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"Diffusible-PEG-Lipid Stabilized Plasmid Lipid Particles"

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ABSTRACT

Many viral and non-viral gene transfer systems suffer from common pharmacological issues that limit their utility in a systemic context. By application of the liposomal drug delivery paradigm, many of the limitations of the first generation non-viral delivery systems can be overcome. Encapsulation in small, long-circulating particles called stabilized plasmid lipid particles (SPLP) results in enhanced accumulation at disease sites and selective protein expression. This work compares the detergent dialysis method of SPLP manufacture with an alternative method, spontaneous vesicle formation by ethanol dilution. The

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pharmacology of SPLP, as determined by monitoring lipid label and quantitative real time PCR, is also presented.

I. INTRODUCTION

Current efforts in gene transfer research focus on the development of genetic drugs capable of treating acquired diseases such as cancer, inflammation, viral infection or cardiovascular disease. The disseminated nature of these diseases requires the development of vector systems capable of accessing distal sites following systemic or intravenous administration. Unfortunately, most vectors have limited utility for systemic applications. Viral vectors, for example, are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver and spleen. In addition, many viruses induce immune responses that compromise potency upon subsequent administration. In the case of most non-viral vectors such as plasmid DNA-cationic lipid complexes (lipoplexes), the large size and positively charged nature of these systems also results in rapid clearance upon systemic administration with the highest expression levels observed in first-pass organs, particularly the lungs (Huang and Li, 1997; Hofland *et al.*, 1997; Templeton *et al.*, 1997; Thierry *et al.*, 1995). In addition, lipoplexes often give rise to significant toxicities both *in vitro* and *in vivo* (Harrison *et al.*, 1995; Li and Huang, 1997; Tousignant *et al.*, 2000, 2003). In spite of these limitations, non-viral gene transfer systems offer specific clinical and commercial advantages as therapeutics. Because non-viral systems use synthetic or highly purified components, they are chemically defined and free of adventitious agents. Non-viral systems can be manufactured under controlled conditions, relatively unconstrained by the biological considerations that define the scale-up of viral production in mammalian cell culture. These advantages have encouraged a number of investigators to focus on the development of non-viral gene transfer systems that have utility in a systemic context (Dzau *et al.*, 1996; Li and Huang, 1997; Templeton *et al.*, 1997; Wheeler *et al.*, 1999; Zhu *et al.*, 1993). Here we will describe one system that specifically attempts to address the inability of current vector systems to overcome the first barrier to systemic gene delivery, delivery to the disease site and the target cell.

II. PROPERTIES OF A PLASMID DELIVERY SYSTEM FOR THE TREATMENT OF SYSTEMIC DISEASE

A. Definition of an appropriate vector

We propose the following definition of an ideal carrier for systemic gene transfer: The ideal vector will (i) be safe and well tolerated upon systemic administration;

(ii) have the appropriate pharmacokinetic attributes to ensure delivery to disseminated disease sites; (iii) deliver intact DNA to target tissue and mediate transfection of that tissue; (iv) be non-immunogenic; and (v) be stable upon manufacture to facilitate production at commercial scale with uniform, reproducible performance specifications.

Gene-based drugs must maximize the benefit to patient health while minimizing the risks associated with treatment. Accordingly, gene transfer systems must be safe and well tolerated. Attempts to bypass the inherent pharmacology of a given vector by invoking elaborate or invasive treatment methodologies are likely to result in an increased, potentially unacceptable, risk to the patient. Methods such as 'hydrodynamic injection' or direct portal vein infusion may continue to generate exciting preclinical results, but translation of these methods to a clinical setting will be limited. Gene-based drugs will be adopted more readily if they can be delivered in a manner analogous to conventional medicines, for example by intravenous injection or in oral form.

The toxicity associated with systemic administration of poorly tolerated compounds is exacerbated by accumulation in non-target tissue and can be reduced by optimizing delivery to the target site. In the case of gene-based drugs, 'delivery' is determined by physical and biochemical properties including stability, size, charge, hydrophobicity, interaction with serum proteins and non-target cell surfaces, as well as the mechanism of action of the nucleic acid payload. In the context of a disease site, effective delivery requires that a vector overcome obstacles associated with heterogeneous cell populations that are often proliferating rapidly, at different stages of the cell cycle and not conforming to the patterns of organization established during the development of normal tissue. As demonstrated in this work, these challenges, and other potential barriers to transfection, can represent opportunities for conferring a degree of selectivity greater than that associated with the use of conventional therapeutics.

B. Overcoming the barriers to transfection

The barriers to transfection include the pharmacological barriers inhibiting delivery to the target cell, and the intracellular barriers that inhibit nuclear delivery and expression of the plasmid DNA construct. An effective delivery system must be able to confer stability to the nucleic acid payload in the blood despite the presence of serum nucleases and membrane lipases. Systemic delivery requires the use of a 'stealthy' delivery system, since indiscriminate interaction with blood components, lipoproteins or serum opsonins, can cause aggregation before the carrier reaches the disease site. This is especially important in the case of systemic delivery systems containing large polyanionic molecules such as plasmid DNA, which have a greater potential for inducing toxicity through interaction with complement and coagulation pathways (Chonn *et al.*, 1991).

Other barriers to gene delivery may include the microcapillary beds of the "first pass" organs, the lung and the liver, and the phagocytic cells of the reticuloendothelial system. Accessing target cell populations requires extravasation from the blood compartment to the disease site. Carriers of appropriate size can pass through the fenestrated epithelium of tumor neovasculature and accumulate at the tumor site via the "enhanced permeation and retention" (EPR) effect (Mayer *et al.*, 1990), also referred to as "passive" targeting or "disease site" targeting. In order to take advantage of the EPR effect, which can result in accumulation of up to 10% of the injected dose per gram of tumor tissue, the gene carriers must be small (diameter on the order of 100 nm) and long-circulating (circulation lifetimes of 5 h or more following intravenous injection in mice). Clearly, nucleic acids require pharmaceutical enablement in the form of appropriate carriers that confer: protection from degradation, an extended circulation lifetime, appropriate biodistribution and delivery facilitation of the nucleic acid payload to the disease site.

While delivery of intact plasmid DNA to a target cell is a *prerequisite*, it in no way *guarantees* transfection. Once at the cell surface, 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, protected by the carbohydrate coating, or glycocalyx, formed by the post-translational glycosylation of transmembrane proteins. 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. This occurs through a number of discrete pathways, reviewed elsewhere in this volume. While there is some evidence that non-viral vectors may be taken up by caveolae, syndecan-mediated endocytosis or other clathrin-independent pathways, the classical endocytic pathway involves the activity of cell surface clathrin-coated pits, invaginations in the plasma membrane that are subsequently pinched off into the cytoplasm (Goldstein *et al.*, 1985). When this occurs, internalized material remains trapped on the exoplasmic side of the internalized vesicle, without direct access to the cytoplasm or the nucleus. Endocytic vesicles undergo a series of biochemical changes that represent escape opportunities for a non-viral vector. The first such change occurs within 5 min of uptake as internalized vesicles form the early endosome containing the "Compartment of Uncoupling of Receptor and Ligand" (CURL) (Geuze *et al.*, 1983). Early endosomes are transiently fusogenic (Dunn and Maxfield, 1992) with a pH close to that of the exoplasm, while late endosomes have a significantly lower luminal pH (Murphy *et al.*, 1993). As endosomes mature to form lysosomes they experience a further decrease in internal pH and an increase in fusogenicity.

Although the process of clatharin-dependent endocytosis has been well characterized, the processing and release of internalized non-viral vectors or their DNA payload is not well understood. Even less clear is the relative import of clathrin-independent uptake through mechanisms that share some, but not all of the features of the classical pathway. Improvements in our understanding of these alternative pathways, and their role in non-viral gene transfer, will be important for the rational design of more effective intracellular delivery strategies for non-viral vectors.

Following uptake, plasmid DNA spends some indeterminate residency time in the cytoplasm prior to gaining entry to the nucleus. Unlike viral systems that have evolved specific mechanisms to traverse this barrier, untargeted non-viral vectors rely on diffusion to facilitate interaction with the nuclear envelope (Kopatz *et al.*, 2004). However the cytoplasm, rather than an empty space, is a highly organized compartment containing networks of cytoskeletal elements and membrane-bound organelles that have the potential to interact with and accumulate vector systems that arrive at the cytosol intact. When plasmid DNA is delivered by direct microinjection into the cytosol of mammalian cells it is rapidly degraded by divalent-cation-dependent cytosolic nucleases (Howell *et al.*, 2003; Lechardeur *et al.*, 1999). This has implications for vector design. Vector systems that either protect the DNA payload from degradation following endosome release or effectively minimize the cytoplasmic residency time are to be expected to yield improved transfection efficiencies.

The final physical barrier to transfection is delivery to the nucleus. The nucleus has evolved as a means of organizing, isolating and protecting the genome of eukaryotic cells from adventitious agents such as viruses or transposons. The nuclear uptake of DNA is limited by the presence of an intact nuclear envelope and as such non-viral transfection is considerably more efficient in highly mitotic cells (Mortimer *et al.*, 1999; Wilke *et al.*, 1996). Strategies to overcome this barrier to transfection 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 quiescent cells by using either nuclear targeting technologies or condensing agents that compact plasmid DNA to a size more amenable to uptake through the nucleopore complex (Blessing *et al.*, 1998; Sebestyen *et al.*, 1998).

C. Proposed mechanism of stabilized plasmid lipid particle mediated transfection

1. Delivery to the target cell

The demands imposed upon vectors used for systemic applications are conflicting. First, the carrier must be stable and long-circulating, circulating long enough to facilitate accumulation at disease sites via the EPR effect.

