treated with TK-SPLP in combination with ganciclovir remained tumor-free after 60 days, while only 15% of the animals treated with TK-SPLP alone survived for this period.

Although these results support the conclusion that SPLP can mediate antitumor effects upon delivery of a therapeutic gene, it is important to control for nonspecific effects associated with systemic administration of plasmid DNA. Figure 6c shows that some antitumor effect is obtained when TK-SPLP are administered in the absence of prodrug treatment. Here a frameshift control plasmid was used to assist in differentiating between gene-specific and nonspecific effects. Cells transfected with the frameshift control plasmid do not produce TK protein. This plasmid serves as a control for the nonspecific effects associated with systemic delivery of bacterial DNA containing unmethylated CpG dinucleotides, which can induce activation and proliferation of B, NK, $CD4^{+}$ T cells, and macrophages (78-82). This nonspecific immune activation must be taken into consideration when interpreting studies that rely on plasmid DNA as a therapeutic agent. Those animals that

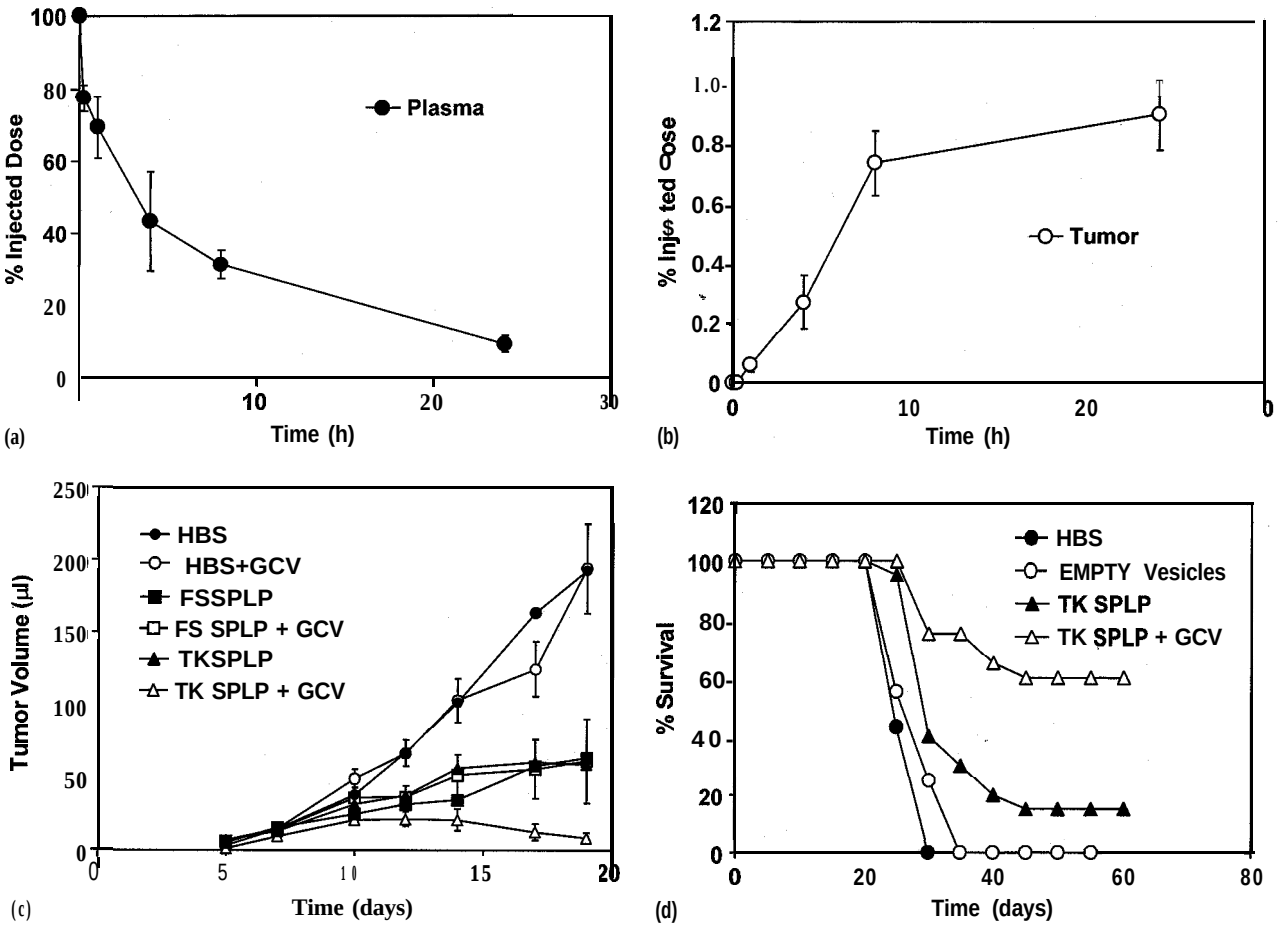


Figure 6 Pharmacology of SPLP. (a) Pharmacokinetics of SPLP. Female C57BL/6 mice were injected subcutaneously in the hind flank with 1 X 10^5 MCA-207 murine fibrosarcoma cells in a total volume of 50 μL PBS. Fourteen days after tumor seeding, 100 μg of SPLP DNA was administered through the tail vein in a total volume of 200 μL HBS. Blood was collected at the indicated time points by cardiac puncture. Plasma was isolated from whole blood and analyzed for ^{3}H-CHE by liquid scintillation. (b) Accumulation of SPLP in distal tumors following systemic administration. Tumors and other organs were removed and frozen at -70°C prior to homogenization and analysis for ^{3}H-CHE by liquid scintillation. (c) Efficacy of systemically administered SPLP: inhibition of tumor growth. Tumors were seeded as in (a). Starting 5 days