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Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties

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Previous work (Wheeler et al, Gene Therapy 1999; **6***: 271–281) has shown that plasmid DNA can be entrapped in 'stabilized plasmid-lipid particles' (SPLP) containing the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), low levels (5–10 mol%) of cationic lipid, and stabilized by a polyethyleneglycol (PEG) coating. The PEG moieties are attached to a ceramide anchor containing an arachidoyl acyl group (PEG-CerC_{20}). These SPLP exhibit low transfection potencies in vitro, due in part to the long residence time of the PEG-CerC_{20} on the SPLP surface. In this work we employed SPLP stabilized by PEG attached to ceramide containing an octanoyl acyl group (PEG-CerC_8), which is able to quickly exchange out of the SPLP, to develop systems that give rise to optimized in vitro and in vivo (regional) transfection. A particular objective was to achieve cationic lipid contents that give rise to maximum transfection levels. It is shown that by performing the dialysis procedure in the presence of increasing concentrations of citrate, SPLP containing up to 30 mol% of the cationic lipid dioleoydimethylammonium chloride (DODAC) could be generated. The SPLP produced could be isolated from empty vesicles by sucrose density gradient centrifugation, and exhibited a narrow size distribution (62 ± 8 nm, as determined by freeze–fracture electron microscopy) and a high plasmid-to-lipid ratio of 65 μg/μmol (corresponding to one plasmid per particle) regardless of the DODAC content. It was found that isolated SPLP containing 20–24 mol% DODAC resulted in optimum transfection of COS-7 and HepG2 cells in vitro, with luciferase expression levels comparable to those achieved for plasmid DNA–cationic lipid complexes. In vivo studies employing an intraperitoneal B16 tumor model and intraperitoneal administration of SPLP also demonstrated maximum luciferase expression for DODAC contents of 20–24 mol% and significantly improved gene expression in tumor tissue as compared with complexes. We conclude that SPLP stabilized by PEG-CerC_8 and containing 20–24 mol% cationic lipid are attractive alternatives to plasmid DNA–cationic lipid complexes for regional gene therapy applications.*

Keywords: *non-viral gene delivery; cationic lipid; detergent dialysis; cancer gene therapy; plasmid encapsulation; liposomes*

Introduction

Non-viral gene delivery systems offer many potential advantages over viral vectors, including avoidance of concerns related to viral immunogenicity and reversion to the infectious phenotype. In addition, well-defined synthetic gene delivery systems offer possible advantages with respect to vector manufacture and characterization. However, the most commonly employed non-viral systems, plasmid DNA–cationic lipid complexes[1–3] formed by adding plasmid to liposomes containing cationic lipids, are not well-defined systems. These complexes are often unstable, are susceptible to aggregation and exhibit transfection potencies that can be sample- and time-dependent. In addition, complexes do not have general applicability. For example, whereas complexes usually exhibit reasonable transfection potency *in vitro*,[4–7] these large systems are generally cleared rapidly following intravenous injection, which limits potential transfection sites to 'first-pass' organs such as the lung, liver and spleen.[8–12] Furthermore, DNA–cationic lipid complexes can also produce toxic side-effects *in vitro*[13] and *in vivo*.[14]

Efforts in our laboratory have focused on the development of small, well-defined lipid-based plasmid carrier systems with general applicability, where the plasmid is fully encapsulated within a lipid envelope. Initial studies have shown that plasmid can be entrapped in 'stabilized plasmid-lipid particles' (SPLP) through a detergent dialysis procedure, resulting in particles of approximately 70 nm in diameter containing one plasmid per particle.[15] These systems contain the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) and a small amount of cationic lipid, and are stabilized in aqueous media by the presence of a polyethylene glycol (PEG) coating. It has also been established that the *in vitro* transfection potency of SPLP is dependent on the hydrophobic ceramide (Cer) group anchoring the PEG polymer to the SPLP, where ceramide groups containing shorter acyl groups exhibit improved transfection properties.[15] This has been attributed to the ability of PEG-Cer molecules with shorter acyl groups to dissociate from the SPLP, thereby destabil-

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izing the particle and improving association with and uptake into target cells. In this regard, it is well established that the presence of a PEG coating can inhibit association and fusion between large unilamellar vesicles (LUVs)[16,17] and between LUVs and biological membranes.[18]