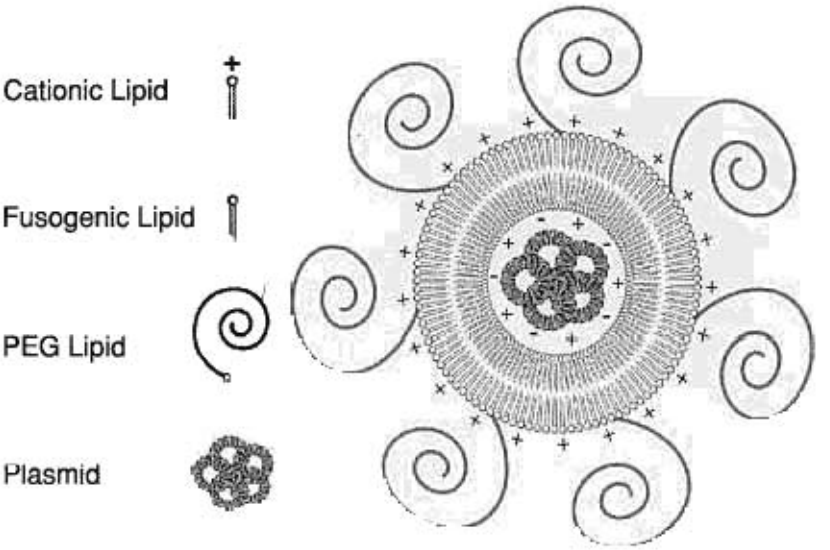

Figure 6.1. Structure of stabilized plasmid lipid particles.

Second, the carrier must interact with—and be taken up by—target cells following arrival at the target site in order to facilitate gene expression. The "stabilized plasmid-lipid particle" (SPLP) attempts to satisfy both of these requirements.

SPLPs consist of a single plasmid encapsulated in a lipid bilayer containing a *diffusible* polyethylene glycol (PEG)-lipid conjugate (Fig. 6.1). The PEG-lipid conjugates in the SPLP play an essential role during the formulation process, stabilizing the nascent particle and preventing aggregation of the particles in the vial. In the blood, the PEG-lipid shields the positive surface charge, preventing rapid clearance following intravenous injection. Following administration, at 37°C and in the presence of sufficient lipid sink, the PEG conjugate dissociates from the SPLP, revealing the positive charge and an increasingly fusogenic lipid bilayer, transforming the particle into a transfection-competent entity. The residency of the PEG conjugate in the SPLP bilayer is determined by the length of the lipid anchor. PEGs with shorter lipid anchors, such as ceramide-C_8 or dimyristoyl-glycerol, dissociate more quickly from the bilayer, quickly 'activating' the SPLP into which they are incorporated. As a result, particles incorporating PEG-lipids with shorter lipid anchors show higher transfection potency *in vitro* than those containing longer lipid anchors (e.g., ceramide-C_{20} or distearoyl-glycerol) (Mok *et al.*, 1999). When injected systemically, PEG conjugates with a larger, more securely fastened anchor and will confer greater stability and extended circulation lifetimes, leading to greater levels of accumulation at disseminated disease sites (Monck *et al.*, 2000; Tam *et al.*, 2000).

2. The role of cationic lipids in promoting intracellular delivery

While the factors facilitating intracellular delivery of non-viral vectors are poorly understood, it is believed that both polycation and cationic lipid-containing systems function, at least in part, by coating plasmid DNA with a positive charge that enables binding of the DNA complex to anionic cell surface molecules, such as cell surface proteoglycans that appear to facilitate transfection both *in vitro* (Mislick and Baldeschwieler, 1996) and *in vivo* (Mounkes *et al.*, 1998). Inhibition of the interaction between the positively charged lipoplex and negatively charged cell surface molecules by pretreatment with polyanionic compounds greatly inhibits lipoplex-mediated transfection while having no effect on electroporation or adenoviral transfection. Intravenous administration of heparinase I, an enzyme specific for the cleavage of heparan sulfate proteoglycans, also inhibits cationic lipoplex-mediated transfection. 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 could yield differences in transfection efficiency. This has certain implications for the design of vector systems for systemic gene therapy. SPLP are transiently charge shielded due to the incorporation of diffusible PEG-lipids. As the PEG-lipid leaves the particle in the blood compartment, the positive charge conferred by the cationic lipid component is revealed. Although systems with a lower surface charge might be expected to benefit from increased circulation time, incorporation of additional cationic lipid in the SPLP lipid bilayer yields an appreciable gain in potency *in vitro* (Zhang *et al.*, 1999).

It is also believed that cationic lipids play a direct role to facilitate intracellular delivery following internalization in the endosome. The proposed mechanism involves the ability of cationic lipids to promote formation of the H_{II} phase in combination with anionic lipids (Hafez *et al.*, 2001), thereby destabilizing the bilayer structure of the endosomal membrane, encouraging fusion with the SPLP bilayer and facilitating the cytoplasmic translocation of the associated plasmid DNA. Clearly, the cationic lipid content of systemic carrier systems must be optimized with a view towards achieving both an extended circulation lifetime and effective intracellular delivery. In particular, a compromise must be made between incorporating high amounts of cationic lipid, which facilitates transfection and the fact that high amounts of cationic lipid result in shorter circulation lifetimes, reducing the amount of the material that arrives at the disease site.

3. 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 (Farhood *et al.*, 1995; Felgner *et al.*, 1994; Gao and Huang, 1995; Hui *et al.*, 1996). Inclusion of lipids, such as unsaturated

phosphatidylethanolamines like dioleoylphosphatidylethanolamine (DOPE), promote destabilization of the lipid bilayer and fusion (Farhood *et al.*, 1995; Hui *et al.*, 1981; Litzinger and Huang, 1992). The fusogenicity of DOPE-containing bilayers is thought to be due to their polymorphic nature. Upon formulation, most lipids adopt the bilayer-forming Lamellar Phase (L_α), while DOPE has a tendency to form the inverse hexagonal (H_{II}) phase (Cullis PR and B., 1978; Koltover *et al.*, 1998). Several researchers have noted that increasing the degree of unsaturation of the lipid hydrophobic domain increases the affinity for the H_{II} phase (Cullis PR and B., 1979; Dekker *et al.*, 1983; Epand *et al.*, 1991; Sankaram *et al.*, 1989; Szule *et al.*, 2002). As a result, the fusogenicity of an SPLP bilayer can be increased by increasing the degree of unsaturation in the hydrophobic domain of either the helper lipid or cationic lipid components (Heyes *et al.*, 2004). Furthermore, certain cationic lipids can function in the absence of fusogenic helper lipids, either alone (Felgner *et al.*, 1994; Gao and Huang, 1995) or in the presence of the non-fusogenic lipid cholesterol (Liu *et al.*, 1995).

The specific role of fusogenic helper lipids in the transfection process, and whether this role is conserved between lipoplex and systems such as SPLP which fully encapsulate plasmid DNA, is not clear. Membrane fusion events could theoretically occur at a number of different stages in the gene delivery process, either at the plasma membrane, endosome or nuclear envelope. In order for fusion with the plasma membrane to occur, positively charged lipid particles must first bypass the negatively charged 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 lipoplex fusion events may resolve with plasmid DNA, formerly attached to the cationic liposome surface, deposited on the outside surface of the plasma membrane. Encapsulated systems differ from lipoplex in this respect. Fusion with the plasma membrane could result in an encapsulated carrier delivering its contents into the cytosol. However, the bulk of both lipoplex- and SPLP-mediated transfection is thought to be by fusion with the endosomal membrane of particles that are taken up intact by endocytosis (Wrobel and Collins, 1995). There is considerable biochemical evidence to support an endosomal route for internalized plasmid DNA. One example is the transient inhibition of endocytosis and concomitant transfection upon treatment of cells with cytochalasin-B, an inhibitor of actin polymerization required in the endocytic process (Hui *et al.*, 1996). Another example utilizes fluorescently labeled lipids to track the fate of lipoplex or SPLP upon delivery to the cell. Fusion of labeled liposomes with the plasma membrane would result in the transfer of lipid label to the membrane. Cells exposed to lipoplex or SPLP containing rhodamine-phosphatidyl-ethanolamine accumulate fluorescent label in endocytic granules, well before plasma membranes become fluorescent (Hui *et al.*, 1996; Palmer *et al.*, 2003). In the absence of a

fusion-induced translocation event, fusion of lipoplex systems with endosomal compartments results in a gradual destabilization and disruption of the endosomal membrane. Encapsulated systems have an advantage over lipoplex in that a single fusion event within an endosomal compartment would be expected to result in efficient delivery of the DNA payload to the cytosol.

The role of fusogenic lipids *in vivo* remains unclear. A number of investigators have reported that replacement of fusogenic DOPE with the less fusogenic lipid cholesterol yields higher levels of gene expression upon systemic administration of either lipoplex or encapsulated systems (Sakurai *et al.*, 2001; Templeton *et al.*, 1997). However, it is important to distinguish the effect of helper lipids on biodistribution from the effect on intracellular delivery. The enhanced gene expression observed upon incorporation of cholesterol in lipoplex or SPLP 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, lipoproteins and the fixed and free macrophages of the mononuclear phagocyte system while in the blood compartment, leading to rapid clearance and decreasing the proportion of carriers that reach target tissue. Incorporation of cholesterol may simply render vectors less promiscuous and thereby improve delivery to the target cell. The implication is that there is further rationale for transiently shielding the fusogenic potential of systemic carriers through the use of diffusible PEG-lipids.

A variety of approaches can be considered for enhancing the endosomal release of internalized liposomes. In addition to the use of fusogenic lipids that are thought to facilitate endosome release, another strategy involves the incorporation of specific lipids that render the liposome pH-sensitive such that it becomes more fusogenic in low pH compartments such as the late endosome and lysosome (Wang and Huang, 1987, 1989; Lee and Huang, 1996). One example of this approach utilizes titratable cationic lipids that become positively charged at the reduced pH values that may be encountered in endosomes. Cationic lipids such as 1,2-dioleoyl-3-(N,N-dimethylamino)propane (AL1) that exhibit pK of approximately 6.6 (Bailey and Cullis, 1994) confer no significant positive charge to carriers at neutral pH yet are fully positively charged at the pH values commonly encountered in endosomes.