after tumor seeding, SPLP were administered through the tail vein once every other day for a total of five treatments. Animals were also treated twice daily with intraperitoneal injection of 200 μL of PBS with or without 1.0 mg ganciclovir. Animals were treated with HBS alone (closed circle), HBS with ganciclovir (open circle), SPLP formulated pCMV-FS-TK alone (closed square), SPLP formulated pCMV-FS-TK with ganciclovir (open square), TK-SPLP alone (closed triangle), or TK-SPLP with ganciclovir (open triangle). Error bars indicate SEM for those groups of eight mice treated with ganciclovir. (d) Efficacy of systemically administered SPLP: survival. Tumors were established as in (d). Animals were treated with HBS alone (closed circle), empty SPLP (open circle), TK-SPLP alone (closed triangle), or TK-SPLP with ganciclovir (open triangle): $N > 20$ in all groups.

received the frameshift SPLP either alone or in combination with ganciclovir demonstrated an inhibition of tumor growth, which corresponded with that observed when animals were treated with TK-SPLP in the absence of prodrug therapy. These results suggest that some nonspecific antitumor efficacy results from the systemic delivery of plasmid DNA, yet support the gene-specific gain in antitumor efficacy associated with the intratumoral delivery of biologically active plasmid DNA in combination with prodrug therapy. It is notable that systemic TK-SPLP administration in combination with prodrug treatment results in little or no toxicity in murine models. This may be a reflection of the preferential accumulation of SPLP in tumor tissue or the low transfection potential in organs such as the liver, which accumulate SPLP yet are not readily transfected due to their low level of mitotic activity. The use of HSV-TK

confers an additional level of selectivity in that it only affects cells that are actively undergoing cell division. It remains to be seen if other antitumor strategies are less tolerable when applied systemically.