This work focuses on the development of SPLP that exhibit optimized transfection properties *in vitro* and that have regional application *in vivo*. This application requires that the PEG coating rapidly dissociates from the carrier, therefore, SPLP have been constructed employing PEG-Cer molecules containing an octanoyl group (PEG-$CerC_8$). Previous work has shown that PEG-$CerC_8$ molecules exhibit half-times ($t_{1/2}$) for dissociation from LUV (in the presence of 'acceptor' LUV) of less than 1.2 min,[18] whereas PEG-$CerC_{14}$ and PEG-$CerC_{20}$ exhibit $t_{1/2}$ values of 1.2 h and greater than 13 days, respectively.[15] Particular attention has been paid to developing SPLP containing optimized amounts of cationic lipid. The previously described SPLP protocol was limited to low (5–10 mol%) cationic lipid contents in order to achieve efficient plasmid encapsulation.[15] In this work, it is shown that SPLP containing up to 30 mol% cationic lipid can be generated by including increasing amounts of citrate in the dialysis medium, and that optimum *in vitro* transfection potencies are obtained at approximately 24 mol% cationic lipid contents. The transfection potencies observed for regional transfection of peritoneal tumors employing these optimized SPLP are superior to those observed using plasmid DNA–cationic lipid complexes.

Results

Plasmid can be encapsulated in SPLP containing high levels of cationic lipid by employing citrate in the dialysis medium

Previous work has shown that plasmid can be encapsulated in SPLP with the lipid composition DOPE, dioleoyldimethylammonium chloride (DODAC) and PEG-$CerC_{20}$ (84:6:10; mol:mol:mol) by a detergent dialysis procedure employing octylglucoside.[15] Under the conditions used, the cationic lipid content was critical, as plasmid encapsulation levels approaching 70% could be achieved for DODAC contents of 6–7 mol%. Little encapsulation was obtained at lower DODAC levels, however, and at DODAC contents above 9% significant aggregation problems were encountered. Similar behavior was observed here for SPLP containing DOPE/DODAC/PEG-$CerC_8$ (78:7:15; mol:mol:mol). Following dialysis against HBS (5 mm HEPES, 150 mm NaCl, pH 7.4), small (diameter $<$70 nm) vesicles exhibiting high encapsulation efficiencies of 80% were formed (see Table 1). The degree of plasmid encapsulation was estimated by determining the relative accessibility of a DNA interchelating fluorescent dye (PicoGreen) to the plasmid in the presence and absence of Triton $\times$-100, as described in Materials and methods. DODAC contents above 10 mol% caused aggregation of the formulation during dialysis.

It has been proposed that SPLP form when plasmids interact with lipid structures exhibiting an appropriate surface charge during the detergent dialysis process.[15] Therefore, it was reasoned that encapsulation into SPLP with higher cationic lipid content might be possible if the ionic strength of the dialysis medium was raised to shield the surface charge on the lipid structures. Initial experiments employed higher NaCl concentrations ranging from 0.15 to 1.0 m NaCl (with 10 mm HEPES, pH 7.2). Increasing the NaCl concentration to 0.5 m was sufficient to prevent aggregation for a formulation containing 10 mol% DODAC (results not shown), but was not effective in a formulation containing 20 mol% DODAC, where aggregation persisted even up to 1 m NaCl. Polyvalent anionic counter-ions such as citrate may be expected to produce stronger shielding effects. A study of plasmid encapsulation as a function of citrate concentration was performed for a lipid mixture composed of DODAC/DOPE/PEG-$CerC_8$ (20:65:15; mol:mol:mol) and the pCMVLuc plasmid. As shown in Figure 1, at concentrations up to 60 mm citrate the dialyzed samples contained large (diameter $>$150 nm) and polydisperse (χ^2 $>$3.0) particles. However, small, monodisperse particles (82 $\pm$ 40 nm diameter as measured by quasi-elastic light scattering; QELS) exhibiting high encapsulation efficiencies of 50–70% were formed when the dialysis medium contained 65–80 mm citrate. Increasing the citrate concentration further also resulted in formation of small particles, but the encapsulation efficiency decreased dramatically.

The results shown in Figure 1 suggest two criteria for determining the optimum citrate concentration for plasmid encapsulation: (1) formation of monodisperse (χ^2 $<$3.0) particles with diameter smaller than 100 nm; and (2) an encapsulation efficiency greater than 50%. Studies to determine citrate concentrations that satisfy these criteria over a range of DODAC concentrations were performed, and the results are summarized in Figure 2. The citrate concentration range giving rise to particles with a diameter smaller than 100 nm and with encapsulation efficiencies of 50% or higher is represented by the solid circles. 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 (χ^2 $>$5). The plasmid encapsulation efficiencies that could be achieved at optimum citrate concentrations for formulations containing up to 30 mol% DODAC are summarized in Table 1.

