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Design and Properties of a Lipid-Based Carrier System for Systemic Gene Therapy

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Abstract. The currently available delivery systems for genetic drugs have only limited utility for systemic applications. Both viral vectors and the non-viral "complex" gene delivery systems formed with cationic liposomes and plasmid DNA are cleared rapidly by the reticuloendothelial system resulting in highest level of activity in the "first-pass" organs, such as the lung liver and spleen. In addition, viruses elicit an immune response compromising transfection activity of subsequent injections. For successful gene therapy for systemic diseases such as tumors, inflammation and infections a carrier system is required that is not readily recognized by macrophages of the reticuloendothelial system. Required characteristics for such a carrier are small size, neutral in charge and highly stable in serum with an extended circulation lifetime. This review presents the concept and construction of a non-viral gene carrier system for systemic gene therapy. The plasmid DNA is encapsulated within a lipid bilayer protecting the DNA from degradation by nucleases. These DNA-lipid particles are on average 70 nm in diameter and stabilized by an exchangeable polyethylene glycol coating. Extended circulation lifetimes can be achieved with significant accumulation of intact plasmid at a distal tumor site. Detection of luciferase activity in the tumor tissue indicates that the plasmid delivered was internalized and transcribed. Approaches to increase transfection activities are discussed.

1. Introduction

Genetic drugs such as plasmids containing therapeutic genes and antisense or ribozyme oligonucleotides have great potential for treatment of human diseases such as cancer, genetic disorders and infections. Recent advances in the understanding of medical diseases generally lead to proposals for genetic interventions, particularly in oncology. The potential advantage of gene therapy over conventional therapy is the ability to regulate gene function in order to achieve specific activity, prolonged response and little toxicity. In oncology, for drugs to be effective, their access and efficacy at metastatic disease sites are necessary, since these are the primary determinant of survival in most cancers. Therefore, for gene therapy to be successful, systemic applications will

generally be required. However, rapid breakdown and clearance from the blood compromise the effectiveness of these molecules for systemic treatment of disease. In addition, **plasmids** are large and highly charged molecules that cannot readily penetrate target cell membranes to reach their sites of action inside the cells. As a result, the development of an efficient delivery system that protects **plasmid** in the circulation and facilitates intracellular delivery into target cells is critical for the clinical success of gene therapy.

1.1. Viral vectors

Presently, the favored **delivery** systems for gene transfer are genetically engineered viruses including retroviruses, adenoviruses, adeno-associated virus (**AAV**) and Herpes virus [1-4]. Engineered viruses are efficient for inserting foreign genes into cells, however, their limitations as a systemic gene delivery system are widely recognized. Viral systems are rapidly cleared from circulation limiting potential target sites to "first-pass" organs such as the lung, liver and spleen. In addition, these systems elicit immune responses compromising the effectiveness of subsequent injections [5,6], and there is the potential that they may become pathogenic [7,8]. Because of these limitations efforts have recently focused on the construction of non-viral delivery systems.

1.2. Lipid-based non-viral gene delivery systems

The most widely and successfully used non-viral delivery systems for gene transfer are lipid-based vectors formed by combining **plasmids** with **cationic** liposomes (lipoplexes) [9-13]. The importance of **cationic** liposomes as gene carriers is reflected in the wide variety of commercially available **cationic** liposome formulations (see Table II in [14]). The vast majority of these formulations consist of a **cationic** lipid mixed with a helper lipid such as DOPE or cholesterol at a 1: 1 molar ratio. The preparation procedure is simple. The **cationic** liposomes, usually vesicles with diameters ≤ 100 nm, are mixed with DNA in a dilute solution. The complexes form spontaneously due to electrostatic charge interactions, leading to liposome **fusion** and aggregation. The interaction between DNA and lipid is difficult to control, producing large complexes that have a very heterogeneous size distribution. Particle sizes range from 250 nm to $>1\mu\text{m}$. The major parameters determining the final product are the charge ratio, the ionic strength of the media and the overall concentration of the reactants. Structural features revealed by electron microscopy include liposome-coated DNA strands (beads on a string), aggregates of liposomes intercalating DNA, DNA entrapped between the lamellae of aggregated multilamellar structures, and tubular structures consisting of **fused** liposomes around DNA [15-18]. The charge of the complexes is slightly positive to allow for interaction with negatively charged cell surfaces, thereby increasing cellular uptake. The transfection efficiency of any given formulation is highly dependent on the cell line, type of **cationic** lipid (liposome formulation) and the ratio of DNA to liposomes used [9,18-20].

These **cationic liposome/DNA** complexes are capable of transporting **plasmid** across cell membranes achieving delivery into a wide spectrum of cells *in vitro*, however, their application *in vivo* is limited. The major drawbacks of these systems are their tendency to aggregate in serum the short circulation lifetime and the limited protection of the **plasmid** from degradation by serum **nucleases** [21,22]. The rapid clearance of the DNA/lipid complexes from the circulation through the reticuloendothelial system (RES) following systemic administration *in vivo* [23] is not surprising, since charged particles are known to bind serum proteins targeting them for removal by the phagocytic cells of the **RES** [24-28]. The clearance characteristics of the **DNA/lipid** complexes indicate that potential transfection sites are limited to these "first-pass" organs [21, 29-32]. The highest expression levels are usually observed in the lung [29, 33-35]. Large complexes and aggregates (2300 nm in diameter) tend to get trapped in the capillary beds of the lung [36].

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This results in an extended exposure of the lung endothelial bed to DNA/lipid complexes and is most likely the reason for the preferential **transfection** observed in the lung following intravenous administration of these complexes [30,37]. The characteristics of the lipoplexes make them potentially **useful** for gene delivery to lung tissue, for example, but they are clearly unsuitable for delivery to a distal tumor site. The need for a gene delivery system for systemic treatment of diseases such as inflammation and tumor is obvious.

In this review we present the general concept, construction and properties of a novel **lipid-based plasmid** carrier system for systemic application.

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2. General Concept and Design of a Systemic Gene Carrier

A successful gene delivery system has to overcome a number of barriers to achieve **plasmid** delivery and expression at a disease site. Different stages are outlined in Figure 1. In principle, a systemic gene carrier should have a prolonged circulation lifetime in the blood to facilitate extravasation at a disease site leading to accumulation in diseased tissue. Once at the disease site, the carrier has to interact with target cells to initiate uptake, e.g. by endocytosis. Subsequently, it will have to destabilize the endosomal membrane to escape degradation in lysosomes and to achieve release of the **plasmid** into the cell cytoplasm. Finally, the **plasmid** has to enter the nucleus for gene expression to occur. The design features for lipid-based delivery systems that preferentially access disease sites such as sites of infection and **inflammation**, as well as tumors are becoming increasingly clear from liposomal drug delivery data.