4. Nuclear delivery

Attempts to improve the nuclear uptake of plasmid DNA must take into consideration the physical constraints of the nucleopore complex that mediates the uptake of plasmid DNA into the intact nucleus. When fully condensed by monovalent detergent counterions, a 5.5 kb supercoiled plasmid DNA molecule becomes a sphere of about 25 nm in diameter (Blessing *et al.*, 1998) while the

passive diffusion channel of the nuclear pore complex has an internal diameter of 9 nm (Ohno *et al.*, 1998). 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. Although there does appear to be considerable potential for improving the nuclear uptake of supercoiled plasmids through attachment of nuclear localization peptides or other nuclear import signals, it remains to be seen if this can be accomplished in a manner that is compatible with large-scale formulation and systemic gene delivery (Sebestyen *et al.*, 1998).

III. 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 easily be "loaded" into preformed liposomes using pH gradients or other similar strategies. Lipid encapsulation of high molecular weight DNA was first demonstrated in the late 1970s, prior to the development of cationic lipid-containing lipoplex (Hoffman *et al.*, 1978; Mannino *et al.*, 1979; Mukherjee *et al.*, 1978). Plasmid DNA has subsequently been encapsulated by reverse-phase evaporation (Cudd and Nicolau, 1985; Fraley *et al.*, 1980; Nakanishi *et al.*, 1985; Soriano *et al.*, 1983), ether injection (Fraley *et al.*, 1979; Nicolau and Rottem, 1982), lipid hydration-dehydration techniques (Alino *et al.*, 1993; Baru *et al.*, 1995; Lurquin, 1979) sonication (Jay and Gilbert, 1987; Ibanez *et al.*, 1997; Puyal *et al.*, 1995), spontaneous internalization into pre-formed liposomes (Templeton *et al.*, 1997) and others methods (Monnard *et al.*, 1997; Szelei and Duda, 1989) (summarized in Table 6.1). Early attempts to encapsulate plasmid DNA yielded mostly large multilamellar vesicles with poor transfection efficiency (Baru *et al.*, 1995; Nicolau *et al.*, 1983; Scaefer-Ridder *et al.*, 1982), while more recently, improvements in formulation technology have resulted in the production of cationic lipid-containing particles with a much greater transfection potential. SPLP initially utilized detergent dialysis, a process in which unilamellar vesicles are formed upon removal of detergent from a DNA:lipid solution. While early efforts to encapsulate plasmid DNA using detergent dialysis yielded low encapsulation efficiencies (Fraley *et al.*, 1979; Nakanishi *et al.*, 1985), these results were significantly improved upon through the use of PEG lipids to stabilize the vesicles during the formulation process (Wheeler *et al.*, 1999). In this way plasmid-containing cationic liposomes are stabilized in a manner analogous to PEGylated liposomal drug formulations that exhibit extended circulation lifetimes (Allen and Chong, 1987; Klibanov *et al.*, 1990; Needham *et al.*, 1992; Papahadjopoulos *et al.*, 1991; Wu *et al.*, 1993). PEG

conjugates sterically stabilize liposomes by forming a protective hydrophilic layer that shields the hydrophobic lipid layer, preventing the association of serum proteins and resulting uptake by the reticuloendothelial system (Gabizon and Papahadjopoulos, 1988; Senior *et al.*, 1991). Although this approach has been investigated with a view towards improving the stability and pharmacokinetics of lipoplex (Hong *et al.*, 1997), lipoplex incorporating PEG-lipids systems suffer from the heterogeneity common to most complexes of plasmid DNA and cationic lipid.

The detergent dialysis method of plasmid encapsulation involves the simultaneous solubilization of hydrophobic (cationic and helper lipid) and hydrophilic (PEG lipid and plasmid DNA) components in a single detergent-containing phase (Fenske *et al.*, 2002;Wheeler *et al.*, 1999). Particle formation occurs spontaneously upon removal of the detergent by dialysis. This technique can result in the formation of small (approximately 100 nm diameter) 'stabilized plasmid lipid particles' (SPLP) containing one plasmid per vesicle in combination with optimized plasmid trapping efficiencies approaching 70%. 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 of >5 mg/ml. These attributes compare favorably with the previously reported plasmid encapsulation processes (Table 6.1). The SPLP method yields the highest plasmid DNA-to-lipid ratio of any method and SPLP are remarkably stable when compared to other encapsulated systems. Although the detergent dialysis process results in 30–50% unencapsulated DNA, free plasmid DNA can be removed by simple ion-exchange chromatography.

Although SPLP shows considerable potential as systemic gene transfer agents, the detergent dialysis method suffers from a number of limitations. Detergent dialysis is exquisitely sensitive to minor changes in the ionic strength of the formulation buffer. Changes as small as 10 mM result in dramatic decrease in encapsulation efficiency (Fenske *et al.*, 2002). Even when SPLP are formed under ideal conditions, the detergent dialysis method results in the formation of large numbers of empty vesicles that are usually separated from SPLP by gradient ultracentrifugation (Fenske *et al.*, 2002). The detergent dialysis method is also difficult to scale to the size required to support preclinical and clinical development of the technology. For these reasons, alternative methods of preparing stable plasmid lipid particles have been explored.

One such method uses ethanol-destabilized cationic liposomes (Maurer *et al.*, 2001). Though this method does not require gradual detergent removal or ultracentrifugation steps, it does require the formation of cationic vesicles prior to the encapsulation of pDNA. Once cationic liposomes of the desired size have been prepared, they are destabilized by ethanol addition to 40% v/v. Destabilization of vesicles with ethanol requires very slow addition of ethanol to a rapidly

Table 6.1. Procedures for Encapsulating Plasmid in Lipid-Based Systems

Procedure	Lipid composition	Length of DNA	Trapping efficiency[a]	DNA-to-lipid ratio[a]	Diameter
Reverse-phase evaporation (Fraley *et al.*, 1980)	PS or PS:Chol (50:50)	SV40 DNA	30 to 50%	<4.2 μg/μmol	400 nm
Reverse-phase evaporation (Soriano *et al.*, 1983)	PC:PS:Chol (40:10:50)	11.9 kb plasmid	13 to 16%	0.23 μg/μmol	100 nm to μm
Reverse-phase evaporation (Nakanishi *et al.*, 1985)	PC:PS:Chol (50:10:40)	8.3 kb, 14.2 kbp plasmid	10%	0.97 μg/μmol	ND
Reverse-phase evaporation (Cudd and Nicolau, 1985)	EPC:PS:Chol (40:10:50)	3.9 kb plasmid	12%	0.38 μg/μmol	400 nm
Ether injection (Fraley *et al.*, 1979)	EPC:EPG (91:9)	3.9 kb plasmid	2 to 6%	<1 μg/μmol	0.1 to 1.5 μm; Aug = 230 nm
Ether injection (Nicolau and Rottem, 1982)	PC:PS:Chol (40:10:50) PC:PG:Chol (40:10:50)	3.9 kb plasmid	15%	15 μg/μmol	ND
Detergent dialysis (Stavridis *et al.*, 1986)	EPC:Chol:stearylamine (43.5:43.5:13)	sonicated genomic DNA (approximately 250,000 MW)	11%	0.26 μg/μmol	50 nm
Detergent dialysis, extrusion (Wang and Huang, 1987)	DOPC:Chol:oleic acid or DOPE:Chol:oleic acid (40:40:20)	4.6 kb plasmid	14 to 17%	2.25 μg/μmol	180 nm (DOPC) 290 nm (DOPE)
Lipid hydration (Lurquin, 1979)	EPC:Chol (65:35) or EPC	3.9 kb, 13 kb plasmid	ND	ND	0.5 to 7.5 μm
Dehydration-rehydration, extrusion (400 or 200 nm filters) (Alino *et al.*, 1993)	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)

Dehydration-rehydration (Baru *et al.*, 1995)	EPC	2.96 kb, 7.25 kb plasmid	35 to 40%	2.65 to 3.0 μg/μmol	to 2 μm
Sonication (in the presence of lysozyme) (Jay and Gilbert, 1987)	Asolectin (soybean phospholipids)	1.0 kb linear DNA	50%	0.08 μg/μmol	100 to 200 nm
Sonication (Puyal *et al.*, 1995)	EPC:Chol:lysine-DPPE (55:30:15)	6.3 kb ssDNA 1.0 kb dsRNA	60 to 95% ssDNA 80 to 90% dsRNA	13 μg/μmol ssDNA; 14 μg/μmol dsRNA	100 to 150 nm
Spermidine-condensed DNA, sonication, extrusion (Ibanez *et al.*, 1997)	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 to 52%	2.53 to 2.87 μg/μmol	400 to 500 nm
Ca^{2+}-EDTA entrapment of DNA-protein complexes (Szelei and Duda, 1989)	PS:Chol (50:50)	42.1 kbp bacteriophage	52 to 59%	22 μg/μmol	ND
Freeze-thaw, extrusion (Monnard *et al.*, 1997)	POPC:DDAB (99:1)	3.4 kb linear plasmid	17 to 50%	ND	80 to 120 nm
SPLP – Detergent Dialysis (Wheeler *et al.*, 1999)	Various	4.4 to 15 kb plasmid	60 to 70%	62.5 μg/μmol	75 nm (QELS); 65 nm (freeze-fracture)
SPLP – Ethanol Dilution (Jeffs *et al.*, 2005)	Various	4.4 to 15 kb plasmid	80 to 95%	70 μg/μmol	100–150 nm (QELS)

[a]Some values calculated based on presented data.
ND = Not determined.

mixing aqueous suspension of vesicles, to avoid localized areas of high ethanol concentration (>50% v/v) that promote fusion and conversion of liposomes into large lipid structures (Maurer *et al.*, 2001). The addition of plasmid must also be accomplished slowly in a drop-wise manner to the destabilized vesicle. The uncontrolled nature of both the vesicle destabilization and nucleic acid addition steps poses challenges for reproducibly preparing SPLP at a scale suitable for clinical evaluation.

