



ACQUIRED DISEASES

RESEARCH ARTICLE

Stabilized plasmid-lipid particles for systemic gene therapy

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The structure of 'stabilized plasmid-lipid particles' (SPLP) and their properties as systemic gene therapy vectors has been investigated. We show that SPLP can be visualized employing cryo-electron microscopy to be homogeneous particles of diameter 72 ± 5 nm consisting of a lipid bilayer surrounding a core of plasmid DNA. It is also shown that SPLP exhibit long circulation lifetimes (circulation half-life >6 h) following intravenous (i.v.) injection in a murine tumor model resulting in accumulation of up to 3% of the total injected dose and concomitant reporter gene expression at a distal (hind flank) tumor site. In contrast, i.v. injection of

naked plasmid DNA or plasmid DNA–cationic liposome complexes did not result in significant plasmid delivery to the tumor site or gene expression at that site. Furthermore, it is shown that high doses of SPLP corresponding to 175 μ g plasmid per mouse are nontoxic as assayed by monitoring serum enzyme levels, whereas i.v. injection of complexes give rise to significant toxicity at dose levels above 20 μ g plasmid per mouse. It is concluded that SPLP exhibit properties consistent with potential utility as a nontoxic systemic gene therapy vector. Gene Therapy (2000) 7, 1867–1874.

Keywords: liposomes; cancer gene therapy; intravenous gene therapy; tumour transfection

Introduction

Gene therapies for systemic diseases such as cancer or inflammatory disorders clearly require systemic vectors. However, currently available vectors for gene therapy have limited utility for systemic applications. Recombinant virus vectors, for example, are rapidly cleared from the circulation following intravenous injection, limiting potential transfection sites to 'first pass' organs such as the liver.^{1,2} Nonviral systems, such as plasmid DNA–cationic liposome complexes, are also rapidly cleared from the circulation, and the highest expression levels are again observed in first pass organs, particularly the lungs.^{3–8}

Intravenous administration of chemotherapeutic drugs encapsulated in small (diameter ≤ 100 nm), long-circulating (circulation half-life $t_{1/2} \geq 5$ h in murine models) liposomes results in preferential delivery of encapsulated drug to distal tumors due to increased vascular permeability in these regions.^{9–11} It therefore follows that intravenous injection of plasmid DNA encapsulated in small, long circulating lipid particles should give rise to preferential delivery of plasmid DNA to tumor sites. Recent work has shown that plasmid DNA can be encapsulated in small (approximately 70 nm diameter) 'stabilized plasmid-lipid particles' (SPLP) that contain one plasmid per particle.¹² These particles contain the 'fusogenic'

lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. Here, we show that the structure of SPLP can be directly visualized employing cryo-electron microscopy to reveal homogeneous particles consisting of plasmid DNA entrapped within a bilayer lipid vesicle. Furthermore, we show that these SPLP exhibit long circulation lifetimes and no evidence of systemic toxicities following i.v. injection in a murine tumor model. Under the experimental conditions employed, approximately 3% of the total injected SPLP dose was delivered to a subcutaneous tumor site and 1.5 % of the total intact plasmid dose could be detected at the tumor site at 24 h. Significant levels of reporter gene expression were observed at the tumor site employing the SPLP system, whereas no expression was observed following i.v. injection of 'naked' plasmid DNA or plasmid DNA–cationic liposome complexes.

Results

SPLP consist of a plasmid trapped inside a bilayer lipid vesicle

Previous work has shown that plasmid DNA can be encapsulated (trapping efficiency approximately 70%) in SPLP by a detergent dialysis procedure employing octylglucopyranoside (OGP).¹² These SPLP are composed of DOPE, 5–10 mol% of the cationic lipid dioleoyldimethylammonium chloride (DODAC) and PEG attached to a

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(PEG-CerC₂₀). SPLP can be separated from non-encapsulated plasmid by ion exchange chromatography and can then be further purified by density gradient centrifugation to remove empty vesicles produced during the dialysis procedure. On the basis of the size and plasmid-to-lipid ratio of these purified SPLP it was determined that each SPLP contained one plasmid molecule.¹²