It was not possible to obtain satisfactory formulations for preparations containing 30 mol% DODAC by varying the citrate concentration. Aggregation persisted in formulations dialyzed in 70–90 mm citrate buffer, while at higher concentrations the plasmid encapsulation was less than 25%. Improved results were achieved, however, by leaving the citrate concentration constant at 100 mm and varying the NaCl concentration. Decreasing the NaCl concentration from 150 mm to 120–140 mm resulted in an increase in the plasmid encapsulation efficiency to 55–70% (Figure 3) while the particle size remained small ($<$100 nm diameter).

Lipid composition, plasmid-to-lipid ratio and size of isolated SPLP

Previous work has shown that SPLP formulations containing 7 mol% DODAC contain empty vesicles and unencapsulated plasmid in addition to SPLP, and that the free plasmid can be removed by passage over a DEAE column, whereas the removal of empty vesicles requires an additional density gradient centrifugation step.[15] In this study it was found that similar procedures could be

Table 1 Physical properties of SPLP formulations containing 7–30 mol% DODAC

DODAC content (mol%)[a]	*After detergent dialysis*			*After isolation by density gradient centrifugation*	
	Diameter (nm)[b]	$\chi^{2\,b}$	*Encapsulation efficiency (%)*[c]	*Diameter*	$\chi^{2\,b}$
7	64 ± 28	1.0	80.5	101 ± 11	0.4
12	68 ± 29	1.1	77.5	91 ± 29	0.6
16	75 ± 34	2.9	68.2	95 ± 21	0.3
20	82 ± 41	0.6	65.9	99 ± 22	0.3
24	97 ± 60	1.7	69.4	94 ± 30	0.9
28	101 ± 59	1.7	47.5	ND	ND
30	81 ± 48	ND	54.4	115 ± 57	1.7

[a]DODAC content in SPLP formulations composed of DODAC/DOPE/PEG-CerC_8 (x:85-x:15; mol:mol:mol).
[b]Mean diameter (± standard deviation) and polydispersity (χ^2) of the SPLP formulations after dialysis as measured by QELS using the volume-weighted vesicle mode.
[c]Plasmid encapsulation efficiency determined by the PicoGreen assay.
ND, not determined.

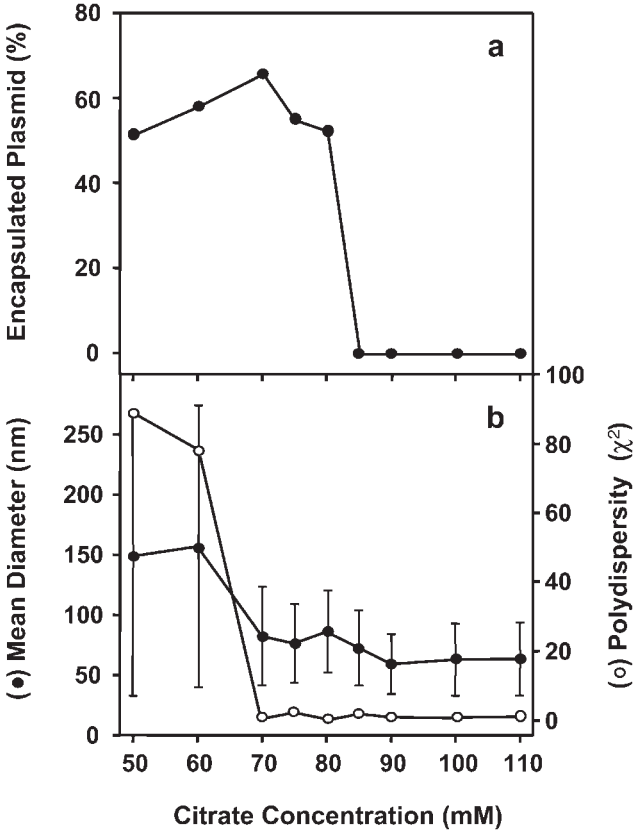

Figure 1 *Plasmid DNA can be entrapped in SPLP containing high levels of DODAC by raising the citrate concentration present during detergent dialysis. (a) The effect of varying the citrate concentration on plasmid encapsulation efficiency following detergent dialysis, as determined by the PicoGreen fluorescence assay (see Materials and methods). (b) The effect of citrate on the diameter (●) and polydispersity, χ^2 (○), of the formulations following detergent dialysis as measured by QELS (volume-weighted vesicle mode). Formulations were composed of DODAC/DOPE/PEG-CerC_8 (20:65:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 200 μg plasmid per ml), and were prepared by detergent dialysis as described under Materials and methods.*