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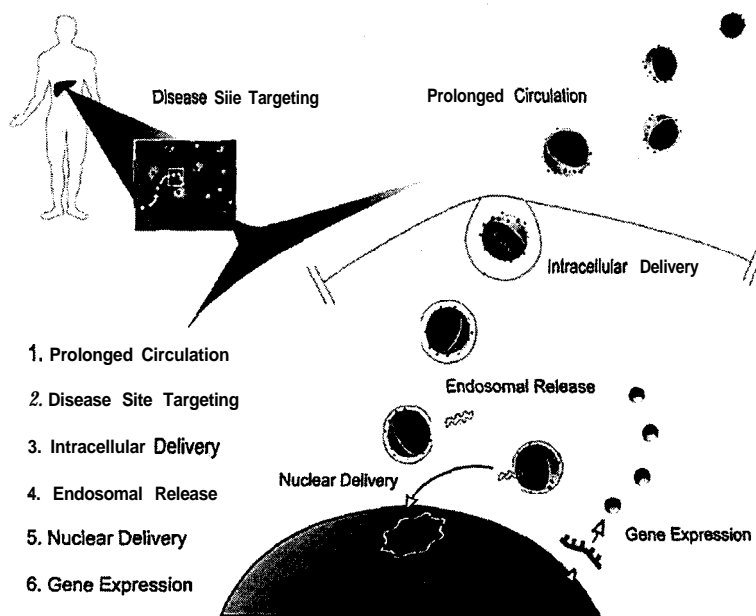


Figure 1. Stages of systemic gene delivery. A carrier of about 100 nm diameter with an extended **circulation** lifetime accumulates at disease sites such as **inflammation** or tumor sites as a consequence of **increased vascular leakage**. It **interacts** with the target **cell** membrane and is taken up by **endocytosis**. **In early stages of endocytosis, the carrier destabilizes the endosomal membrane and releases the plasmid** DNA into the cytoplasm. For gene expression to occur the **plasmid** needs to enter the nucleus.

2.1. Properties of liposomal drug delivery systems

It is now generally recognized that preferential delivery of anticancer drugs to tumor sites, following intravenous injection, can be achieved by encapsulation of these drugs in large unilamellar vesicles (**LUVs**) that exhibit a small size of approximately 100 nm diameter. The accumulation of long circulating liposomes at disease sites, which includes sites of infection, **inflammation** and tumors, has been attributed to enhanced permeability of the local vasculature in diseased tissue [38] allowing extravasation of small particles into the surrounding tissue. **Non-charged 100 nm PC/Chol vesicles** have been shown to evade rapid removal from the circulation by the reticuloendothelial system resulting in extended circulation lifetimes (circulation half-life in mice > 5 h) and to accumulate at tumor [39-42] and inflammation sites [46]. The circulation lifetime of lipid vesicles in the blood can also be increased by coating their surface with polyethylene glycol (PEG). The polymer acts as a steric barrier and reduces the level of plasma protein binding to the liposome surface and thereby avoiding their rapid recognition and uptake by the Kupffer cells in the liver [39, 40, 43-45]. As much as 10 % of the injected dose of PEG-coated liposomes per g of tumor tissue has been detected in tumors following systemic application [40, 43]. The increased accumulation of sterically stabilized liposomes loaded with anticancer drugs in tumor tissues translates into enhanced antitumor efficacy.

2.2. Properties of cationic lipid complexes

Most of the **cationic** lipid formulations presently used employ DOPE as helper lipid for optimal activity. The membrane-destabilizing activity of DOPE [10, 47-50] is believed to enhance the release of **plasmid** DNA into the cytoplasm. DOPE in isolation, adopts the inverted hexagonal phase (H_2) at temperatures above 10°C [51] but can be stabilized in a bilayer structure in the presence of other lipids such as PC's, **cationic** lipids, PEG-lipid conjugates and detergents. The effect of DOPE in the **plasmid/lipid** complex on **plasmid** delivery into the cell cytoplasm and expression of a marker gene is shown in Figure 2. Although the lipid uptake is the same for both the PE and PC containing systems, the **transfection** activity observed with PE complexes is much higher than with PC complexes. The number of intact **plasmids** detected in the cytoplasm and consequently also in the nucleus is higher for PE than for PC systems. Since the route of entry of DNA / **cationic** lipid complexes is via endocytosis [52-55], this establishes a direct correlation between **plasmid** release from endosomes and PE in the complexes.

2.3. Design features of a lipid-based gene carrier for systemic application

Combining the information on liposomal drug carriers and lipoplexes leads to the following design principles for the ideal liposomal gene carrier.

- (i) **Plasmid** encapsulated in small size lipid vesicle/particle of approximately 100 nm diameter
- (ii) Highly stable particles providing **full** protection of the **plasmid** from degradation by serum **nucleases**
- (iii) Particles that do not interact with blood components and evade uptake by the RES, leading to circulation longevity and accumulation at disease sites
- (iv) Following arrival at the disease site, the ability of the particles to interact with and enter into target cells
- (v) Efficient intracellular delivery of **plasmid**, e.g. via endocytosis and release of its content into the cytoplasm

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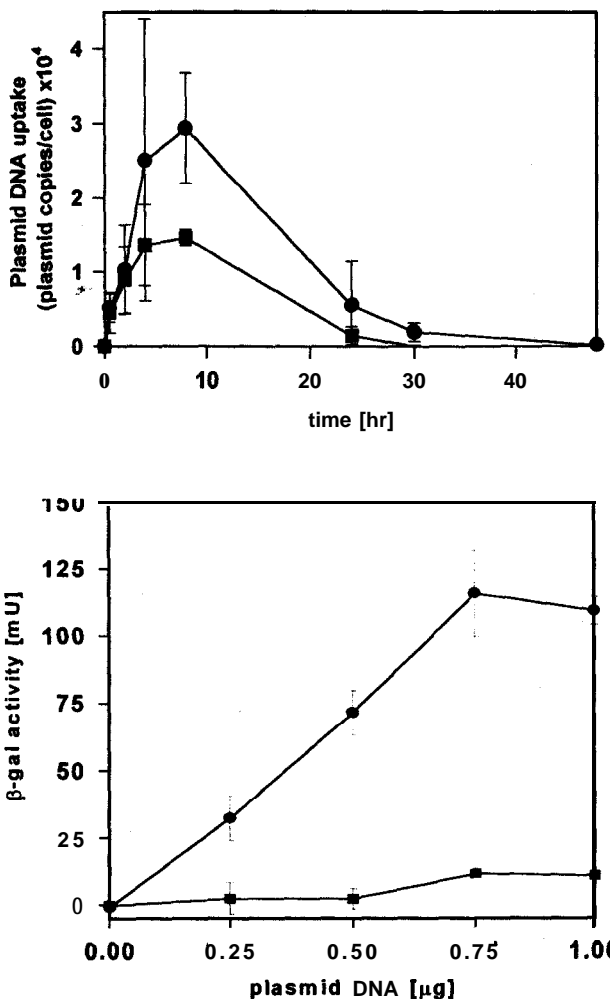