VII. ENGINEERING FUTURE GENERATIONS OF SYNTHETIC VIRUS

A. The Role of Cationic Lipids in Promoting Intracellular Delivery

The factors affecting intracellular delivery of nonviral vectors are poorly understood. It is believed that both polycations and cationic lipids function by surrounding plasmid DNA with a net positive charge, which in turn enables binding of the DNA complex to anionic cell surface molecules. Increasing our understanding of this process is important not only to improve the efficiency of gene transfer but also to facilitate control of the site of transfection. One approach to elucidating the mechanism of nonviral gene delivery is to identify the molecules on the cell surface that interact with and are responsible for the uptake of cationic gene-transfer agents. Obvious candidates for interaction with cationic vectors would be the most abundant anionic cell surface molecules: sulfated proteoglycans and sialic acids. Proteoglycans appear to enhance polyplex-mediated transfection both in vitro (83) and in vivo (84). In vitro treatment of HeLa cells with sodium chlorate, an inhibitor of proteoglycan sulfation, reduced polylysine-mediated gene expression by 69%. Mutant, proteoglycan-deficient CHO cells yield less than 2% of the reporter gene expression of wild-type cells transfected with polylysine. Both mutant and sodium chlorate-treated cells demonstrated a reduction in the uptake of polylysine DNA complex measured at 37°C and a reduction in binding of DNA to the surface of cells at 4°C (83). Cationic lipoplex systems also rely on proteoglycans to facilitate intracellular delivery. Raji cells lack proteoglycans and as such are poorly transfectable by lipoplex. When stably transfected with cDNA coding for the proteoglycan syndecan- 1, Raji cells are rendered transfectable by DOTIM-cholesterol lipoplex (84). Inhibition of the interaction between the positively charged lipoplex and negatively charged cell surface molecules by pretreatment with polyanionic compounds also inhibits lipoplex-mediated transfection. In vitro treatment of B16 murine melanoma cells with the anionic polysaccharide fucoidan inhibited cationic lipoplex-mediated transfection while having no effect on electroporation or adenoviral transfection. Anionic cell surface proteins are also implicated in transfection following intravenous administration of cationic lipoplex. Treatment of mice with fucoidan prior to lipoplex administration dramatically inhibits transfection of the lung, the primary target tissue for this vector, but has little effect on the more moderate levels of transfection observed in the heart liver and spleen (84). Intravenous administration of heparinase I, an enzyme specific for the cleavage of heparan sulfate proteoglycans, also inhibited cationic lipoplex-mediated transfection of lung, spleen, and heart. This supports the concept that intact proteoglycans are required for the efficient delivery of cationic lipoplex to cells in vivo.

The precise role of proteoglycans in the transfection process remains to be determined. Proteoglycans may interact with cationic vectors directly and be internalized as a complex, or they may serve to anchor cationic vectors for presentation to secondary receptors that in turn undergo specific receptor-mediated endocytosis. Given that the basis of the interaction between proteoglycans and cationic vectors appears to be electrostatic, differences in charge and charge density between vector systems ‘should yield differences in transfection efficiency, This has certain implications for the design of vector systems for systemic gene therapy.

B. The Role of Helper Lipids in Promoting Intracellular Delivery

The majority of cationic lipids require the addition of a fusogenic helper lipid for efficient in vitro gene transfer (85-88). Fusogenic liposomes are thought to facilitate the intracellular delivery of complexed plasmid DNA by fusing with the membranes of the target cell. Inclusion of lipids that preferentially form nonbilayer phases, such as unsaturated phosphatidylethanolamines like DOPE, promote destabilization of the lipid bilayer and concomitant fusion (89). However, certain cationic lipids can function in the absence of fusogenic helper lipids either alone (86,87) or in the presence of the nonfusogenic lipid cholesterol (90). This would suggest that these lipids may have properties that promote transfection through a mechanism that does not require membrane fusion. Recent results which suggest that lipoplex-containing fusogenic lipids are actually less effective than nonfusogenic lipoplex when delivered intravenously bring into question the specific role of fusogenic helper lipids in the transfection process and whether this role is conserved between lipoplex and systems that fully encapsulate plasmid DNA.

Membrane fusion events may occur at a number of different stages in the gene-delivery process, at either the plasma membrane, endosome, or nuclear envelope. In order for fusion with the plasma membrane to occur, lipid particles must first bypass the glycocalyx. Fusion of lipoplex systems with the plasma membrane would be expected to be a particularly inefficient method of introducing

DNA into the cytosol since these systems are topographically challenged. The original model for lipoplex formation suggests that it occurs by the electrostatic interaction between performed cationic liposomes and plasmid DNA, which become attached to the surface of the liposome (91). Lipoplex fusion events are expected to resolve with plasmid DNA, formerly attached to the liposome surface, deposited on the outside surface of the plasma membrane. Encapsulated systems differ from lipoplex in this respect. Upon fusion with the plasma membrane, encapsulated carriers deliver their contents into the cytosol. The major mode of lipoplex-mediated transfection may be either the inefficient translocation of plasmid DNA following fusion of the cationic liposome with the membrane of the target cell or postendocytic fusion of lipoplex that are taken up intact by endocytosis (92). Again, encapsulated systems have an advantage over lipoplex in that fusion with endosomal compartments would be expected to deliver the DNA payload to the cytosol. However, even in the absence of a fusion-induced translocation event, fusion of lipoplex systems with endosomal compartments results in a destabilization and disruption of the endosomal membrane.