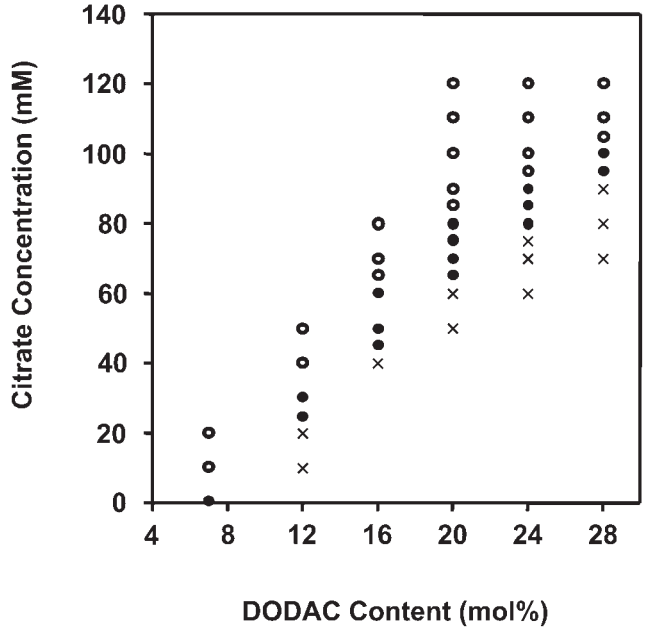

Figure 2 *Determination of the optimal citrate concentration range as a function of SPLP DODAC content to achieve maximum plasmid encapsulation in combination with minimum aggregation. Formulations were composed of DODAC/DOPE/PEG-CerC_8 (x:85-x:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 100–200 μg 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, χ^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, χ^2 <3). The crosses (x) indicate polydisperse formulations with large size distributions (diameter >100 nm, χ^2 >3).*

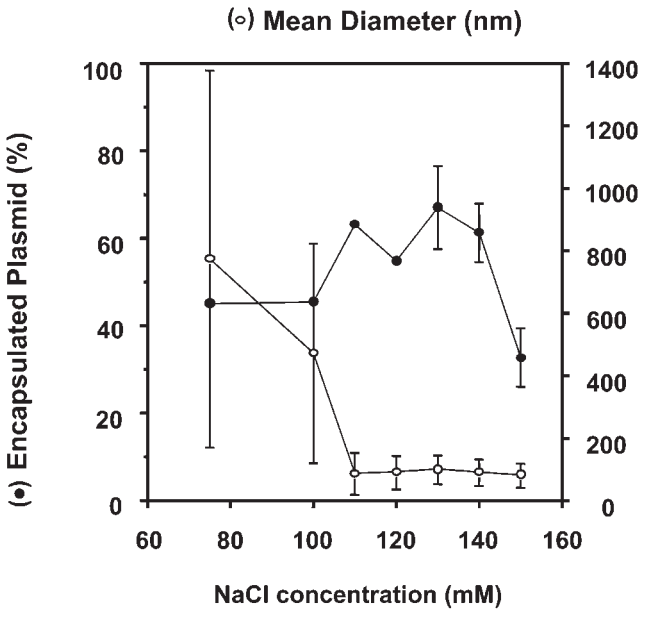

Figure 3 *Plasmid encapsulation in SPLP at a given DODAC content and citrate concentration can be optimized by varying the NaCl concentration. The formulations were composed of DOPE/DODAC/PEG-CerC_8 (55:30:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 200 μg plasmid per ml), and were prepared by detergent dialysis where the dialysate contained varying amounts of NaCl and 100 mm sodium citrate, 10 mm HEPES (pH 7.2). The closed circles (●) indicate the plasmid encapsulation efficiencies determined employing the PicoGreen fluorescence assay. The open circles (○) indicate the mean diameter (± standard deviation) of the formulations following dialysis, as measured by QELS (volume-weighted vesicle mode).*