Figure 2. Fusogenic lipid DOPE increases plasmid delivery and transfection activity of cationic lipid/DNA complexes. The time course of intracellular plasmid delivery using DNA/cationic lipid complexes formed with DODAC/DOPE (1:1; mol:mol) (●) and DODAC/DOPC (1:1; mol:mol) (■) vesicles at a charge ratio of 1.5 (+/-) is shown in (a). Complexes containing 2 μg p-gal plasmid were added to BHK cells in complete DMEM medium. Following incubation of up to 4 hours, the transfection medium was replaced with complete DMEM medium. At times indicated cells were lysed and the extracted DNA characterized by dot blot analysis following hybridization with ³²P-labeled plasmid-specific probe. The amount of intracellular plasmid DNA was quantified using a standard curve generated with the identical plasmid. In (b) dose dependent transfection using complexes formed with DODAC/DOPE (1:1; mol:mol) (●) and DODAC/DOPC (1:1; mol:mol) (■) vesicles with a charge ratio of 1.5 (+/-). Cells were incubated with DNA-lipid complexes for 4 h and the medium replaced with complete DMEM medium. p-gal assay using the CPRG substrate was performed 24 h post-transfection.

This places two potentially conflicting demands on the **plasmid** delivery system. Initially, the **plasmid** carrier must be stable to achieve an extended circulation lifetime but later should bind to target cells and 'exhibit membrane-destabilizing "**fusogenic**" characteristics to facilitate intracellular delivery of **plasmid**. Using PEG-lipids that can dissociate from the membrane with a controllable exchange rate could in principle solve these conflicting demands. PEG-lipids could stabilize a lipid particle with encapsulated DNA and a high content of the **fusogenic** lipid DOPE. It is expected that these polymer-coated particles could initially evade uptake by the RES, resulting in prolonged circulation lifetime and accumulation at disease sites. As the PEG coating exchanges out of the particle, cellular uptake can occur and the carrier with its large content of the **fusogenic** lipid DOPE will become increasingly destabilized. The exchange rate of the PEG-lipid out of the membrane can be controlled, since it is dependent on the acyl chain length and their degree of saturation [56, 57]. As shown in an *in vitro* model system, an increase in the acyl chain length of the ceramide anchor for PEG, which increases its hydrophobicity, results in a lower exchange rate of PEG [58]. Typical exchange half times of PEG-ceramide with acyl chain lengths of 8 to 20 carbons range from minutes to days [59]. This permits the adjustment of the rate at which the stabilizing coating dissociates from the plasmid-lipid particle.

3. Construction and Physicochemical Properties of Stabilized Plasmid-lipid Particles

3.1. Encapsulation of plasmid in 100 nm lipid particles

Plasmid DNA has been encapsulated by a variety of methods, including reverse phase evaporation [60-62], ether injection [63, 64], detergent dialysis in the absence of PEG stabilization [62, 64], lipid hydration and dehydration-rehydration techniques [65-67] and sonication [68-70] among others. The characteristics of these protocols are summarized in a table given in Wheeler et al. [71]. None of these procedures yields small, serum-stable particles at high **plasmid** concentrations and **plasmid**-to-lipid ratios in combination with high **plasmid**-encapsulation efficiencies.

Entrapment of supercoiled **plasmids** in 80-100 nm diameter vesicles presents a difficult packing problem because of their large size. The dimensions of a 4.4 kb **plasmid** from an electron micrograph [72] in comparison to a 100 nm liposome are shown in Figure 3. The **plasmid** exhibits an extended length of about 520 nm and on average (in two dimensions) a diameter in the range of 350 nm. This would suggest that a vesicle with an average diameter of 400 nm would be required to encapsulate a 4.4 kb **plasmid**. Clearly, due to the large **plasmid** size passive encapsulation in liposomes is very inefficient. Efficient entrapment requires the interaction of the lipid components with the **plasmid** with a concomitant reduction in DNA size. **Cationic** lipids could fulfill these requirements [73-75]. Neutralization of the negative phosphate charges through association with **cationic** lipids decreases repulsion between DNA segments and allows bending of DNA and a reduction in size. A 4.4 kb **plasmid** bound to a positively charged surface of a 100 nm liposome would occupy approximately 15 % of the total surface area [50].

A detergent dialysis process was developed based on the observation that incubation of **plasmid** DNA with **cationic** lipids can result in hydrophobic **plasmid** DNA-cationic lipid particles, which are soluble in organic solvent [76]. It relied on the hypothesis that the **plasmid** combined with the **cationic** lipid would form a hydrophobic "inverted micellar" structure that could be stabilized in aqueous media by detergent. The addition of phospholipid and subsequent removal of detergent by dialysis was expected to lead to the exchange of the solubilizing detergent with phospholipid, resulting in the deposition of a phospholipid monolayer around the hydrophobic intermediate, yielding particles which are stable in aqueous suspension. Initial experiments failed, since the strong electrostatic interaction between DNA and **cationic** lipid was difficult to control,

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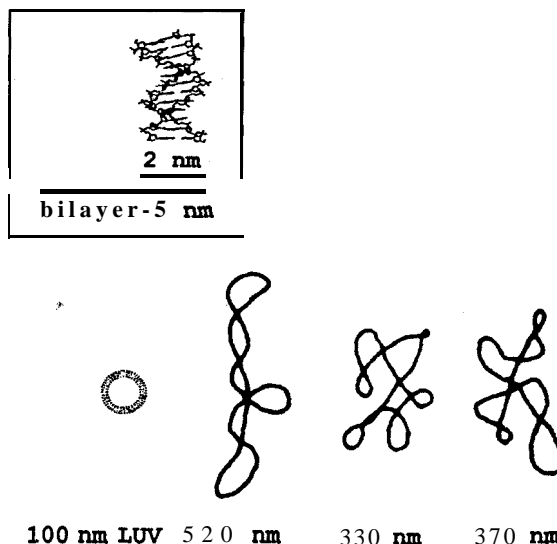


Figure 3. Comparison of the dimensions of a 100 nm LUV and a 4.4 kbp plasmid. Three different structures of the same 4.4 kbp plasmid are shown, together with their longest dimension. The bilayer thickness and DNA cross-section are not plotted according to their relative dimensions. They are shown on a correct scale in the inset. The plasmid structures were reproduced from electron micrographs published in [72].

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resulting in membrane fusion and aggregation, leading to the formation of large heterogeneous aggregates. Therefore, regulatory components, which allow the control of these processes, were required.