In spite of attempts to elucidate the role of fusogenic lipids in facilitating intracellular delivery of plasmid DNA, it remains to be determined at which stage in the gene-transfer process membrane fusion occurs. Two factors confound investigators attempting to address this issue. First, attempts to modulate transfection efficiency by effecting the fusogenicity of lipoplex have the potential to affect fusion with the plasma membrane or the endosomal membrane, either of which would be expected to have an affect on gene expression. Second, attempts to follow the fate of plasmid DNA as it travels through the cell to the nucleus have been limited by technical difficulties. This has precipitated an investigative approach by which the process of lipoplex uptake and intracellular trafficking is biochemically dissected in an attempt to identify the critical barriers to transfection. An example of this approach is the transient inhibition of endocytosis by treating cells with cytochalasin-B, an inhibitor of actin polymerization (85). Actin polymerization is required in order to establish the formation of microfilaments that mediate the cytoskeleton-controlled lateral membrane movements that precede endocytosis. When cells in culture are pretreated with cytochalasin-B prior to exposure to cationic lipoplex, transfection is inhibited; This observation supports a major role for endocytosis in lipoplex-mediated transfection. Another approach utilizes fluorescently labeled liposomes to track the fate of lipids upon delivery to the cell. Fusion of labeled liposomes with the plasma membrane results in the transfer of lipid label to the membrane. Treatment of cells with DOTAP:DOPE lipoplex containing rhodamine PE results in the accumulation of fluorescent label in endocytic granules, which become visible at early time points, well before plasma membranes become fluorescent (85). This is additional evidence in support of an endocytic uptake mechanism for lipoplex systems and implies that the role of fusogenic lipids in intracellular delivery is limited. The benefits of incorporating fusogenic lipids in lipoplex systems may be manifest at the level of enhancing fusion and release from endosomal compartments. It remains to be seen if this is also true with encapsulated gene-delivery systems.

Attempts to address the in vivo role of fusogenic lipids in cationic liposome-mediated gene delivery have had confounding results. It has been suggested that lipoplex containing fusogenic lipids are actually less effective than nonfusogenic lipoplex when delivered intravenously. A number of investigators have reported that replacement of fusogenic DOPE with the nonfusogenic lipid cholesterol yields higher levels of gene expression upon systemic administration. It is important to distinguish the effect of fusogenic lipids on gene delivery from the effect on the transfection process. The enhanced gene expression observed upon incorporation of cholesterol in lipoplex formulations may be a result of either an increase in transfection efficiency or improved pharmacokinetics and delivery to the target cell. Fusogenic formulations are more likely to interact with the vascular endothelium, blood cells, and lipoproteins while in the blood compartment. Incorporation of cholesterol in cationic lipoplex may simply render them less promiscuous and thereby improve delivery to the target cell. The implication is that there may be an advantage to transiently shielding the fusogenic potential of systemic carriers. Encapsulated systems provide a platform that may facilitate this approach.