A more simple, robust and fully scalable method for the encapsulation of plasmid DNA in stable plasmid lipid particles has been developed. This method, termed 'stepwise ethanol dilution,' produces SPLP with the same desirable properties as those prepared by detergent dialysis (Jeffs *et al.*, 2005). Lipid vesicles encapsulating plasmid DNA are formed instantaneously by mixing lipids dissolved in ethanol with an aqueous solution of DNA in a controlled, stepwise manner (Fig. 6.2). Combining DNA and lipid flow streams results in rapid dilution of ethanol below the concentration required to support lipid solubility. Using this method, vesicles are prepared with particle sizes less than 150 nm and DNA encapsulation efficiencies as high as 95%. Although analysis by transmission electron microscopy shows that SPLPs prepared by stepwise ethanol dilution are a more heterogenous population of unilamellar, bilamellar and oligolamellar vesicles than those prepared by detergent dialysis, extensive analysis reveals that this more diverse morphology has little effect on the stability or activity of SPLPs *in vitro* or *in vivo*.

The ethanol dilution method represents an effective solution to the issues confounding SPLP preparation by detergent dialysis (Wheeler *et al.*, 1999). The efficiency of the ethanol dilution method obviates the requirement for an ultracentrifugation purification step (Fig. 6.3). Another benefit of the method is that it facilitates the rapid preparation of SPLP samples, allowing formulation development to proceed at a much faster pace. An SPLP formulation can be prepared in a few hours with ethanol dilution, whereas it takes days to prepare SPLP by detergent dialysis (Fenske *et al.*, 2002). Furthermore, the improved method enables the accelerated optimization of formulation composition (e.g., lipid molar ratios) and process parameters (e.g., buffer concentration or pH). The ethanol dilution method has been used to formulate a 4.5 g batch of plasmid DNA under current Good Manufacturing Practices (cGMP) (Fig. 6.4). The properties of SPLP from this batch were identical to batches prepared at 1/10 and 1/100 of this scale using smaller process steps. Until now, an issue plaguing the development of non-viral vectors has been their less than predictable formulation and performance characteristics, particularly when manufactured at large scale. Preparation of SPLP batches at a scale suitable for clinical evaluation or commercialization is possible using this novel approach.

The ability of the ethanol dilution method to rapidly prepare liposomes of desirable size and encapsulate plasmid DNA with high efficiency is thought to

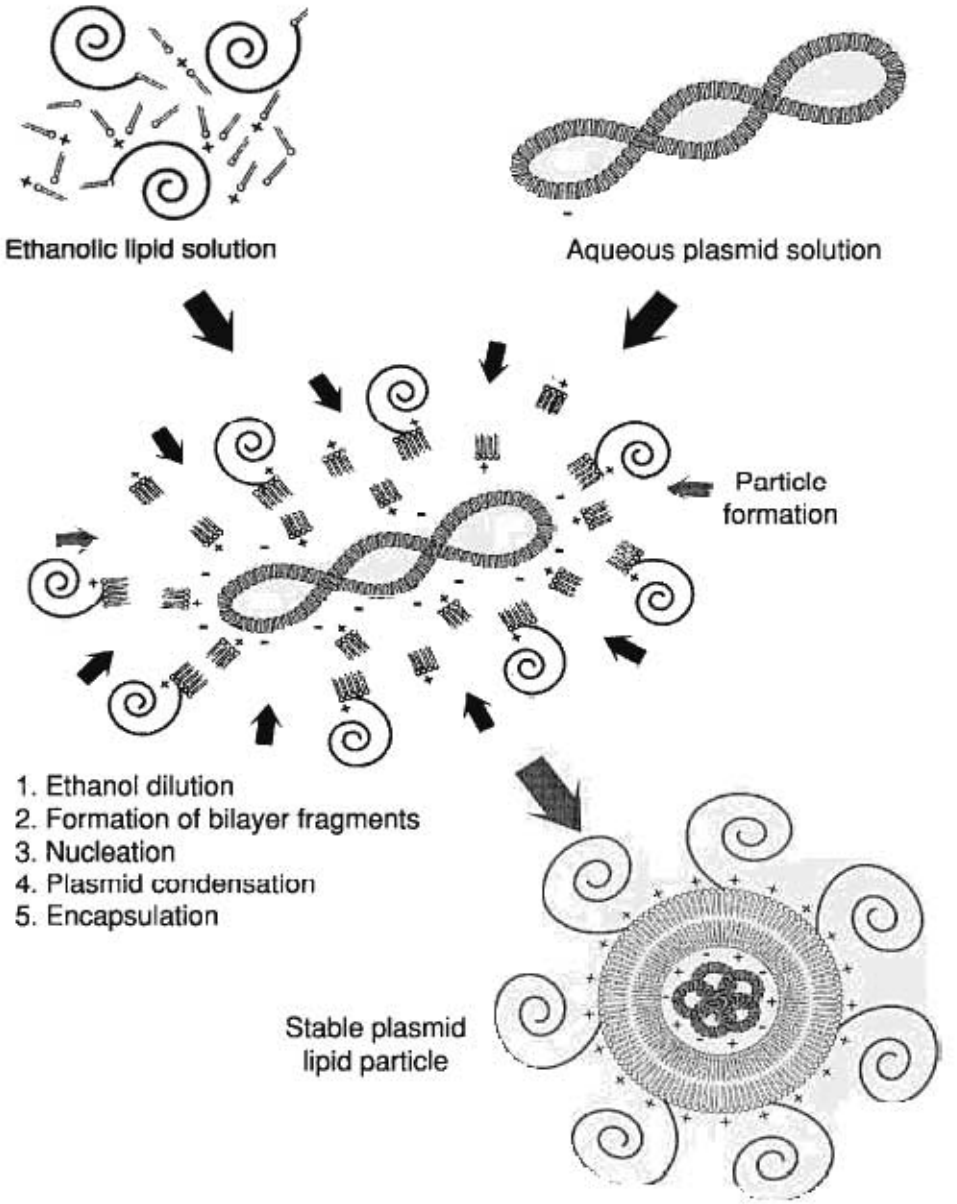

Figure 6.2. A model for spontaneous vesicle formation by ethanol dilution.

result from the precise control of the conditions under which the lipids enter the aqueous environment, self arrange into lipid bilayer fragments and then form liposomes. Several parameters have been shown to be critical for SPLP formation and plasmid encapsulation when using detergent dialysis (Wheeler *et al.*, 1999; Zhang *et al.*, 1999). Ionic strength, cationic lipid and PEG lipid content must be optimized to maximize plasmid entrapment and minimize aggregation or the formation of empty vesicles (Wheeler *et al.*, 1999). 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 to these bilayer

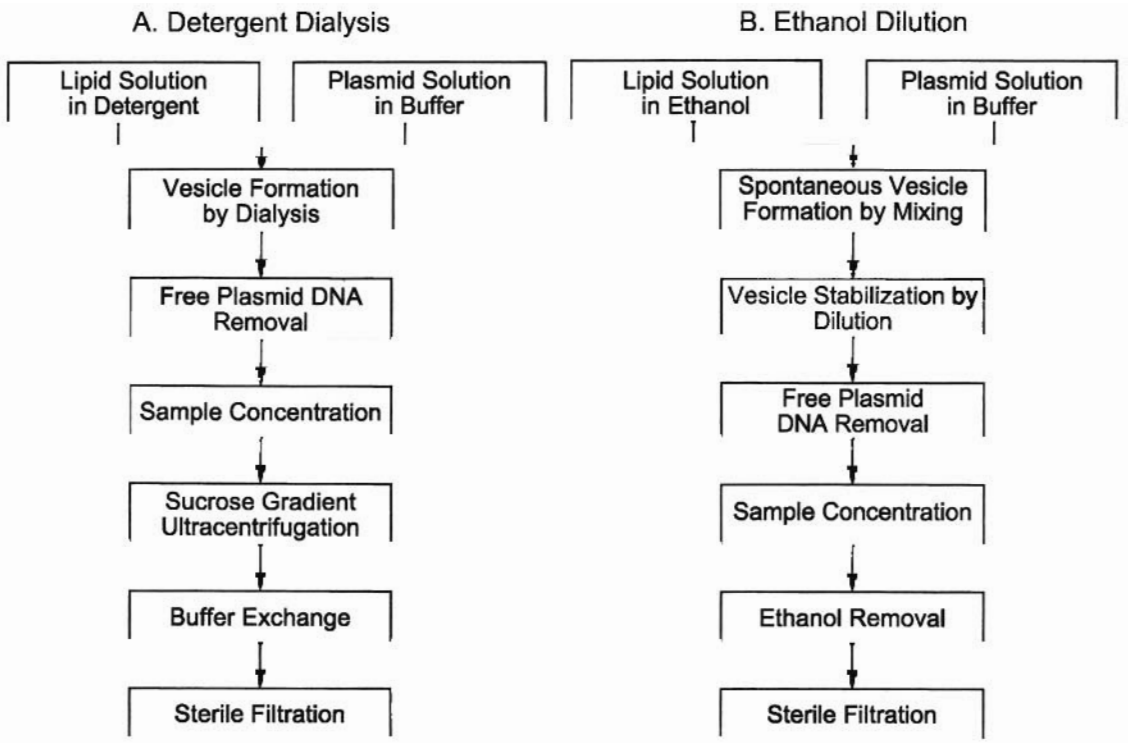

Figure 6.3. Process flow diagram: detergent dialysis vs. spontaneous vesicle formation by ethanol dilution.

fragments by electrostatic attraction. If the cationic lipid content is too low, the 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 additional lipid leads to the formation of vesicles containing encapsulated plasmid (Wheeler *et al.*, 1999). Similar to detergent dialysis, SPLP formation by ethanol dilution is optimized by balancing ionic strength, cationic lipid and PEG lipid content. However the ethanol dilution method appears much more robust than detergent dialysis, with optimal results achieved through a wide range of formulation conditions.