Here, we further characterize SPLP structure employing cryo-electron microscopy. Following the procedures summarized in Materials and methods, purified SPLP were prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) and pCMVluc, whereas large unilamellar vesicles (LUV) with the same lipid composition were prepared by extrusion of the hydrated lipid mixture through 100 nm pore size filters. As shown in Figure 1a, the cryo-electron micrographs clearly reveal SPLP to con-

sist of a lipid bilayer surrounding an internal structure consistent with entrapped plasmid DNA molecules. Small (diameter approximately 30 nm), empty vesicles formed during the detergent dialysis process¹³ that were not removed by density centrifugation do not exhibit such internal structure (see arrows in Figure 1a). This internal structure is also not observed in the LUV produced by extrusion (Figure 1b). It may also be noted that SPLP as detected by cryo-electron microscopy have a remarkably homogeneous size (diameter 72 ± 5 nm), in close agreement with measurements of SPLP diameter employing freeze-fracture electron microscopy (diameter 64 ± 9 nm).¹² The homogeneous size and morphology of SPLP contrasts with the irregular morphology and large size distribution of the extruded vesicles. The narrow size distribution of SPLP was also reflected by quasi-elastic light scattering (QELS) measurements (data not shown) which indicated a mean diameter of 83 ± 4 nm. Plasmid DNA-cationic liposome complexes made from DOPE:DODAC (1:1; mol:mol) LUV exhibited a large, heterogeneous size distribution as determined by QELS (diameter 220 ± 85 nm, data not shown).

SPLP exhibit extended circulation lifetimes, preferential accumulation at tumor sites, and low systemic toxicities following intravenous injection

The next set of experiments was aimed at characterizing the pharmacokinetics and biodistribution of SPLP following i.v. injection into tumor-bearing mice. SPLP were prepared with trace amounts of the lipid label, ³H-cholesteryl hexadecylether (³H-CHE) and were injected at a dose level equivalent to 100 μg plasmid DNA per mouse into C57Bl/6 mice bearing a subcutaneous Lewis lung carcinoma (approximately 200 mg) in the hind flank. The clearance of SPLP from the circulation as assayed by the lipid label (Figure 2a) corresponds to a first order process with a *t*_{1/2} of 6.4 ± 1.1 h. Relatively low levels of uptake by the lung and liver are observed (Figure 2b and c) whereas approximately 3% of the injected SPLP dose accumulates at the tumor site over 24 h (Figure 2d). Such tumor accumulation levels are comparable with those achieved for small, long-circulating liposomes containing conventional drugs such as doxorubicin, where approximately 5% of the injected dose can be found at 24 h in larger (>0.5 g) tumors.¹⁴ In contrast to the behavior of the SPLP system, ³H-CHE-labeled plasmid DNA-cationic liposome complexes were rapidly cleared from the circulation (*t*_{1/2} < 15 min), appearing predominantly in the lung and liver, and less than 0.2% of the injected dose was found at the tumor site at 24 h. The biodistribution of ³H-CHE labeled SPLP and complexes 4 and 24 h following injection are summarized in Table 1. Only trace amounts were detected in kidney, heart and lymph nodes.

The levels of intact plasmid DNA in the circulation and tumor tissue following i.v. injection of naked plasmid DNA, plasmid DNA-cationic lipid complexes and SPLP were analyzed by Southern blot hybridization (Figure 3a, b and c, respectively) and quantified by phosphorimaging analysis (Figure 3d and e). For naked plasmid, less than 0.01% of the injected dose remained intact in the circulation at 15 min, and no intact tumor-associated plasmid could be observed at any time. For plasmid administered in complexes, only a small fraction (<2%) was still intact in the circulation at 15 min and less than