Previous studies have shown that incorporation of PEG-lipid conjugates into the liposomal membrane can inhibit Ca^{+2} - induced aggregation and fusion between LUVs [57]. However, the use of the standard PEG-phosphatidylethanolamine (PEG-PE) as a stabilizing factor was contraindicated, because the PEG-PE molecule bears a net negative charge and could displace the cationic lipid from the plasmid, as has been noted for other negatively charged lipids [77]. Therefore, PEG, was linked to ceramide as the hydrophobic anchor to produce a neutral molecule. Incorporation of 10 mol% PEG-CerC₂₀ in the detergent mixture with DOPE, DODAC and plasmid DNA prevented precipitation during detergent dialysis and >50% of the plasmid was encapsulated in systems termed "stabilized plasmid-lipid particles" (SPLP).

As shown in Figure 4, the trapping efficiency is a sensitive function of the cationic lipid content, with encapsulation levels of up to 70% or higher at about 6-7% DODAC. At higher DODAC concentrations significant aggregation was observed. As a mechanism for encapsulation it was proposed that during dialysis of the lipid mixture the plasmids initially interact with macromolecular lipid structures, such as cylindrical micelles and lamellar sheets, bearing an appropriate surface charge. The formation of such structures during the formation of lipid vesicles by detergent dialysis is well-established [78-80]. A low concentration of cationic lipid in these structures would result in little association of plasmid resulting in little or no plasmid entrapment following detergent dialysis. At higher concentrations of cationic lipid, intermediate structures may be expected to associate with the plasmid and, if the cationic lipid content is too high, plasmid-lipid-plasmid association could dominate as dialysis proceeds, leading to the formation of aggregates. If the cationic lipid content is at a critical level, the positive surface charge on the

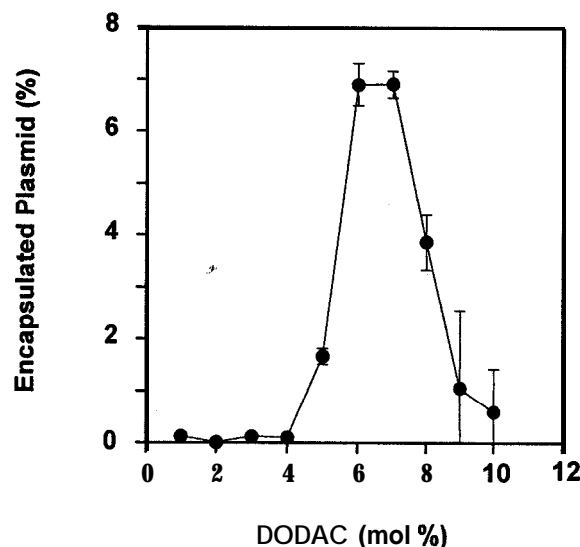


Figure 4. Effect of cationic lipid concentration on the encapsulation efficiency of plasmid DNA (pCMV-CAT) in DOPE/DODAC/PEG-ceramide₂₀ SPLP. 10 mg lipid were dissolved in 200 mM octylglucoside and mixed with 50 μ g plasmid DNA in a total volume of 1 ml to form an optically clear solution. This was then placed in a dialysis tube and dialyzed against HBS for 36 hrs at 20°C. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography. DNA was quantified using either ³H-labeled plasmid or the DNA intercalating fluorescent dye, PicoGreen (Molecular Probes). Lipid concentrations were determined by chromatography or with radiolabeled lipids. This figure is published in [71].

plasmid-associated intermediates will be reduced below that needed to associate with other plasmids, due to charge neutralization. This would mitigate against further aggregation and results in the formation of SPLP as outlined in [71].

2.2. Encapsulation of plasmid in lipid particles composed of different cationic lipid concentrations

As the encapsulation relies on an electrostatic interaction between the intermediate lipid structures and plasmid, it should be highly dependent on the ionic strength of the medium. Therefore, it is expected that increasing the ionic strength of the dialysis buffer, shielding the surface charge on the lipid structures could permit formulations using higher cationic lipid concentrations. Both the magnitude of the surface potential and the extension of the electrical effects away from the liposomal surface are reduced at high salt concentrations. Simply increasing the NaCl concentration was not sufficient to prevent aggregation in preparations containing more than 10 mol% DODAC. As predicted by the Gouy-Chapman theory, the screening effect is much more potent with multivalent ions [81] and sufficient shielding was obtained with polyvalent anionic counter-ions such as citrate [59] and phosphate [82]. Figure 5 demonstrates the effect of the citrate concentration on the encapsulation for a formulation with 20 mol% DODAC (DODAC/DOPE/PEG-CerC₈; 20:65:15; mol:mol:mol) and the pCMVLuc plasmid. At concentrations up to 60 mM citrate the dialyzed samples contained only large (diameter > 150 nm) and polydisperse particles. However, between 65 and 80 mM citrate, small monodisperse particles (82 \pm 40 nm diameter) were formed with encapsulation efficiencies of 50-70%. Increasing the citrate concentration further also resulted in the formation of small particles, but the encapsulation efficiency decreased dramatically. Similar