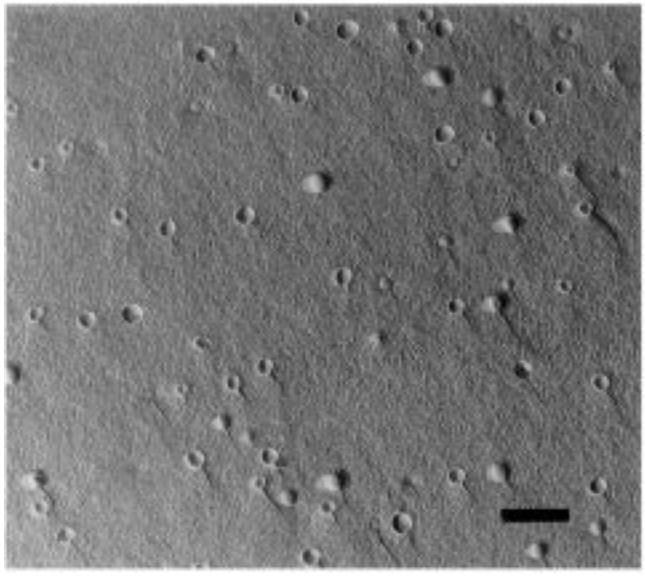

Figure 4 *Freeze–fracture electron micrograph of isolated SPLP. An SPLP formulation composed of DOPE/DODAC/PEG-CerC_8 (64:21:15; mol:mol:mol) and plasmid (pCMVLuc) was prepared as indicated in the caption for Figure 1 and non-encapsulated plasmid was removed by DEAE column chromatography. SPLP were separated from empty vesicles on a discontinuous sucrose density gradient. The gradient consisted of 2 ml 10% sucrose, 6 ml 5% sucrose, 1 ml 1% sucrose and was centrifuged at 160 000 g for 12 h. The bar indicates 200 nm. For details of sample preparation and electron microscopy, see Materials and methods.*

applied to SPLP formulations containing PEG-CerC_8 and higher cationic lipid contents. Nearly 90% of the plasmid loaded on to the gradient was recovered in the fraction containing the SPLP (located at the 5–10% interface of the density gradient), whereas less than 20% of the total lipid was associated with this fraction. The lipid composition of purified SPLP was found to be similar to the initial lipid composition, with a slight enrichment (1–3%) in the DODAC content. Importantly, regardless of the initial plasmid-to-lipid ratio or relative DODAC content, the final plasmid-to-lipid ratios in these purified SPLP were consistently in the range of 65 μg/μmol, which corresponds to one plasmid per SPLP.[15]

Particle size and morphology were characterized by QELS and by freeze–fracture electron microscopy techniques. The isolated SPLP (Table 1) were highly monodisperse ($\chi^2 < 1$) and had a consistent mean diameter of 90–110 nm with a narrow size distribution (standard deviation of approximately 20–30%) as determined by QELS (volume-weighted vesicle mode). Note, the mean diameter of isolated SPLP detected by QELS in 'solid-particle' mode was 70–90 nm. The empty vesicle fraction following density centrifugation contained very small particles with diameters of 30–60 nm. The morphology and size distribution of isolated SPLP containing 21 mol% DODAC as determined by freeze–fracture electron microscopy revealed highly uniform spheres of 62 ± 8 nm in diameter as shown in Figure 4. The morphology of these SPLP is similar to that observed for bilayer LUV systems,[19] consistent with an SPLP structure consisting of plasmid trapped within a lipid bilayer. Similar results were observed for a SPLP formulation containing 15 mol% DODAC (data not shown). Freeze–fracture studies on the empty lipid fraction revealed small vesicles less than 40 nm in diameter.

SPLP containing high cationic lipid contents are stable against serum degradation and storage

SPLP stability in serum provides a rigorous test, as serum proteins associate with lipid vesicles,[20] resulting in leakage and potential exposure of encapsulated plasmid to serum nucleases. Isolated SPLP (DODAC/DOPE/PEG-CerC_8; 21:64:15, mol:mol:mol) containing ^{3}H-pCMVLuc were incubated in 90% serum for 1 h at 37°C and passed over a size exclusion chromatography column. Over 85% of the plasmid in SPLP was intact and eluted in the void volume (Figure 5). This may be compared to the behavior of free plasmid, which was completely degraded in serum and the plasmid fragments eluted in the included volume.

The stability of the isolated SPLP during storage was assessed by monitoring size and plasmid encapsulation. No significant changes in size and plasmid encapsulation were observed for the two SPLP formulations tested (containing 15 mol% and 20 mol% DODAC) during storage at 4°C for a period of 5 months (results not shown).