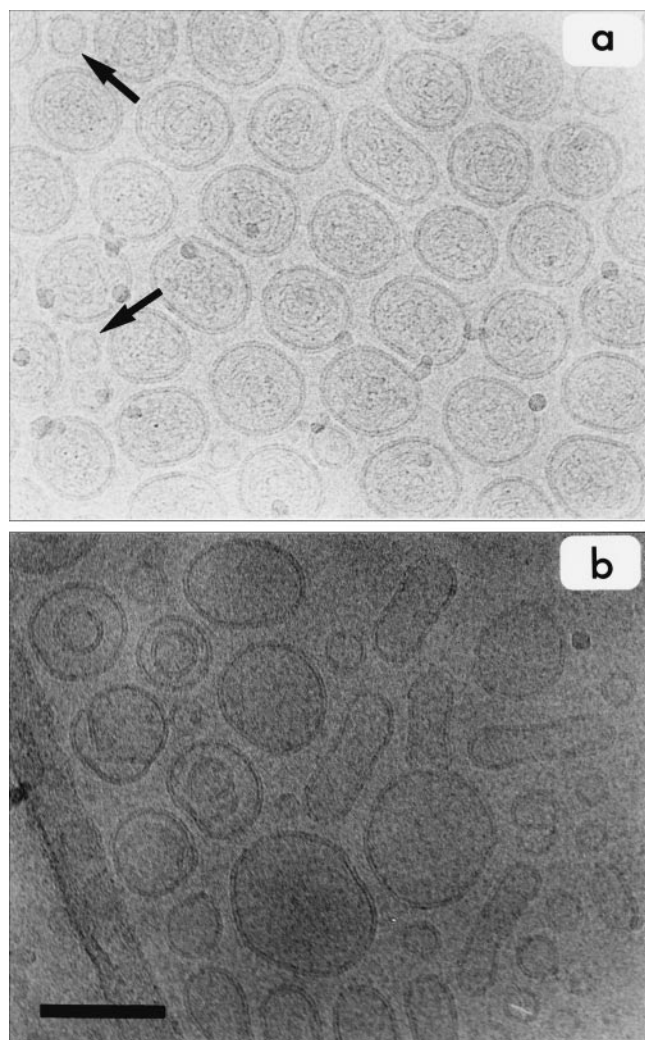


Figure 1 Cryo-electron micrographs of (a) purified SPLP and (b) LUV prepared by extrusion. SPLP were prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) and pCMVluc and purified employing DEAE column chromatography and density gradient centrifugation. LUV were prepared from DOPE:DODAC:PEG-CerC₂₀ (83:7:10; mol:mol:mol) by hydration and extrusion through filters with 100 nm diameter pore size. The arrows in panel (a) indicate the presence of residual 'empty' vesicles formed during the detergent dialysis process that were not removed by the density centrifugation purification step. The bar in panel (b) indicates 100 nm. For details of sample preparation and cryo-electron

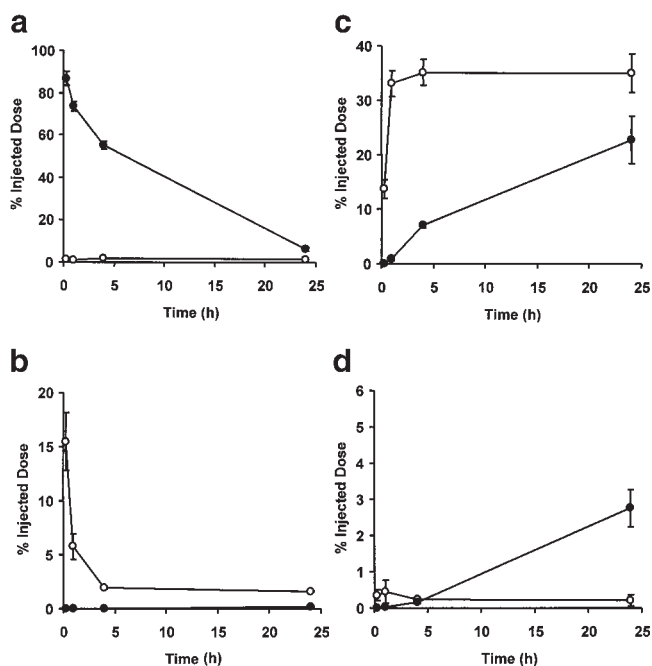


Figure 2 Pharmacokinetics, tissue distribution and tumor accumulation of SPLP and plasmid DNA-cationic liposome complexes following intravenous administration in tumor-bearing mice as reported by the ³H-CHE lipid marker. The levels of complexes (○) and SPLP (●) in the circulation, the lung, the liver and in Lewis lung tumor tissue are shown in panels (a), (b), (c) and (d), respectively. The accumulations in liver, lung and tumor were corrected for plasma contributions²⁹ and are expressed as a percentage of the total injected dose.