C. Dissociating PEG

Although PEG-containing SPLP are promising with respect to their ability to deliver intact plasmid DNA to disease sites, improvements are required in order to increase levels of gene expression. In particular, SPLP exhibit relatively low transfection efficiencies in vitro (5). This is mainly due to the ability of the PEG coating to inhibit cell association and uptake of PEG-containing liposomes Q&93,94). An ideal carrier would incorporate PEG-lipid conjugates that have the ability to dissociate from the carrier in the blood and transform the SPLP from a stable particle to a transfection-competent entity at the target site (Fig. 7). The feasibility of this approach has been confirmed (5). PEG-ceramide molecules differing in the length of the ceramide acyl chain (CerC14 or $CerC_{20}$) were incorporated into SPLP, and the resulting particles were assayed for in vitro transfection activity. SPLP containing PEG-

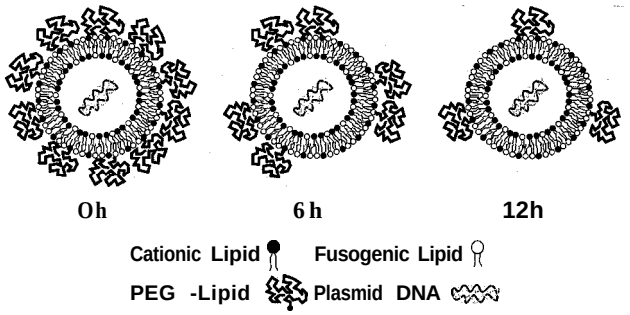


Figure 7 Dissociating PEG coatings for systemic carrier systems. PEG-containing systems exhibit relatively low transfection efficiencies in vitro due to the ability of the PEG coating to inhibit cell association and intracellular delivery. The use of diffusible PEG-ceramides facilitates the formulation of stable particles, which become increasingly fusogenic as the PEG-ceramide dissociates from the particle. This approach may help to resolve the two conflicting demands imposed upon carriers for systemic gene therapy. First, the carrier must be stable and circulate long enough to facilitate accumulation at disease sites. Second, the carrier must be capable of interacting with target cells in order to facilitate intracellular delivery.

$CerC_{20}$ demonstrated little if any transfection activity in vitro, consistent with the stability of the PEG in the particle. When the transfection properties of SPLP containing PEG-$CerC_{20}$ were compared to SPLP containing PEG-$CerC_{14}$, the SPLP containing the shorter-acyl-chain PEG exhibited substantially higher levels of activity than those containing PEG-$CerC_{20}$. This increase in transfection activity correlated with a more rapid dissociation rate when the rate of PEG-ceramide dissociation from egg phosphatidylcholine vesicles was measured in vitro. The use of diffusible PEG-ceramides facilitates the formulation of stable particles containing a high percentage (79-84 mol%) of the fusogenic lipid DOPE. As the PEG-ceramide dissociates from the particle, it is expected to become increasingly fusogenic This approach may help to resolve the two conflicting demands imposed upon carriers for systemic gene therapy. First, the carrier must be stable and circulate long enough to facilitate accumulation at disease sites. Second, the carrier must be capable of interacting with target cells in order to facilitate intracellular delivery. The use of PEG coatings that dissociate from the carrier at the disease site is a potential solution to this problem.

D. Active Targeting

Active targeting is distinguished from passive disease-site targeting, which results in the accumulation of appropriately designed carriers in disease sites such as tumor tissue. Active targeting refers to processes that aim to increase the accumulation, retention, or internalization of targeted carriers through the addition of cell-specific ligands to the carrier surface. Targeting has been successfully applied to liposomal drug formulations and generally has the effect of improving the therapeutic index of.the liposomal drug. Gene-delivery systems stand to benefit from targeting in two ways: through improving the accumulation and binding of formulations to target cells and by facilitating intracellular delivery through endocytosis. Targeting has been achieved through the use of molecules as diverse as antibodies (95,96), proteins (97-l00), vitamins (l0l), and glycolipids (102,103). Liposomal systems modified to include antibodies directed against cell-surface proteins exhibit increased transfection in vitro when compared to untargeted controls (100,104). Targeting of liposomal systems to the asialoglycoprotein receptor has been achieved through the use of either asialofetuin (98) or simple galactosylated lipids (105). Likewise, the addition of transferrin to lipoplex systems results in an increase in both uptake and gene expression in vitro (106). In vitro studies demonstrate the potential advantage of targeted gene delivery at the level of increasing intracellular delivery but do not address the issue of enhanced accumulation at disease sites. It is unlikely that addition of targeting ligands to gene-transfer systems that are rapidly removed from the circulation will result in delivery exceeding that achieved by systems that display passive disease-site targeting. For this reason a number of investigators have pursued approaches involving the addition of targeting ligands to sterically stabilized systems containing PEG lipids, hoping that active and passive targeting effects will be at least additive. In one case PEG lipids were modified to contain folate, a ligand for the cell surface folate receptor (101). PEG lipids were used in the formation of anionic liposomes, which were added to precondensed polyplex. The resulting lipopolyplex demonstrated a significant increase in intracellular delivery when applied to KB cells, yet only a modest increase in gene expression in vitro. Nonetheless, this study demonstrates the potential for the application of targeting technology to sterically stabilized systems. It remains to be seen if encapsulated systems containing targeting ligands can achieve disease-site targeting following systemic administration. The benefits of active targeting may be outweighed by the deleterious effect on serum clearance rates and increased immunogenicity that often accompanies the addition of targeting ligands.