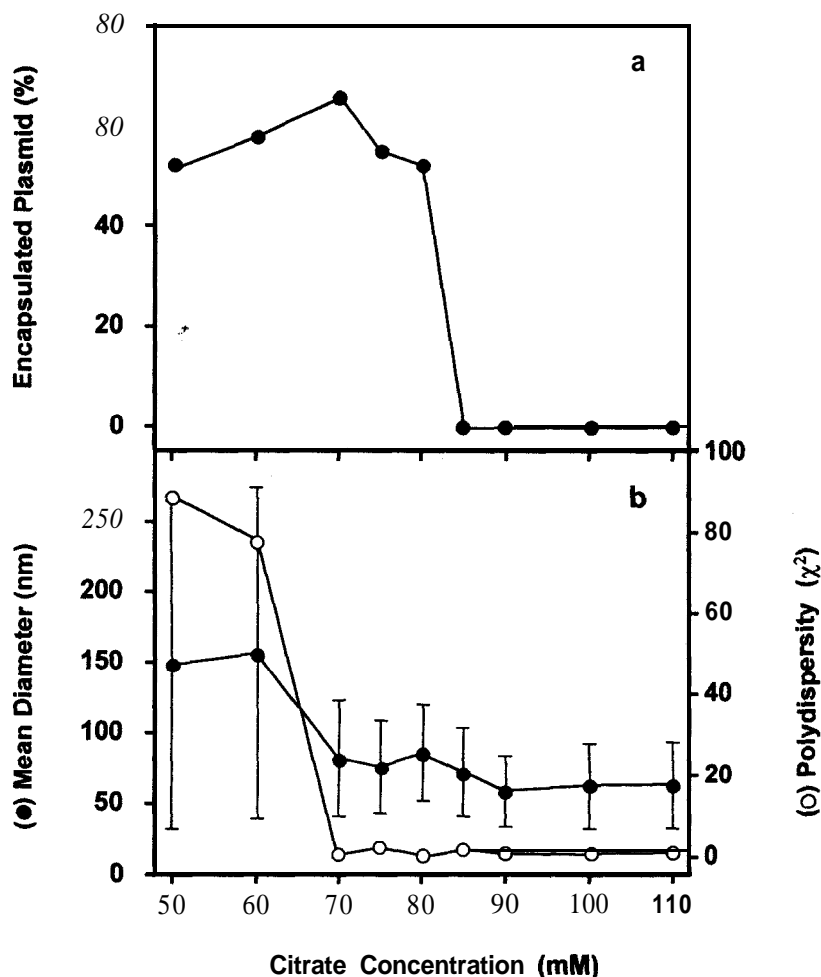


Figure 5. Plasmid DNA can be entrapped in SPLP containing high levels of DODAC by raising the citrate concentration present during detergent dialysis. Panel (a) indicates the effect of varying the citrate concentration on plasmid encapsulation efficiency following detergent dialysis, as determined by plasmid accessibility to the DNA-intercalating dye PicoGreen. Panel (b) indicates the effect of citrate on the diameter (\bullet) and polydispersity, χ^2 (\circ), of the formulations following detergent dialysis as measured by QELS (volume-weighted vesicle mode). Formulations were composed of DODAC/DOPE/PEG-CerC₃ (20:65:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 200 μ g plasmid per ml). This Figure is published in [59].

results were obtained with phosphate as counter-ion [82]. The optimal ionic strength (citrate or phosphate and NaCl) required for efficient encapsulation was determined for a DODAC concentration range of 6 to 50 mol% using two criteria: (1) formation of monodisperse particles with diameter smaller than 100 nm, and (2) an encapsulation efficiency greater than 50%. Typically, citrate or phosphate concentration was varied first followed by NaCl concentration to fine-tune and maximize encapsulation. The results are summarized in Figure 6 (for citrate) and Table 1 (for phosphate). The citrate concentration range, giving rise to particles with diameter smaller than 100 nm and with encapsulation efficiencies of 50% or higher, is represented by the

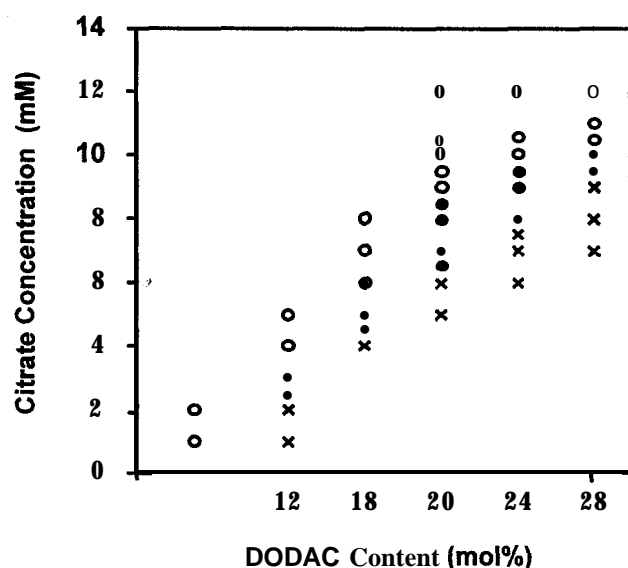


Figure 6. Optimal citrate concentration range as a function of SPLP DODAC content for maximum plasmid encapsulation in combination with minimum aggregation. Formulations were composed of DODAC/DOPE/PEG-CerC₃ (x: 85-x: 15; mol:mol:mol) and pCMVLuc (10 mg lipid and 100-200 μ g plasmid per ml) and were prepared by detergent dialysis, where the dialysate contained the indicated sodium citrate concentrations as well as 150 mM NaCl, 10 mM HEPES (pH 7.2). The solid circles (●) indicate formulations that exhibited plasmid encapsulation efficiencies greater than 50% and a small, monodisperse size distribution as determined by QELS (diameter < 100 nm, $c^2 < 3$). The open circles (○) indicate formulations that exhibited plasmid encapsulation efficiencies of less than 40% in combination with a small, monodisperse size distribution (diameter < 100 nm, $\chi^2 < 3$). The crosses (x) indicate polydisperse formulations with large size distributions (diameter > 100 nm, $\chi^2 > 3$). Figure published in [59].

Table 1. Optimal Phosphate Buffer Concentration for Plasmid Encapsulation

SPLP DODAC Concentration (mol%)	Optimal Detergent Dialysis Buffer Concentration
7	150 mM NaCl
20	90 mM Na ₂ HPO ₄
24	110 mM Na ₂ HPO ₄
30	140 mM Na ₂ HPO ₄
34	150 mM Na ₂ HPO ₄ + 50 mM NaCl
38	150 mM Na ₂ HPO ₄ + 90 mM NaCl
42.5	150 mM Na ₂ HPO ₄ + 140 mM NaCl

solid circles (Figure 6). Higher citrate concentrations give rise to low **plasmid** encapsulation efficiencies of 30% or less, whereas citrate concentrations below the optimum levels resulted in large, polydisperse aggregates. Varying the citrate concentration in the dialysis medium permitted the formation of SPLP containing up to 25 **mol% cationic lipid** [59], whereas with phosphate as counter-ion SPLP containing 6 to 50 **mol% cationic lipid** could be formed (Table 1) [82].

The encapsulation relies on a delicate balance between DODAC concentration and ionic strength of the medium, and this supports the previously proposed mechanism. A scheme for the encapsulation mechanism is presented in Figure 7 with citrate as counter-ion. The surface charges on the intermediate lipid structures formed during dialysis have to be shielded just enough to permit binding of only one **plasmid** molecule. The resulting screening of the positive lipid headgroups by the negatively charged **plasmid** could cause a reduction in the surface area of one of the lipid leaflets and preferential closure with trapping of the **plasmid** inside the lipid vesicle. By using the appropriate amount of citrate or phosphate the positive charge on the lipid structures is shielded, reducing the **affinity** for **plasmid** to levels compatible with good entrapment. Within this model, citrate (or phosphate) concentrations below the optimum range for a particular charge density do not result in adequate shielding of the positively charged lipid structures, resulting in