Table 1 Biodistribution of SPLP and plasmid DNA-cationic liposome complexes in mice 4 and 24 h following i.v. injection

Tissue	% Injected dose (s.e.m.)			
	SPLP		Complexes	
	4 h	24 h	4 h	24 h
Plasma	55.0 (1.7)	6.4 (1.0)	1.7 (0.2)	1.4 (0.3)
Liver	7.0 (0.6)	23.0 (4.3)	35.2 (2.3)	35.1 (3.5)
Lung	0.0 (0.1)	0.2 (0.1)	1.8 (0.8)	0.5 (0.0)
Spleen	0.4 (0.1)	1.6 (0.1)	0.2 (0.2)	0.1 (0.3)
Tumor	0.2 (0.0)	2.8 (0.5)	0.2 (0.1)	0.3 (0.2)

Both SPLP and complexes contained pCMVLuc as well as trace levels of the ¹⁴C-labeled CHE lipid marker and were administered at a dose level of 100 μg plasmid per mouse. The biodistribution was measured employing the CHE lipid marker. s.e.m., standard error of the mean.

contrast, following i.v. injection of SPLP, approximately 85% of the injected plasmid DNA remained in intact form in the circulation at 15 min, and progressively higher levels of intact plasmid accumulated at the tumor site over the time-course of the experiment. The levels achieved at 24 h correspond to approximately 1.5% of the total injected plasmid DNA dose. The circulation half-life of intact plasmid DNA following injection of SPLP was calculated to be 7.2 ± 1.6 h, in good agreement with the circulation half-life of ³H-CHE-labeled SPLP, confirming the highly stable nature of SPLP in the circulation.

(ALT) or aspartate aminotransferase (AST) were assayed for evidence of toxicity following i.v. administration of SPLP and plasmid DNA-cationic liposome complexes. Elevated ALT and AST levels are usually associated with liver damage, although elevated AST levels can also indicate systemic tissue damage. Mice receiving SPLP at dose levels as high as 175 μg plasmid DNA per mouse did not have significantly elevated serum levels of ALT and AST (Figure 4a). However, mice receiving doses of plasmid DNA-cationic liposome complexes corresponding to plasmid doses above 20 μg per mouse exhibited progressively higher serum levels of ALT and AST, reaching levels 100-fold above normal levels at plasmid doses of 75 μg (Figure 4b).

Intravenously administered SPLP promote gene expression in a distal tumor

It is of obvious interest to determine whether SPLP-mediated delivery of intact plasmid to the tumor site results in transgene expression at that site. Luciferase gene expression in tumor tissue was therefore monitored following i.v. injection of SPLP, naked plasmid DNA and plasmid DNA-cationic liposome complexes at dose levels corresponding to 100 μg plasmid DNA per mouse. This dose level corresponded to the maximum tolerated dose of complexes as evidenced by animal morbidity and mortality. As shown in Figure 5, administration of SPLP results in reporter gene expression at the tumor site, with maximum levels corresponding to 32 pg luciferase per gram of tumor tissue at the 48 h time-point and significant gene expression extending to 96 h after injection. Injection of free plasmid DNA or plasmid DNA-cationic liposome complexes, on the other hand, resulted in no detectable gene expression at the tumor site. It is of interest to note that i.v. administration of complexes did result in transfection in the lung, liver and spleen, whereas administration of SPLP did not result in detectable levels of gene expression in these organs (data not shown). In an attempt to understand why SPLP did not give rise to significant gene expression in the liver, the levels of intact plasmid in the liver 24 h after injection of SPLP into C57BI/6 mice (100 μg plasmid per mouse) bearing a subcutaneous Lewis lung carcinoma were analyzed by Southern blot hybridization. No intact plasmid could be detected in the liver whereas intact plasmid was readily detected at the tumor site (results not shown). This suggests that the ability of SPLP to transfect cells at the tumor site but not in the liver may reflect relatively rapid breakdown of SPLP and associated plasmid following uptake into liver phagocytes (Kupffer cells), which play a dominant role in clearing liposomal systems from the circulation.¹⁵ Lower gene expression in the liver may also reflect the finding that nonviral vectors such as SPLP transfect dividing cells much more efficiently than non-dividing cells¹⁶ or that Kupffer cells are less readily transfected than tumor cells.