E. Endosomal Release

A variety of approaches can be considered for enhancing the endosomal release of internalized liposomes. We have

already described the use of fusogenic lipids, which are thought to facilitate endosome release. Another strategy involves the incorporation of specific lipids, which render the liposome pH sensitive such that it becomes more fusogenic in low pH compartments such as the late endosome and lysosome (10 1,104). Alternatively, nonfusogenic liposomes can be rendered fusogenic by the addition of viral coat proteins, which promote intracellular delivery and endosomal release. The uptake and endosomal release of lipoplex are enhanced when associated with intact replication-deficient adenovirus (107,108). An extension of this approach has been used to form "virosomes" consisting of the major envelope glycoprotein, hemagglutinin, embedded in a phospholipid-cholesterol bilayer derived from the viral envelope (109,110). Sendai virus may be directly fused with preformed lipoplex to yield virosomes with improved in vitro transfection characteristics (111,112). This approach has also been applied to encapsulated systems. Virosomes containing the F (fusion) protein of Sendai virus, but devoid of hemagglutinin, were prepared by a detergent dialysis process, which results in the encapsulation of plasmid DNA within the lipid envelope. Virosomes prepared in this manner were found to mediate increased gene expression when compared to lipoplex systems (113). Sendai virus F protein is known to effect transfection through two independent mechanisms. Galactosylated F protein is a ligand for the cell surface asialoglycoprotein receptor. F protein also behaves as a membrane fusogen. The fusogenic activity of F protein-containing liposomes can be abrogated by a brief heat treatment without affecting the galactose-mediated endocytic pathway. Heat treatment results in a substantial decrease in the overall transfection efficiency but at the same time increases the rate of virosome accumulation in the endosomal compartments of the cell. Unlike cationic lipoplex, the preferred route of entry for F protein-coated virosomes appears to be direct fusion with the plasma membrane. This result has implications for other encapsulated gene-delivery systems. It implies that there may be significant advantages to increasing the fusogenicity of encapsulated systems such that they fuse with the plasma membrane and deliver their contents to the cytosol, bypassing the lysosomal degradation pathway. However, the addition of viral proteins to lipid-delivery systems is likely to increase their immunogenicity and thus compromise their utility in applications that require repeat administration. A number of peptide derivatives of fusogenie proteins have been described, which may provide a strategy to enhance the fusogenicity of encapsulated systems without the generation of a compromising immune respqnse (114).

F. Nuclear Delivery

The kinetics of gene expression resulting from cytoplasmic injection of plasmid DNA-polyethylenimine polyplex is similar to that observed when the polyplex is administered to cell culture medium, implying that nuclear uptake is the rate-limiting step in the transfection process (115). In an effort to improve upon existing gene-delivery technology, a number of investigators have directed their attention to "the last 200 nm" (11) and the factors affecting the nuclear uptake and expression of gene-therapy vectors. The last stages of the transfection process are generally regarded as the least efficient, and in practice this may be due to the fact that compositions optimized for stability outside the cell may contain components that inhibit transport into the nucleus and/or inhibit transcription. Pollard et al. (115) determined that cationic lipids inhibit transgene expression when reporter plasmids were injected directly into the nucleus of COS-7 cells in association with cationic liposomes. Polycations had no such effect. In contrast, polyethylenimine was found to enhance nuclear uptake and gene expression when polyplex were injected into the cytoplasm. Nuclear uptake of polyplex DNA was independent of charge ratio, implying that nuclear uptake is mediated by DNA compaction rather than net positive charge. These results suggest that neither lipoplex or polyplex systems are capable of simultaneously maximizing gene delivery and gene expression.