In summary, while a variety of techniques are available for encapsulating plasmid DNA into lipid-based systems, only the SPLP approach—employing PEG-lipid conjugates during formulation—satisfies the demands of generating small (diameter ~100 nm), well-defined (one plasmid per particle) stable systems with high encapsulation efficiencies (>50%) and high plasmid-to-lipid ratios (>0.1 mg plasmid DNA/mg lipid) that exhibit the extended circulation lifetimes required to achieve preferential accumulation at disease sites such as

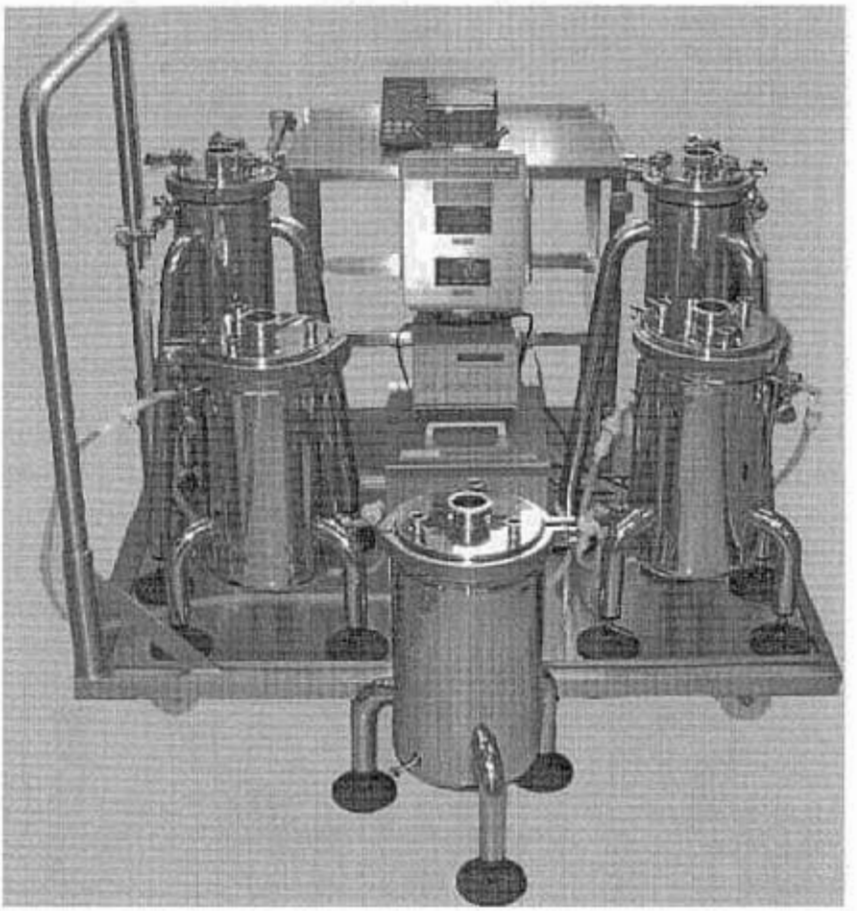

Figure 6.4. Apparatus for the manufacture of clinical grade SPLP.

solid tumors. Among the methods for producing SPLP, the stepwise ethanol dilution approach most adequately satisfies demands related to scalability and reproducibility.

IV. PHARMACOLOGY OF ENCAPSULATED PLASMID DNA

As described above, SPLP were designed to take advantage of the EPR effect whereby small, long-circulating particulate carriers preferentially accumulate at disease sites such as tumors. The importance of such an approach is profound. For example, liposomal vincristine formulations that have these characteristics facilitate the accumulation of 50- to 100-fold higher amounts of drug at a tumor site compared to injection of the same amount of free drug (Boman *et al.*, 1994; Mayer *et al.*, 1993) resulting in significantly improved efficacy (Webb *et al.*, 1995). In the case of SPLP, accumulation of the plasmid carrier at a tumor site contributes to a remarkable and unexpected benefit, in that preferential gene

expression is observed at the tumor site as compared to expression in normal tissues resulting in tumor-selective protein expression. The pharmacokinetics, tumor accumulation and transfection properties of SPLP have been extensively characterized in several murine models and will be described here in more detail (Fenske *et al.*, 2001, 2002; Monck *et al.*, 2000; Tam *et al.*, 2000).

A. Biodistribution following systemic administration of SPLP

Following intravenous injection into mice, the clearance of SPLP can be assessed by lipid and/or DNA markers (Monck *et al.*, 2000; Tam *et al.*, 2000). Previous experience shows that, due to the stability of the SPLP, the lipid and DNA components are cleared from the blood compartment at the same rate and the plasmid DNA remains intact while encapsulated within the SPLP lipid bilayer (Tam *et al.*, 2000). Because the SPLP remains intact in the blood compartment, the biodistribution of a non-exchangeable lipid marker (Stein *et al.*, 1980) incorporated into an SPLP is representative of the biodistribution of the entire particle, including the plasmid DNA component, at early time points. This finding is applied to analysis of SPLP clearance and biodistribution up to 24 h after administration.

An example of the clearance of an SPLP from the circulation of tumor-bearing mice is shown in Fig. 6.5. SPLP was formulated containing DSPC, cholesterol, DODMA and PEG-disterylglycerol (20:55:15:10 mol%, respectively), encapsulating plasmid DNA containing the luciferase reporter

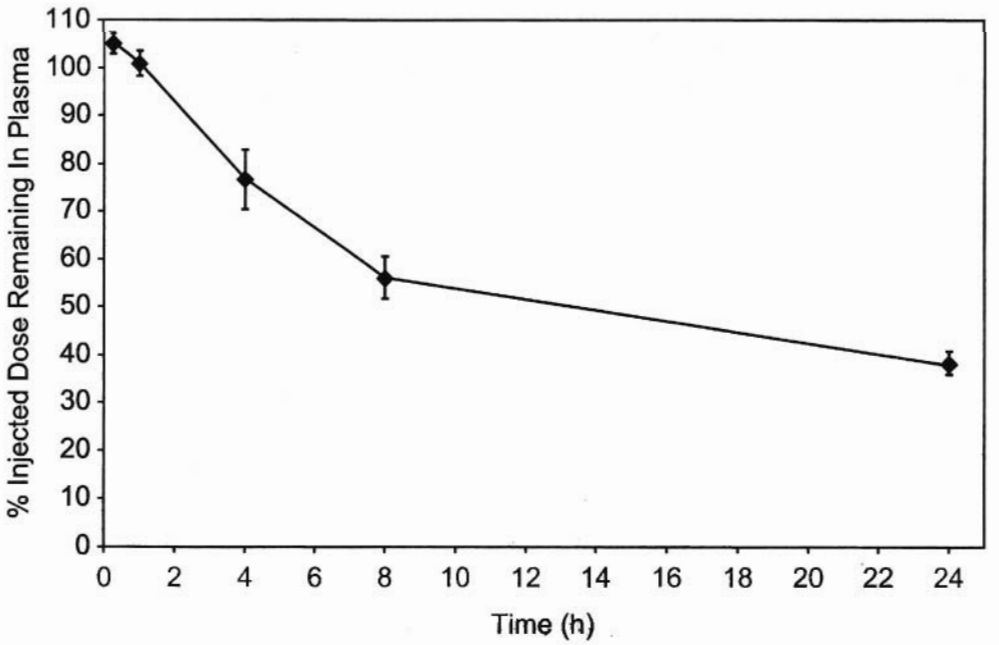

Figure 6.5. Plasma clearance of SPLP following a single intravenous administration in neuro-2a tumor-bearing male A/J mice.

gene. Trace amounts of radiolabelled lipid marker ([^{3}H]-cholesteryl hexadecyl ether (CHE)) were included in the SPLP to quantify SPLP in the plasma and tissue samples.

SPLP was administered intravenously and blood was subjected to analysis for ^{3}H-CHE lipid. Twenty-four hours after intravenous administration of SPLP, forty percent of the injected dose remains in the plasma with a half-life of 13 h. This result is typical of numerous such experiments in normal and tumor-bearing mice (Monck *et al.*, 2000). SPLP, regardless of the DNA encapsulated, remain in the circulation for many hours with 15–40% of the injected dose in circulation at 24 h following injection. The serum half-life of unprotected plasmid DNA is known to be <5 min (Monck *et al.*, 2000; Thierry *et al.*, 1997).

The extended circulation of SPLP results in the accumulation of particles in tumors following intravenous administration. The accumulation of SPLP at a distal Neuro-2a tumor site is shown in Fig. 6.6. The amount of SPLP delivered to the tumor is substantial, in this experiment corresponding to >8% of the total injected dose per gram of tumor at 24 h. This result is also typical of numerous experiments in tumor-bearing mice. SPLP, regardless of the DNA encapsulated, achieve significant levels of accumulation, with 8–15% of the injected dose per gram of tumor at 24 h following injection (Fenske *et al.*, 2001, 2002; Monck *et al.*, 2000; Tam *et al.*, 2000).