Discussion

This study demonstrates that SPLP consist of plasmid DNA encapsulated in a bilayer vesicle, and that systemic administration of SPLP results in significant accumulation and transfection at a distal tumor site. There are three important features of these results. The first con-

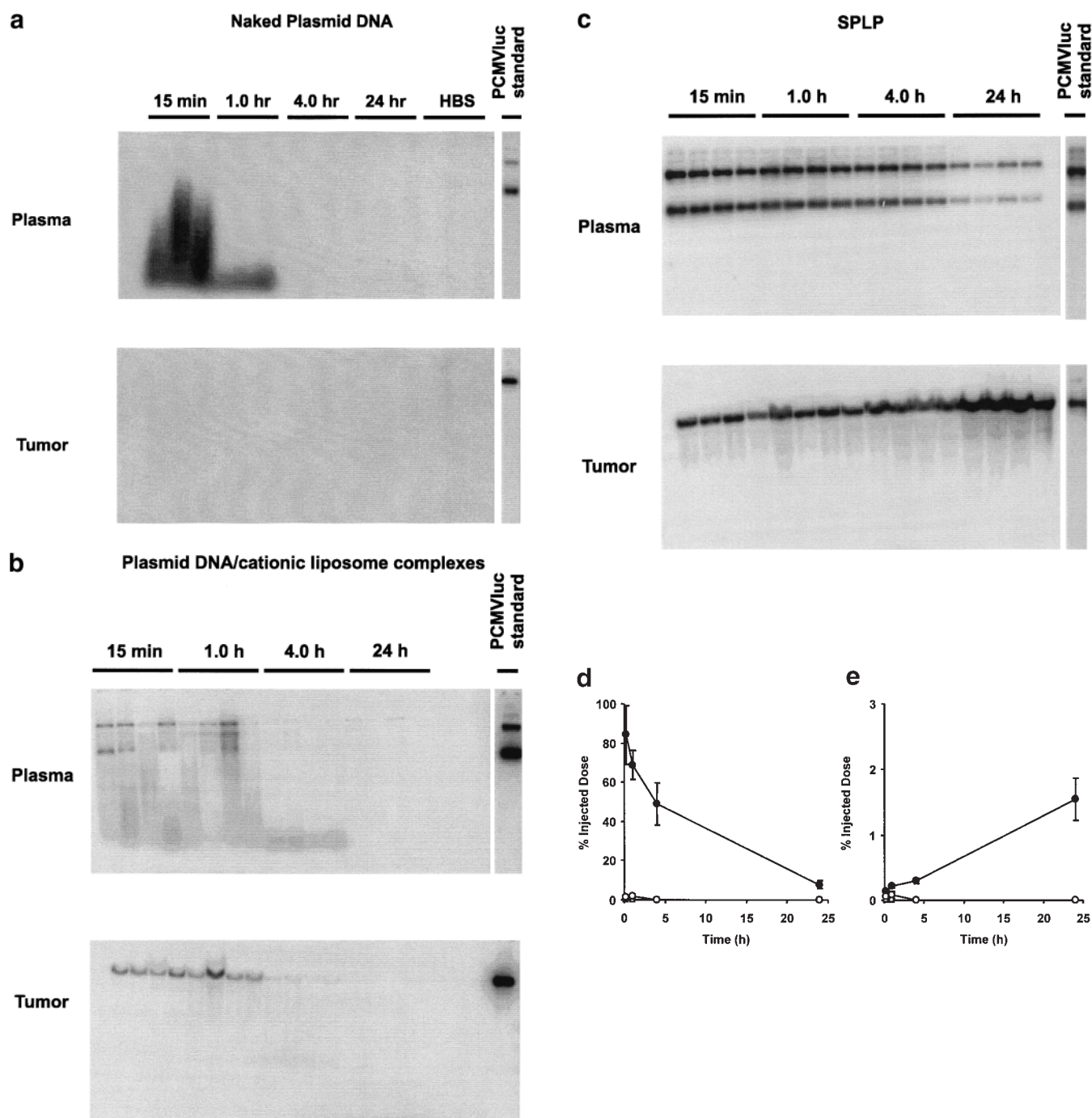


Figure 3 Pharmacokinetics and tumor accumulation of plasmid DNA following intravenous administration of naked plasmid, plasmid DNA–cationic liposome complexes and SPLP as reported by a Southern blot analysis. The Southern blot hybridizations shown in panels (a), (b) and (c) result from plasmid DNA isolated from blood and tumor tissue of mice injected with naked plasmid DNA, plasmid DNA–cationic lipid complexes and SPLP, respectively. Each panel shows pCMVluc (2 ng) to indicate the position of intact plasmid DNA. The levels of intact plasmid resulting from i.v. injection of naked plasmid DNA (□), plasmid DNA–cationic liposome complexes (○) and SPLP (●) were quantified for plasma (panel d) and tumor tissue (panel e) by phosphor-imaging