Attempts to improve the nuclear uptake must take into consideration the physical constraints of the nucleopore complex that mediates the uptake of plasmid DNA. When fully condensed by monovalent detergent counterions, a 5.5 kb supercoiled plasmid DNA molecule becomes a sphere of about 25 nm in diameter (117). The passive diffusion channel of the nuclear pore complex has an internal diameter of 9 nm (116). The diameter of the activated nuclear pore complex through which active transport occurs and therefore the size limit for signal-mediated nuclear import is 25 nm. Clearly there may be an advantage to invoking active transport mechanisms for the uptake of plasmid DNA. Wolff et al. recently described results obtained using plasmid DNA covalently modified to contain multiple copies of the SV-40 large T antigen nuclear localization signal peptide (NLS) (118). NLS sequences bind to the nucleopore component protein importin alpha-triggering activation of the nucleopore complex (116). Wolff observed a significant enhancement in nuclear uptake upon treatment of digitonin-permeabilized cells with NLS plasmid conjugates (118). When these same conjugates were injected directly into the cytoplasm of normal cells no increase in nuclear uptake was observed. J. P. Behr has constructed a model

that may explain these findings (119). His interpretation of these results is that multiple copies of NLS sequences may actually be inhibitory to nucleopore-mediated DNA uptake. In a separate study cells were treated with linearized restriction fragments, which had been capped with hairpin-loop oligonucleotides. One of these oligonucleotides was covalently modified to contain a single copy of the SV-40 large T antigen NLS or a mutant NLS. Transfection results were compared to those obtained using uncapped linear DNA. NLS-specific transfection enhancements of 10-1000 times were observed in a variety of cell types when plasmid DNA contained a single copy of NLS peptide (119). Behr proposes a model whereby plasmid DNA is rapidly incorporated into chromatin-like structures upon entry into the nucleus. The initial nucleopore interaction is specific and can be NLS mediated, whereas the remaining events are driven by interactions with histones and basic nuclear matrix proteins. The inclusion of multiple copies of NLS peptides on a single plasmid may inhibit nuclear translocation by enabling competing interactions with multiple nucleopores. The implication of these findings with respect to the uptake of supercoiled plasmid DNA are not known, however, there appears to be considerable potential for improving the nuclear uptake of supercoiled plasmids through attachment of NLS peptides. It remains to be seen if this can be accomplished in a manner that is compatible with formulation and systemic gene delivery.

VIII. CONCLUSION

There is clear rationale for the development of a synthetic virus that can be used for systemic administration. First-generation delivery systems have been developed that possess the minimum set of viral functions necessary to transfect eukaryotic cells in vitro and in vivo, namely the ability to protect DNA from nuclease digestion long enough to facilitate association with cells and transit the membrane systems blocking entry to the nucleus. The efficiency, reproducibility, and pharmacology of these systems do not as yet justify their clinical utilization for treatment of systemic diseases. The ability to capitalize on advances in the molecular biology of gene expression systems and fulfill the promise of sustained high-level regulated gene expression will require careful consideration of the pharmacology and formulation of systemically administered gene-delivery systems. Assessment of the performance of the first-generation systems indicates that there are a number of critical physiological barriers that limit their effectiveness. The next generation of delivery systems must balance the often contradictory requirements of formulation stability and bioavailability. Lessons from the formulation of conventional chemotherapeutic drugs suggest that optimal formulations that encapsulate therapeutic payloads can provide significant improvements in pharmacology, yielding increases in potency and reduced toxicity. The recent development of a number of encapsulated systems should improve the pharmacology of gene drugs and provide a platform for the systematic incorporation of additional viral functions designed to confer cellular tropism and maximize potency through enhanced intracellular delivery and gene expression. Ultimately, the goal is to develop synthetic gene-delivery systems with real clinical utility, systems that can be prescribed and administered in a manner analogous to conventional drugs.

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