In addition to tumor accumulation, the biodistribution of SPLP in various other tissues has been studied. The accumulation of SPLP in the tumor, liver, spleen, lymph nodes and small intestine increases in the first 24 h after

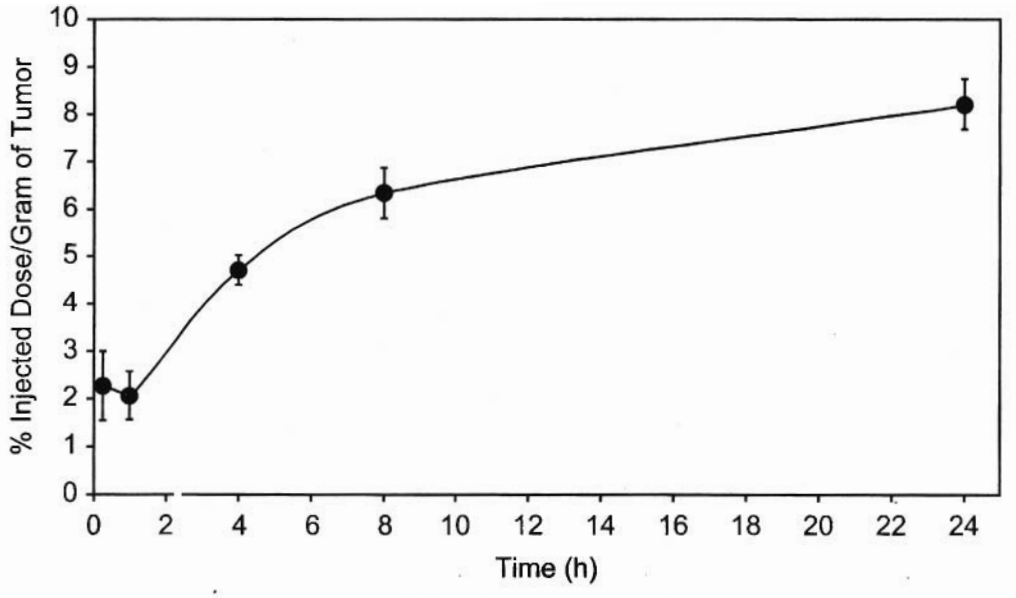

Figure 6.6. Tumor accumulation of SPLP following a single intravenous administration in neuro-2a tumor-bearing male A/J mice.

Table 6.2. Biodistribution of [^{3}H]-CHE Labeled SPLP Following A Single Intravenous Administration in Neuro-2a Tumor-Bearing Male A/J Mice. Data is expressed as percent injected dose per gram of tissue. SEM is the standard error of the mean

Tissue	0.25 h	1 h	4 h	8 h	24 h
Spleen	7.94 ± 2.70	10.6 ± 1.86	10.1 ± 0.95	0.97	44.1 ± 1.28
Liver	4.16 ± 0.30	6.18 ± 0.90	10.7 ± 1.01	19.0 ± 1.90	25.8 ± 2.67
Adrenal Glands	7.92 ± 1.99	11.5 ± 1.92	20.8 ± 2.25	19.9 ± 3.88	14.1 ± 0.99
Small Intestine	1.92 ± 0.42	2.65 ± 0.40	5.35 ± 1.58	8.60 ± 1.20	12.4 ± 1.61
Tumor	2.25 ± 0.74	2.05 ± 0.51	4.70 ± 0.31	6.32 ± 0.52	8.19 ± 0.54
Mesenteric L.N.	1.56 ± 0.24	2.19 ± 0.27	5.03 ± 0.55	6.85 ± 1.65	7.23 ± 0.80
Thymus	9.31 ± 1.38	4.87 ± 0.54	4.28 ± 0.87	4.87 ± 0.15	5.72 ± 1.90
Large Intestine	1.81 ± 0.27	1.46 ± 0.14	1.86 ± 0.46	2.31 ± 0.56	4.47 ± 0.75
Kidneys	9.41 ± 1.84	9.81 ± 0.37	7.17 ± 0.48	7.36 ± 1.09	4.27 ± 0.13
Lungs	3.76 ± 1.27	5.10 ± 1.91	4.21 ± 1.96	4.62 ± 1.46	4.14 ± 0.39
Heart	5.02 ± 1.03	3.88 ± 0.43	4.23 ± 1.03	3.14 ± 0.45	3.54 ± 0.35
Bone Marrow	1.77 ± 0.21	2.10 ± 0.22	2.54 ± 0.20	3.23 ± 0.24	2.44 ± 0.41
Testes	0.87 ± 0.11	0.95 ± 0.21	0.82 ± 0.26	1.02 ± 0.23	1.65 ± 0.20
Brain	1.49 ± 0.20	0.89 ± 0.06	0.97 ± 0.23	0.69 ± 0.08	0.60 ± 0.08

administration. The amount of SPLP in other tissues, including the bone marrow, testes, and the brain, decreases or is maintained at low levels during this period (Table 6.2). The spleen and liver consistently demonstrate the highest levels of SPLP accumulation (44% and 26% of the injected dose per gram, respectively); whereas, the testes and brain accumulate the least amount of SPLP (Fig. 6.7).

To determine the pattern of biodistribution and rate of clearance of the plasmid DNA component of SPLP, pharmacokinetics have been evaluated based upon the detection of the plasmid DNA by quantitative real-time polymerase chain reaction (QPCR). This approach allows for analysis at later timepoints than would be appropriate using a lipid label. It also allows for the determination of intact plasmid payload, rather than a lipid marker for the SPLP.

Peak levels of plasmid accumulation occur on Day 1, 24 h after intravenous administration of SPLP. The greatest concentration of plasmid at this time is found in the blood (Fig. 6.8). At 6.5×10^{10} copies/μg, plasmid DNA accounts for greater than one-third of the total DNA extracted from whole blood (corresponding to a ratio of 300,000 plasmid copies per diploid cell). Plasmid, in both supercoiled and open circular conformations, was observed upon conventional electrophoretic analysis of DNA extracted from blood

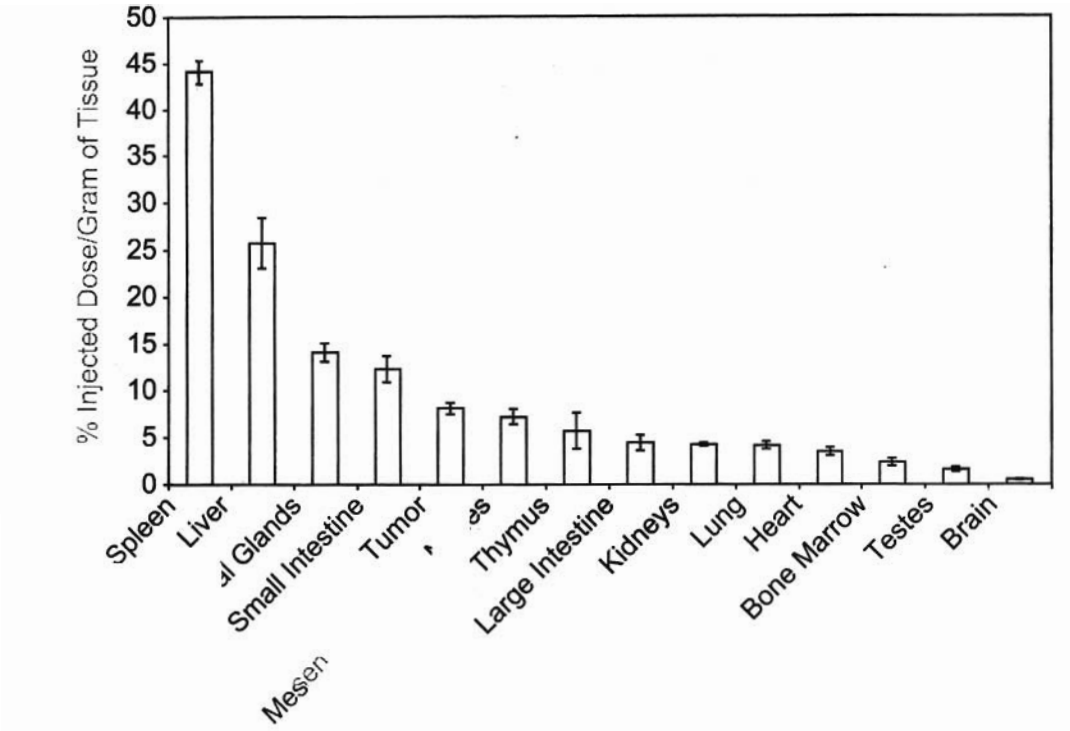

Figure 6.7. Biodistribution of [^{3}H]-CHE labeled SPLP following a single intravenous administration in neuro-2a tumor-bearing Male A/J mice.