analysis and converted to mass quantities of plasmid DNA by comparison to a standard curve made from known amounts of plasmid DNA. Tumor accumulations of plasmid were corrected for plasma contributions and expressed as a percentage of the total injected plasmid DNA dose.²⁹

advance for plasmid encapsulation in liposomal delivery systems. Second, it is of interest to compare the properties of the SPLP system for systemic gene delivery and distal tumor transfection with the properties of other

capabilities for further optimization of the SPLP system are of interest. We discuss these areas in turn.

The cryo-electron microscopy results presented here establish the structure of SPLP as a plasmid surrounded

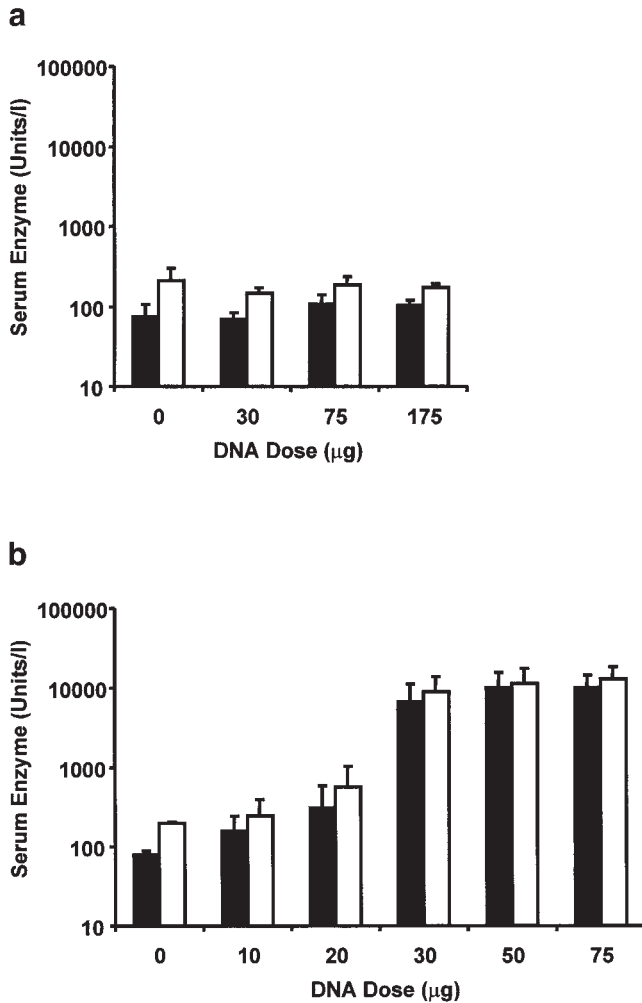


Figure 4 Toxicity resulting from i.v. injection into mice of varying amounts of SPLP (panel a) and plasmid DNA-cationic lipid complexes (panel b) as assayed by determining serum levels of the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (ASP). The serum levels of AST (□) and ALT (■) were measured 24 h after injection.