SPLP contain a trapped aqueous volume

The volume of one encapsulated plasmid is not sufficient to fill the interior volume of a particle of approximately 70 nm diameter, suggesting that SPLP contain an interior aqueous volume.[15] It is of interest to measure this entrapped volume directly. In this regard, the measurement of the trapped volume of liposomes usually employs a radiolabeled membrane-impermeable marker,

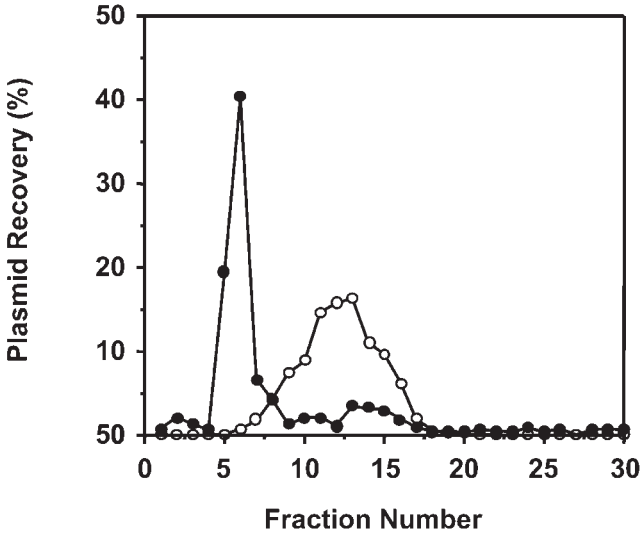

Figure 5 *Plasmid in SPLP containing 21 mol% DODAC is protected from serum nuclease degradation. An SPLP formulation composed of DOPE/DODAC/PEG-CerC_8 (64:21:15; mol:mol:mol) and plasmid (pCMVLuc) was prepared as indicated in the caption for Figure 1. The non-encapsulated plasmid was removed by DEAE column chromatography and the SPLP isolated by sucrose density gradient centrifugation. Free plasmid and SPLP-encapsulated plasmid was incubated in 90% mouse serum for 1 h at 37°C. The sample was then loaded on to a 5 ml Sepharose CL-4B gel filtration column and eluted with HBS. Fractions (0.5 ml) were analyzed for ^{3}H-labelled plasmid by scintillation counting. The open circles represent the elution profile of the 'free plasmid' sample and the closed circles the SPLP-encapsulated plasmid preparation.*

such as inulin or sucrose.[21,22] Before using sucrose to measure the trapped volume of SPLP, it was important to show that sucrose would not leak out during the time required to isolate the particles. The release rate of ^{14}C-sucrose from LUV with the same lipid composition as the SPLP was therefore measured as indicated in Materials and methods. Two LUV formulations composed of DODAC/DOPE/PEG-CerC_8, containing 7:78:15 and 20:65:15 molar ratios, respectively, were employed. The measured half-life for sucrose retention at 20°C was found to be in the range of 300–700 h (results not shown). The time required for sucrose density gradient centrifugation was 10–20 h, thus allowing ^{14}C-sucrose to be used as a membrane-impermeable marker to determine the trapped volumes of SPLP. Trapped volumes of 2.2 and 2.0 μl/μmol lipid were obtained following the procedures detailed in Materials and methods for the isolated SPLP formulations containing 7 and 20 mol% DODAC, respectively.

Influence of cationic lipid content on the transfection potency of SPLP in vitro

The effect of cationic lipid content in the SPLP on *in vitro* transfection was investigated in COS-7 and HepG2 cell lines using SPLP formulations that had been purified by DEAE chromatography only and SPLP isolated by both DEAE chromatography and sucrose density gradient centrifugation. The luciferase activities detected in COS-7 cells following transfection are shown in Figure 6. Luciferase expression was dramatically increased in cells incubated with the isolated SPLP (Figure 6b), as compared with SPLP that had not undergone density gradient centrifugation (Figure 6a). In addition, the transfection activity was strongly dependent on the DODAC content in the SPLP. Luciferase activity was low for isolated SPLP containing 7–8 mol% DODAC but increased substantially to reach a plateau value between 20 and 24 mol% DODAC. Luciferase expression remained essentially unchanged for further increases in DODAC content. Importantly, the luciferase activities detected for isolated SPLP containing high DODAC levels are comparable with those obtained for the plasmid–lipid complexes formed with pCMVLuc and DODAC/DOPE (1:1; mol:mol) liposomes. Similar results were also observed for the HepG2 cell line (results not shown). The charge ratio of 1.5:1 (+/–) used to form plasmid DNA–cationic lipid complexes resulted in optimal transfection in these cell lines.