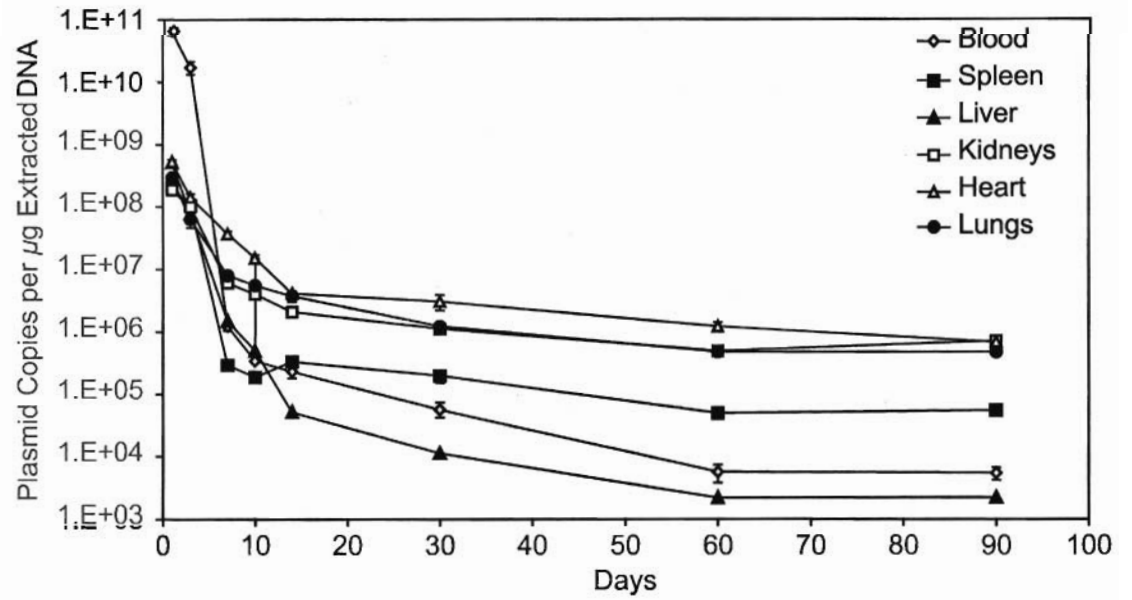

Figure 6.8. Clearance of plasmid DNA from the blood and first pass organs after SPLP administration in A/J mice.

samples. Plasmid concentrations in tumor and non-target tissues are generally two to three orders of magnitude lower than in blood, with the brain accumulating the least plasmid DNA at four orders of magnitude lower than in blood. Differences in plasmid concentration between male and female tissues are found only in the brain, femur (bone marrow) and reproductive organs. The target tissue in this test system is the subcutaneous Neuro-2a tumor. Tumor tissue is found to accumulate roughly the same relative plasmid concentration as the liver on Day 1, however, plasmid clearance from the tumor is much slower than from non-target tissues and blood (Fig. 6.9). By Day 7, plasmid concentration in tumor tissue has decreased, on average, 5.5-fold; whereas it has declined at least 21-fold and, on average, 196-fold in the non-target tissues. At Day 10, the most extended time-point for tumor-bearing mice, the tumor plasmid concentration has decreased, on average, 60-fold, compared to an average decline of 320-fold in the non-target tissues. Comparison of plasmid distribution in tumor-bearing and non-tumored animals shows that the pattern of tissue distribution is qualitatively similar yet plasmid concentrations in blood and non-target tissues of non-tumored animals are generally found to be higher than in the tumor-bearing animals (Lee and MacLachlan, 2004).

Ninety days after administration, plasmid concentrations in blood are 5.4×10^3 copies/μg, corresponding to 1–2 copies per 100 diploid cells (Fig. 6.8). The liver, followed by blood and brain tissue, contains the least plasmid DNA. The tissue containing the highest concentration of plasmid DNA after ninety days is the heart at 6.7×10^5 copies/μg, corresponding to 2 copies per diploid cell.

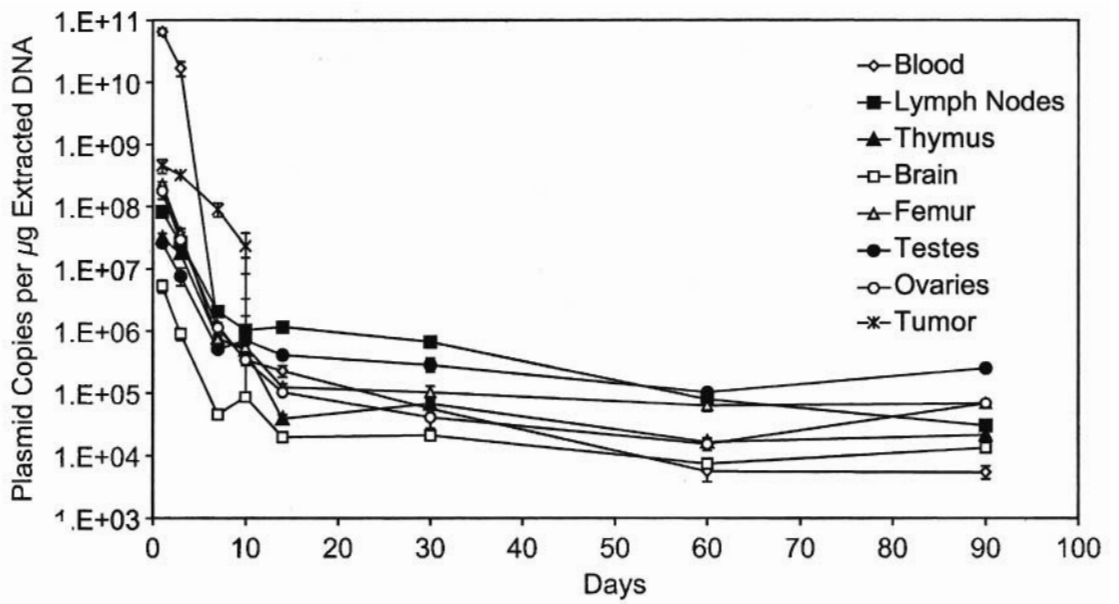

Figure 6.9. Clearance of plasmid DNA from the tumor and other tissues of interest after SPLP administration in A/J mice

This represents a decrease of less than three orders of magnitude from peak levels observed on Day 1, the slowest rate of clearance of all the tissues assayed.

The greatest change in plasmid concentration occurred in the blood compartment (decreasing seven orders of magnitude, Fig. 6.8) followed by liver tissue (decreasing five orders of magnitude), whereas plasmid levels decreased less than 1,000-fold between Day 1 and Day 90 in the kidneys, heart, lungs, brain and testes. The remaining analyzed tissues (lymph nodes, thymus, spleen, femur and ovaries) displayed moderate decreases in plasmid concentration of four orders of magnitude or less over the time period examined.

The rate of SPLP plasmid DNA clearance, from all tissues, is initially rapid between Day 1 and Day 7, and then slows considerably beyond Day 14. This pattern is reminiscent of that expected in a two-compartment model of pharmacokinetics. Two-compartment models assume that a drug, in this case plasmid DNA, distributes between two compartments, moves between the two compartments in proportion to its concentration, and is eliminated from the first compartment in proportion to its concentration (Fig. 6.10). Two-compartment models are often used to describe the behavior of a drug as it is distributed from a more accessible compartment, such as the systemic circulation or the more perfused tissues, to less perfused tissues such as the adipose tissue, skin or brain. In the case of SPLP, each individual tissue exhibits two-compartment behavior. One may speculate that each tissue contains a less accessible compartment, perhaps intracellular, that acts to sequester plasmid DNA delivered by SPLP and that this remarkably stable compartment is responsible for the more gradual clearance observed at later time-points. However, this is purely speculation. In compartmental analysis, the term 'compartment' refers to a mathematically distinct kinetic pool that does not necessarily correspond to any physical location or process. It remains to be determined experimentally if the second compartment responsible for the long-term sequestration of SPLP plasmid DNA following the initial distribution phase is a real physical compartment or merely a mathematical construct.

The extent of plasmid DNA distribution in tissues following SPLP administration is markedly greater than has been observed in other non-viral gene delivery systems (Lew *et al.*, 1995). This can be attributed to the extended blood circulation lifetimes of SPLP formulations and their ability to protect encapsulated plasmid DNA from degradation, greatly extending the available timeframe for plasmid delivery to—and accumulation within—tissues. While SPLP formulations provide plasma half-lives for intact plasmid DNA of 7 to 13 h (Tam *et al.*, 2000), 'naked' DNA and cationic lipoplex have half-lives of 30 min or less (Lew *et al.*, 1995; Ogris *et al.*, 1999; Thierry *et al.*, 1997). However the persistence of distributed plasmid DNA following SPLP administration is not entirely dissimilar to that observed in other non-viral systems. Plasmid has been detected by conventional PCR in mice up to six months after intravenous

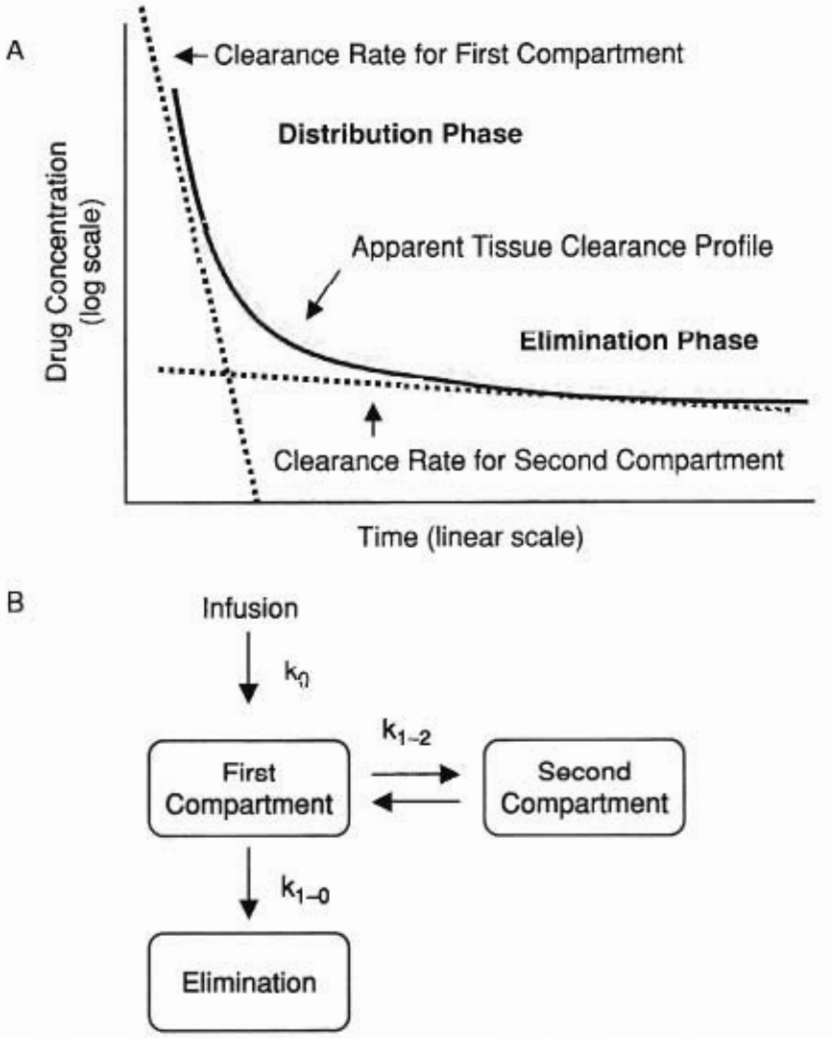

Figure 6.10. The two-compartment model of pharmacokinetics.