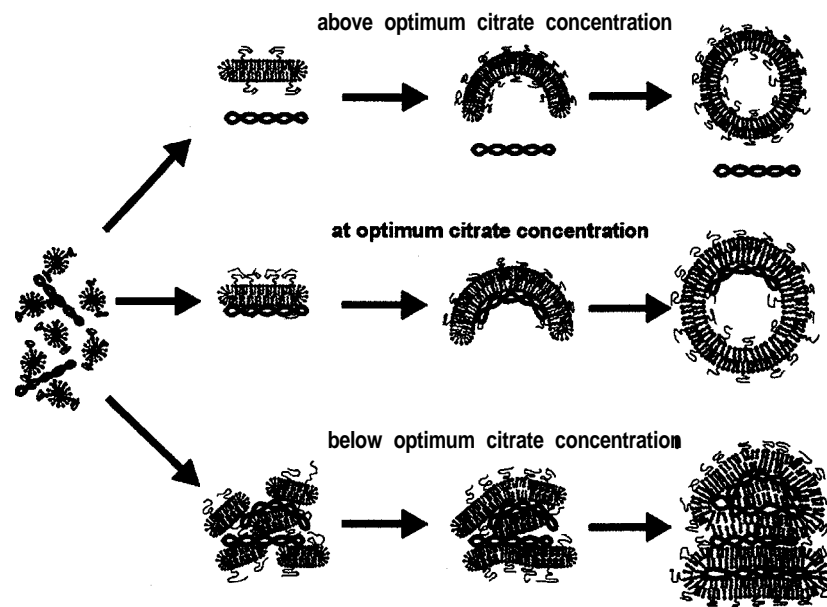


Figure 7. Model of SPLP formation during dialysis. In the first stage of dialysis, formation of macromolecular lipid intermediate structures are expected to occur in the form of lamellar sheets, cylindrical micelles or leaky vesicles [79, 80]. If the citrate concentration is too high (top), the charges on the lipid structure are shielded and there is little or no plasmid association with these intermediate lipid structures and as dialysis proceeds, empty vesicles are formed and **plasmid** remains free in solution. At a lower citrate concentration, **plasmid** associates with the lipid intermediates illustrated as **plasmid** attached to a bilayer sheet (middle). At optimum citrate concentration the net charge of the **plasmid** is not strong enough for association of additional lipid intermediates. The **plasmid** reduces the net positive charge of the lipid intermediate at the point of contact possibly resulting in bilayer curvature around the **plasmid** leading to encapsulation of the **plasmid** as dialysis proceeds and additional lipid condenses on this plasmid-lipid structure. At too low citrate concentration (bottom) the net charge of the initial plasmid-lipid intermediate structure is strong enough to attract additional lipid intermediate structures leading to the formation of larger aggregates.

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crosslinking by **plasmids** and aggregate formation. **At** citrate concentrations above the optimum range on the other hand, the positive charge on the lipid structures is shielded to the extent that interaction with **plasmid** is inhibited, resulting in little or no entrapment. At the critical citrate concentrations, the shielded charge on the lipid structure is just sufficient to bind **plasmid**, and encapsulation can then proceed as outlined in Figure 7.

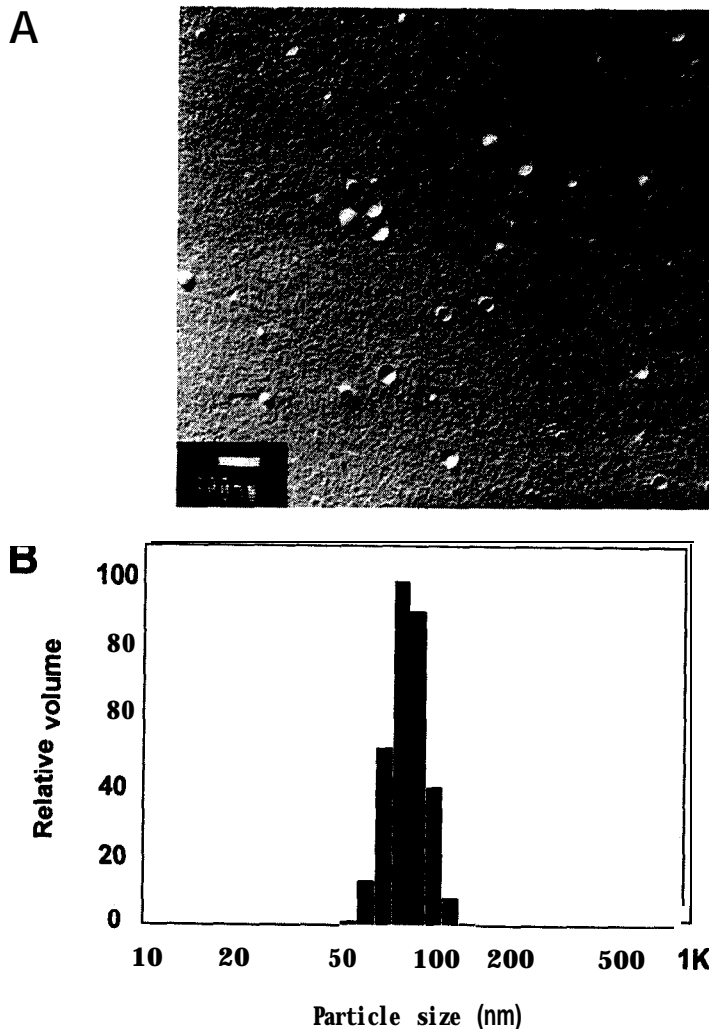


Figure 8. Freeze-fracture electron microscopy and size distribution of SPLP. Freeze-fracture electron micrograph of SPLP, isolated by sucrose density gradient centrifugation is shown in (A). In (B) the same sample is analyzed by quasielastic light scattering using a Nicomp sub-micron particle sizer operating in the volume-weighted vesicle mode.

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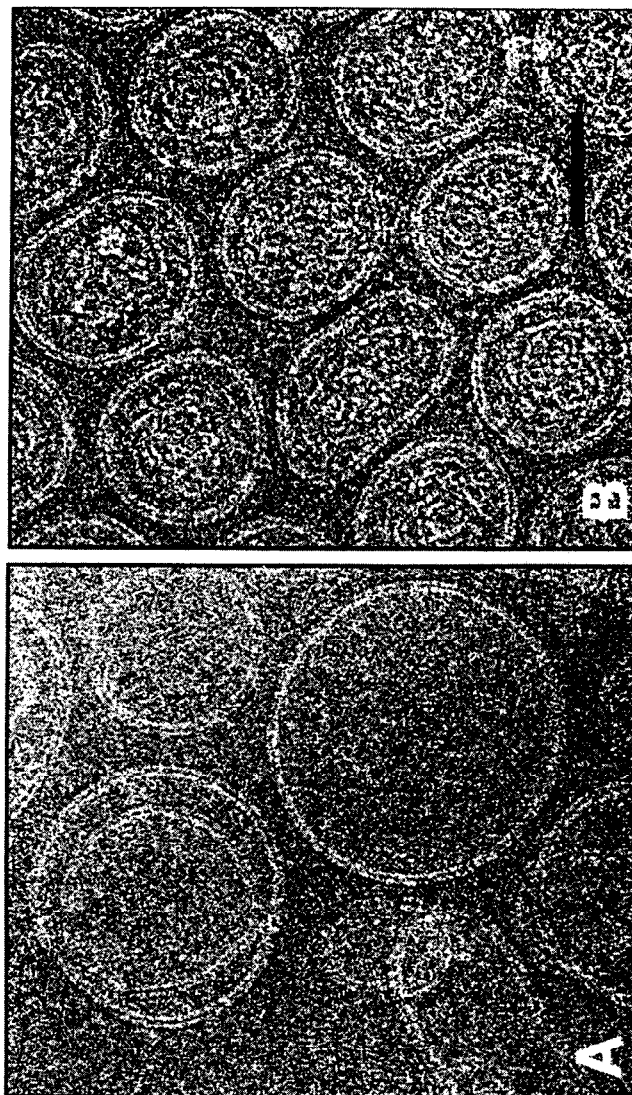


Figure 9. Cryo-electron micrographs of SPLP. LUVs (A) were prepared by hydration and extrusion through polycarbonate filters with 100 nm pore size. The lipid concentration of the formulations was adjusted to about 2 mg/ml. Isolated SPLP (B) were prepared as described in Figure 4 with 7 mol% DODAC. The bar in panel (B) indicates 50 nm. Microscopy was done by Holger Stark, Imperial College London.