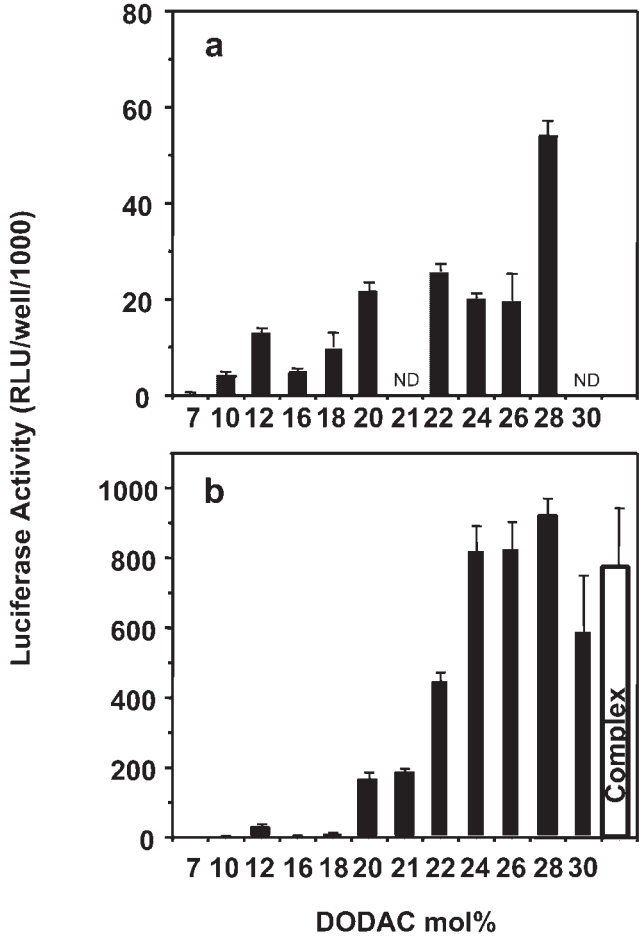

Figure 6 *Effect of DODAC content in SPLP on transfection activity in vitro. Transfection activity is shown with SPLP before (a) and after (b) isolation by density gradient centrifugation. Note the different y-axis scales for panels a and b. Plasmid (pCMVLuc) was encapsulated in SPLP containing 7–30 mol% DODAC, as described in the caption for Figure 2. SPLP isolation by density gradient centrifugation was conducted as described in the caption for Figure 4. SPLP were added to COS-7 cells (1 μg plasmid per well) and then incubated for 24 h. The luciferase activity was determined as described in Materials and methods. The 'complex' bar illustrates the transfection activity achieved with complexes of pCMVLuc and DODAC/DOPE (1:1; mol:mol) LUV at a charge ratio of 1.5:1 (+/–). ND, not determined.*

Influence of cationic lipid content on SPLP transfection potency in vivo

The ability of SPLP containing different levels of DODAC to transfect a tumor following regional injection *in vivo*

was evaluated in an intraperitoneal (i.p.) tumor model. B16BL-6 tumor cells were seeded in the peritoneal cavity of C57BL/6 mice. After 7 days isolated SPLP containing pCMVLuc were injected intraperitoneally at a dose of 30 μg plasmid per mouse. The tumors were collected after 24 h and assayed for luciferase activity. The mean luciferase expression was found to increase with increasing DODAC content in the SPLP (Figure 7) as was observed in the *in vitro* studies. The highest levels of luciferase expression, approximately 1.1 ng/g tumor, were observed for SPLP with DODAC concentrations ranging between 20 and 30 mol%. In this model system, luciferase expression at 24 h was 10 times higher than that observed with DOPE:DODAC (1:1; mol:mol) LUVs complexed with pCMVluc. These complexes were constructed at a 3:1 cationic lipid-to-DNA charge ratio, which was optimal for *in vivo* transfection in this model. Also, expression following transfection with complexes followed a different time-course than that observed for transfection with SPLP, and maximum transfection levels of 100–200 pg luciferase per gram of tumor were observed 12 h after injection with the complex system. For SPLP the highest expression levels were observed 24 h following transfection as compared with 12 and 48 h.

As an indicator for systemic toxicity of SPLP, aspartate aminotransferase (AST) levels in the serum were analyzed 24 h after i.p. injection of the preparations. It was found that administration of SPLP containing 6 to 30 mol% DODAC at a lipid dose of 120 mg/kg did not result in significantly elevated enzyme levels (data not shown).