demonstration that plasmid can be entrapped in small (diameter approximately 70 nm), well defined vesicular systems containing a single plasmid per vesicle. Entrapment of a plasmid such as pCMVluc, which contains 5650 bp, in a supercoiled configuration in a 70 nm diameter vesicle represents a solution for a difficult packing problem. For example, electron micrographs of supercoiled 4.4 kbp plasmids reveals extended lengths of approximately 500 nm and average (two dimensional) diameters in the range of 350 nm, suggesting an average diameter for free supercoiled pCMVluc of approximately 400 nm.¹⁷ The detergent dialysis process clearly involves a partial condensation of entrapped plasmid to allow encapsulation in a 70 nm diameter vesicle. The mechanism of entrapment is not understood in detail, but appears to proceed via association of plasmid with lipid structures formed as intermediates in the detergent dialysis process.¹²

SPLP exhibit extended circulation lifetimes ($t_{1/2}$ approximately 7 h) following i.v. injection, can deliver significant

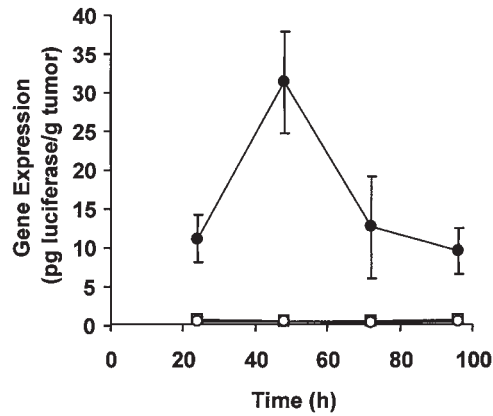


Figure 5 Transgene expression at a distal tumor site following intravenous injection of naked plasmid DNA (□), plasmid DNA-cationic lipid complexes (○) and SPLP (●). Mice bearing subcutaneous Lewis lung tumors were injected i.v. with doses containing 100 μg of pCMVluc. Tumors were harvested at the indicated times and assayed for luciferase activity. The level of transgene expression reported is normalized for the weight of the tumor tissue.

enable transgene expression corresponding to 30 pg luciferase per gram tumor at that site with no evidence of toxicity (as indicated by serum enzyme levels). Delivery of approximately 3% of the injected dose of SPLP at a 200 mg tumor site corresponds to more than 1000 plasmid copies per tumor cell, assuming a cell density of 1×10^9 per milliliter. It is of interest to compare these properties with the behavior of other gene delivery systems. In the case of viral vectors, there have been three reports of transgene expression in liver metastases and in a distal tumor following systemic administration of a recombinant vaccinia virus^{18,19} and a selectively replicating adenovirus.²⁰ These viral vectors are replication incompetent in normal nondividing cells but can selectively replicate in tumor cells resulting in transgene expression in tumors and antitumoral efficacy. The major drawback of these viral vectors is the immune response, which occurs within 6 days. In the case of nonviral vectors such as plasmid DNA-cationic polymer 'polyplexes', there is only one report showing transfection of distal tumors following i.v. injection.²¹ This work utilized a PEG-containing polyplex that exhibits plasmid circulation half-lives of less than 0.5 h following intravenous injection and gave rise to transfection at a distal tumor site, achieving transfection levels corresponding to approximately 250 pg/g tumor, approximately eight-fold higher than the levels reported here.

With regard to plasmid DNA-cationic liposome complexes ('lipoplexes'), a number of studies have characterized transfection properties following i.v. administration,³⁻⁷ however, only two studies by Xu and co-workers have demonstrated transfection at a distal tumor site.^{22,23} In the initial study,²² less than 5% of the cells at the tumor site were transfected as indicated by immunohistochemical staining, whereas in the second study using transferrin targeted complexes 20-30% of the cells were transfected. The levels of gene expression could not be related to the levels observed here. Issues related to circulation lifetimes, plasmid tumor accumulation and toxicity were not addressed. An additional study²⁴ has demonstrated the presence of complexes at a

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