3.3. Physico-chemical properties of SPLP

Sucrose density gradient ultracentrifugation shows that the detergent dialysis process not only resulted in plasmid-containing particles but also in a considerable proportion of empty lipid vesicles. The isolated SPLP exhibit a narrow size distribution with a mean diameter of 75 nm as determined by dynamic light scattering and 64 ± 9 nm based on freeze-fracture electron microscopy (Figure 8). The plasmid-to-lipid ratio in these isolated SPLP of approximately 60 g plasmid per mol lipid was independent of the cationic lipid concentration employed. This ratio corresponds to one plasmid per SPLP for an SPLP diameter of 70 nm (the average of the freeze-fracture electron microscopy and QELS results), assuming a lipid molecular area [83] of 0.67 nm^2 and an average nucleotide molecular weight of 330.

The plasmid DNA in SPLP is fully protected from degradation by DNase I and serum nucleases in contrast to plasmid in cationic liposome/DNA complexes. The cryo-electron micrograph of SPLP [82] (Figure 9B) showing a lipid bilayer surrounding an internal electron-dense structure is consistent with plasmid DNA encapsulated in a lipid particle. The internal structure is not seen in LUVs prepared by extrusion (Figure 9A). The homogeneous well-defined structure of SPLP is remarkable and demonstrates that plasmid can be entrapped in small well-defined lipid vesicles. The structure is clearly distinct from any structures described for plasmid/cationic lipid complexes.

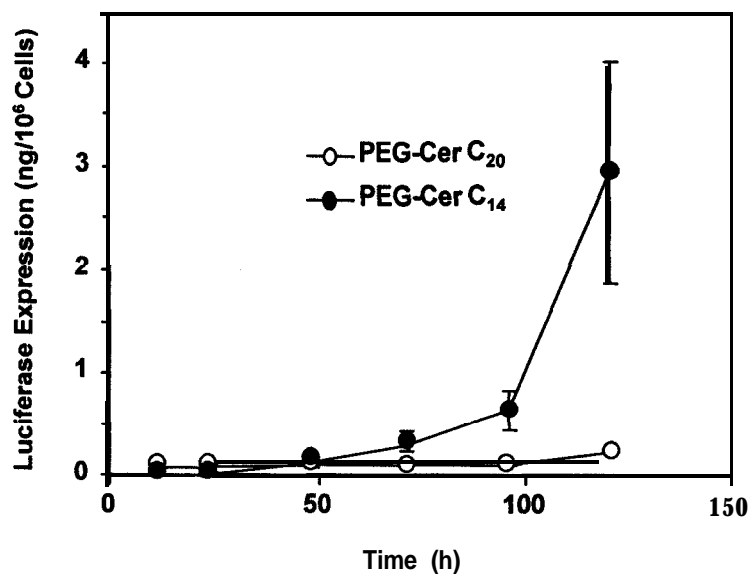


Figure 10. Effect of PEG-ceramide coating of SPLP on transfection activity *in vitro*. Plasmid (pCMVLuc) was encapsulated in SPLP (DOPE/DODAC/PEG-Cer; 84:6:10; mol/mol/mol) containing PEG-CerC₂₀ (○) or PEG-CerC₁₄ (●). The SPLP preparation (1 μg plasmid) was then added to COS-7 cells at a density of 2×10^4 per 24-well plate. The cells were incubated with the SPLP for the times indicated, and luciferase activity was determined. This Figure was published in [71].

3.4. Pharmacokinetics and Tumor accumulation / transfection

As discussed previously, there are two conflicting demands on the carrier system. Initially the particles have to evade uptake by the RES for extended circulation lifetime and accumulation at disease sites. But once at the disease site the particles have to interact with target cells and efficiently deliver its payload to the cell cytoplasm. The solution proposed was a particle with a high concentration of the fusogenic lipid DOPE and stabilized by a polymer coating that slowly exchanges out of the membrane resulting in highly unstable particles. The polymer coating is expected to initially protect the particles from uptake by the RES.

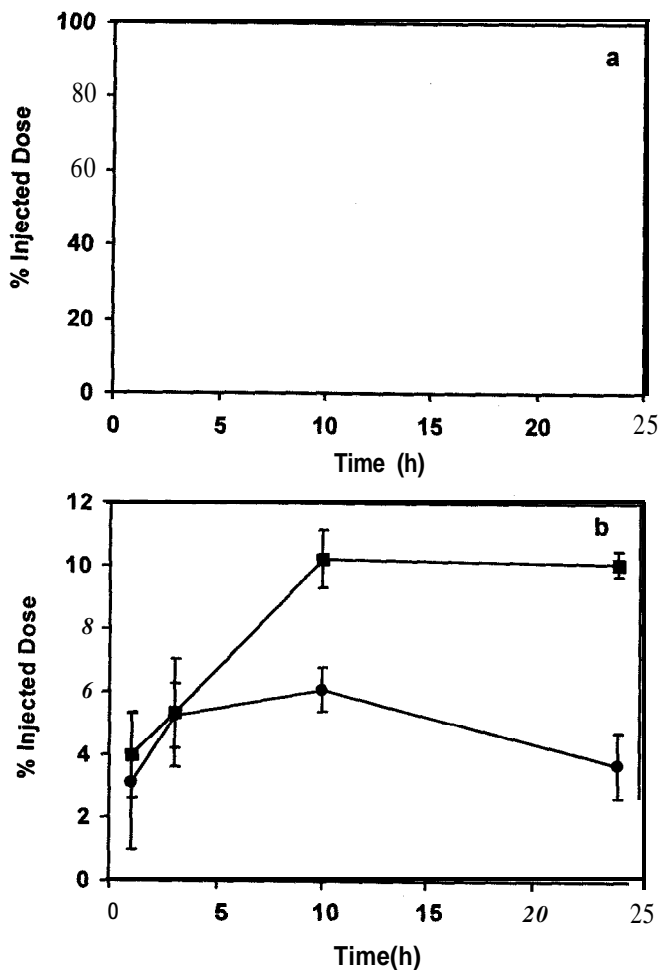


Figure 11. Tumor accumulation and plasma clearance of SPLP in BDF-1 mice bearing Lewis hmg tumor. Mice were seeded with tumor cells and after 14 days injected with DOPE-SPLP at a dose of 30 μ g plasmid DNA and \sim 2 mg lipid. Animals were sacrificed 1, 3, 10 and 24 hours post-injection. Plasma samples (a) and tumor tissue (b) were analyzed for %-lipids by scintillation counting and for intact plasmid by Southern hybridization analysis. The amount of lipid (■) and intact plasmid (●) recovered from tumor tissue, and blood are given as percent injected dose and plotted as a function of time following injection.