injection of lipoplex DNA (Lew *et al.*, 1995). Plasmid has also been demonstrated to persist in mice at least 19 months after intramuscular injection of naked DNA (Wolff *et al.*, 1992), and 1.5 years after three intraperitoneal doses of lipoplex (Xing *et al.*, 1998). With SPLP, residual plasmid DNA has been detected as long as 360 days after a single intravenous injection of SPLP at a dose level equivalent to 100 μg for a mouse weighing 20 grams. The implications of these findings should be assessed in conjunction with other relevant information. In particular, it is important to note that the initial plasmid distribution pattern and persistence of a gene delivery system is distinct from its gene expression pattern, as has been demonstrated for SPLP, DNA/lipid complexes (Lew *et al.*, 1995; Osaka *et al.*, 1996; Xing *et al.*, 1998) and naked DNA (Wolff *et al.*, 1992).

B. Biodistribution of protein expression following systemic administration of SPLP

Encapsulation of plasmid DNA coding for luciferase facilitates analysis of the biodistribution of protein expression resulting from intravenous administration of SPLP. Fig. 6.11 shows that the accumulation of SPLP following intravenous administration leads to significant levels of expression in Neuro-2a tumors, with other tissues yielding much lower levels of luciferase. With the exception of the adrenal glands, with one order of magnitude less than the tumor, all other tissues express 2 or 3 orders of magnitude less luciferase than the tumor. The liver and the brain demonstrate the least amount of expression. These results confirm that SPLP are capable of preferential disease-site targeting and expression, a conclusion supported by a number of preclinical studies in which expression has been observed in tumors following intravenous administration of various other SPLP formulations. In each of these studies, intravenous administration of SPLP leads to protein expression in the tumor on the hind flank of the animal.

Tumor-selective protein expression is achieved with SPLP in the absence of tumor-specific promoters, targeting ligands or any other so called 'targeting' technology. In fact, the results compare favorably with the degree of selectivity conferred to protein therapeutics, small molecule drugs or viral

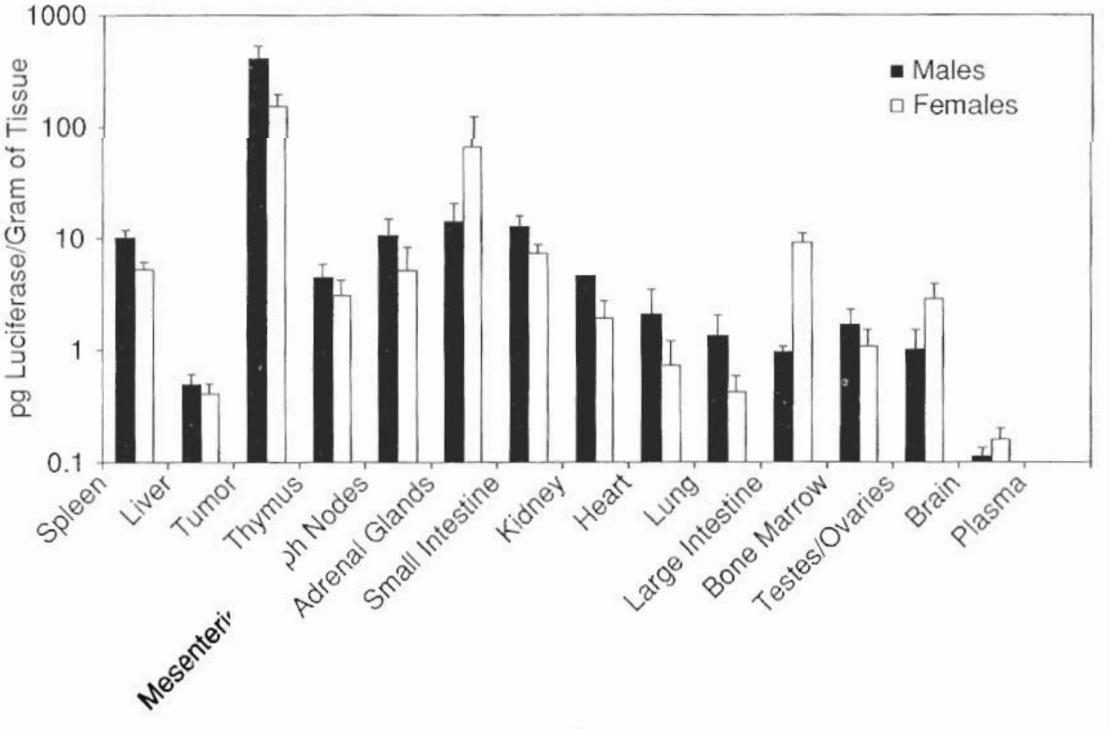

Figure 6.11. Biodistribution of gene expression following a single intravenous administration of SPLP in neuro-2a tumor bearing A/J mice.

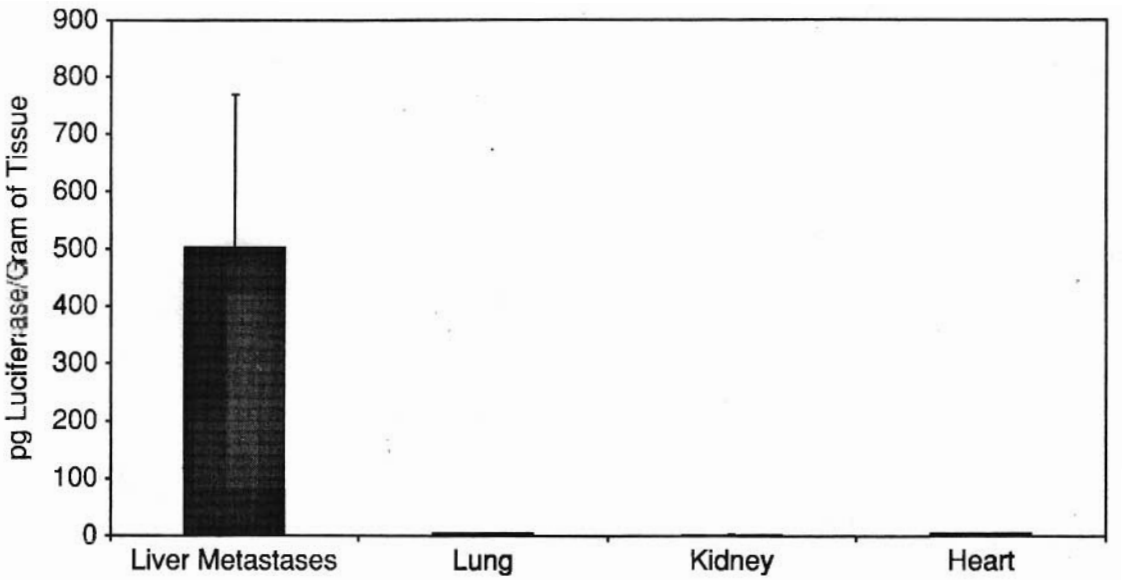

Figure 6.12. Luciferase gene expression 48 h after administration of SPLP in female balb/c mice bearing CT26 liver metastases.

vectors conjugated to monoclonal antibodies or peptides directed toward tumor cells in an attempt to convey tumor selectivity.

In an effort to further explore the potential of disease site targeting and tumor selective protein expression, the SPLP gene expression pattern has been evaluated in murine models of metastatic disease. One such model is the CT26 liver metastases model. Murine CT26 colon carcinoma cells are injected into the mouse spleen 10 min prior to splenectomy. Twenty-four days after tumor inoculation, mice are treated with SPLP by injection in the lateral tail vein. Forty-eight hours later, tissues are collected for luciferase analysis. In this experiment, and other models of liver metastasis, gene expression is greatest in the liver metastases (Fig. 6.12). Although previous studies have shown that significant amounts of SPLP are delivered to the liver in both normal and tumor-bearing mice, it is important to note that gene expression in this instance is increased by more than 100-fold in the presence of hepatic tumor nodules.

V. CONCLUSION

Many viral and non-viral gene transfer systems suffer from common pharmacological issues that limit their utility in a systemic context. Attempts to address these issues through manipulation of the virology or molecular biology of these systems have met with limited success. By application of the liposomal drug delivery paradigm, many of the limitations of the first generation non-viral

delivery systems can be overcome. Encapsulation in small, long-circulating particles results in enhanced accumulation at disease sites. The selectivity of nucleic acid payloads is further enhanced by their mechanism of action. Plasmid DNA must overcome a number of intracellular barriers to reach the nucleus of the cell where gene expression occurs. Since these barriers are more readily overcome in transformed cells relative to their more quiescent, healthy counterparts the barriers to transfection actually represent opportunities to confer additional selectivity in the gene expression pattern.

Although lipid-mediated systemic gene delivery and expression was reported by Zhu *et al* in 1993 (Zhu *et al.*, 1993), progress in developing lipoplex systems capable of delivering plasmid DNA to distal disease sites has been slow. The ability of SPLP to mediate tumor-selective protein expression in disseminated tumor sites represents a promising foundation upon which to build targeted molecular therapeutics for oncology, inflammation and infectious disease. The pharmacology of these systems is currently being evaluated in a Phase I clinical trial.

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