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The feasibility of this approach has been confirmed. The SPLP systems described above rely on the stabilizing effects of PEG coatings and become progressively destabilized as the PEG coating dissociates from the liposomes accompanied by a progressive exposure of the positive surface charges. The rate of exchange of PEG-lipid conjugates from the SPLP can be adjusted and depends on the acyl chain lengths. Typical exchange half-times of PEG-ceramides with acyl chain lengths from 8 to 20 carbons from SPLP range from minutes to days [59]. In the absence of PEG-ceramide the SPLP become highly unstable and fusogenic, as expected. Figure 10 shows that SPLP with PEG-CerC₂₀ coating, which has a long residence time in lipid bilayers, exhibits poor transfection properties, whereas greatly improved transfection is observed for the SPLP containing a PEG-CerC₁₄ coating, which can dissociate from lipid bilayers more rapidly. Also, the *in vivo* clearance of SPLP is directly dependent on the acyl chain length. Circulation half lifetimes of approximately 10 hrs were achieved with SPLP containing PEG-CerC₂₀ while SPLPs with PEG-CerC₁₄ were cleared from the blood with a half-time of approximately 1 hr [84]. The prolonged circulation lifetimes and the protection of the plasmid facilitate the accumulation of intact plasmid DNA in disease sites such as tumors following intravenous injection. As shown in Figure 1 la, 80% of the lipid with roughly the same amount of intact plasmid DNA remained in circulation after 1 hr and slowly decreased to 10-20% over the course of 24 hrs [84]. Using a mouse tumor model and SPLP with PEG-CerC₂₀, 6% of the injected dose (30 µg plasmid, 2 mg lipid) was detected in the tumor as intact plasmid 10 h following injection (Figure 1 lb). The amount of intact plasmid delivered to the tumor is substantial, but the transfection activity observed in tumor tissue was quite low (Figure 12). The low levels of gene expression appeared to be related to low cellular uptake of SPLP and / or limited release of plasmid into the cell cytoplasm.

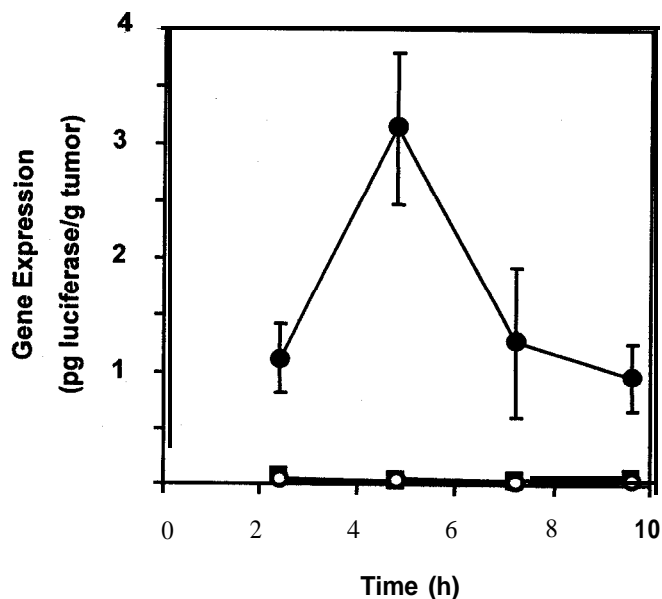


Figure 12. Transgene expression at a distal tumor site following intravenous injection of plasmid encapsulated in SPLP (●) and naked plasmid (■). Mice bearing a subcutaneous Lewis lung carcinoma (-200mg) in the hind flank were injected with SPLP (dose containing 100 µg of pCMVLuc). Tumors were collected at times indicated and assayed for luciferase activity. The level of transgene expression is normalized to weight of tumor tissue. Figure is taken from [86].

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In vitro, cellular uptake and transfection activity could be increased a 1000 fold by increasing the **cationic** lipid concentration in the SPLP and employing a PEG coating with a rapid dissociation rate of minutes (**PEG-CerC₈**) compared to SPLP with 6 mol% DODAC. A similarly increase in transfection activity was observed *in vivo* in a regional tumor model using SPLP with 24 mol% DODAC and **PEG-CerC₈** [59, 82].

4. Conclusions

This first-generation gene carrier system described here meets the first two requirements of a carrier system for systemic gene therapy as outlined in Figure 1. It exhibits circulation longevity and accumulation at a distal tumor site with delivery of intact **plasmid**. These stabilized **plasmid-lipid** particles (SPLP) are small in size with a high plasmid-to-lipid ratio and provide **full** protection of the **plasmid** from degradation by serum **nucleases**. Their small uniform size permits filter-sterilization. The stability of the SPLP observed, showing no changes in size and no loss of transfection activity over a one-year period is remarkable. The exchangeable PEG coating solved in part the conflicting demands of a highly stable carrier that initially can evade RES uptake to achieve extended circulation lifetimes, but following accumulation at the disease site, become **fusogenic** to promote target cell interaction, cellular uptake and delivery of **plasmid** to the cytoplasm. The transfection activity observed at the tumor site was low, even so, the amount of intact **plasmid** delivered was high and reflected the lipid accumulation. **Transfection** experiments *in vitro* and in a regional tumor model, using SPLP containing different concentrations of **cationic** lipid demonstrate that the transfection activity can be increased significantly by increasing the **cationic** lipid content in the SPLP. The highest transfection activity was observed with SPLP containing approximately 25 mol% **cationic** lipid. The increased transfection potency was attributed to an increased electrostatic interaction between the higher positively charged SPLP and target cells, leading to more efficient cellular uptake. This indicates that the barriers for efficient **plasmid** delivery are presently the cellular uptake of SPLP and release of intact **plasmid** into the cytoplasm of target cells. The next generation carrier must overcome these limiting steps. The highly flexible formulation process and the modular nature of the SPLP permit substitution and evaluation of individual carrier components that may affect the limiting steps for efficient intracellular **plasmid** delivery. Targeting components to initiate selective cellular uptake at the disease site and **fusogenic** components such as **pH-sensitive** lipids, **peptide** derivatives of **fusogenic** proteins, that become exposed following exchange of the polymer coating, may be required to trigger the efficient release of **plasmid** from endosomes and these are presently under investigation.

Acknowledgement

This work was supported by the Medical Research Council of Canada and Inex Pharmaceuticals together with the National Science and Engineering Research Council of Canada through the Collaborative Research and Development Program.

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