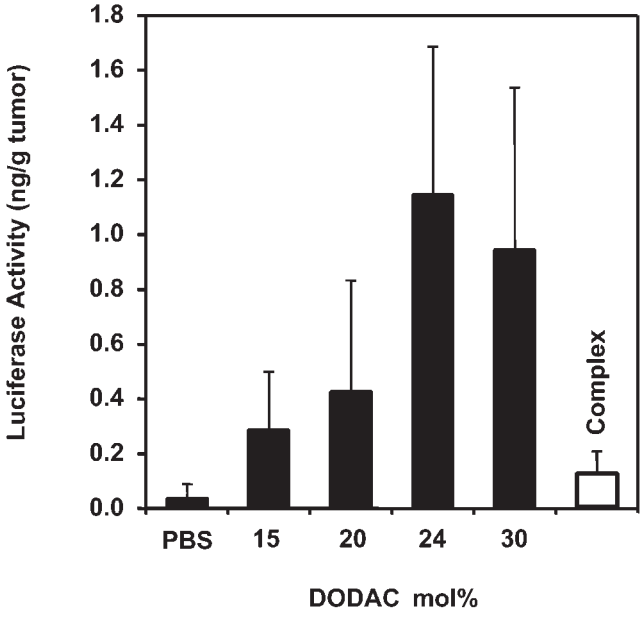

Figure 7 *Transfection of B16 tumors in vivo following regional (i.p.) injection of isolated SPLP is dependent on the DODAC concentration in SPLP. B16BL-6 cells (100 000) were seeded in C57BL/6 mice i.p 7 days before i.p. administration of the different formulations (30 μg plasmid in 500 μl). Tumors were collected 24 h after transfection and assayed for luciferase activity (n = 4). The 'complex' bar shows the transfection activity obtained with cationic lipid–plasmid DNA complexes formed with pCMVLuc and DODAC/DOPE (1:1; mol:mol) LUVs at a charge ratio of 3:1 (+/–).*

Discussion

This study demonstrates that SPLP with a dissociable PEG coating and elevated levels of the cationic lipid DODAC exhibit improved transfection potency *in vitro* and *in vivo*. There are three interesting aspects of these results. The first concerns the improved transfection properties of SPLP containing PEG-CerC_8 molecules that can rapidly dissociate from the carrier. The second concerns the mechanism whereby citrate facilitates formation of SPLP with higher cationic lipid contents and the *in vitro* and *in vivo* transfection potency of SPLP with optimized cationic lipid contents. The final point relates to the small, stable, well defined nature of SPLP. We discuss these aspects in turn.

The physical properties of the SPLP described here differ significantly from the properties of the SPLP described previously.[15] A major difference concerns the PEG-CerC_8 molecule used to stabilize the SPLP. In the previous work SPLP containing PEG-CerC_{20} were generated and it was shown that these SPLP exhibited poor transfection properties *in vitro*. Improved transfection potency could be achieved by using PEG-CerC_{14} as the stabilizing component. This improvement was attributed to the ability of the PEG-CerC_{14} to diffuse away from the SPLP due to the much faster dissociation rate of PEG-CerC_{14} ($t_{1/2}$ = 1.2 h) as compared with PEG-CerC_{20} ($t_{1/2} \geqslant 13$ days), thus rendering the particle more able to interact with target cells. It has been shown elsewhere that the presence of a PEG coating can inhibit interaction and fusion between lipid vesicles.[16,17] The SPLP employed in the present study contained PEG-CerC_8 as the stabilizing agent, which exhibits a considerably faster dissociation rate ($t_{1/2} \leqslant 1.2$ min), thus maximizing the *in vitro* transfection potency required for the *in vitro* studies performed here. It should be noted that these systems would not be suitable for intravenous delivery with the aim of reaching sites of disease such as distal tumor sites, as they will be highly unstable following interaction with biological fluids. More stable SPLP containing PEG-CerC_{20} which diffuses away over much longer times, are better suited to such applications.[23]

With regard to the mechanism whereby citrate facilitates formation of SPLP with higher cationic lipid contents, previous work conducted at a fixed ionic strength showed that the plasmid entrapment efficiency was a sensitive function of the cationic lipid content, with maximum entrapment in the range of 5–10 mol% DODAC.[15] It was proposed that plasmid first interacts with macromolecular structures composed of DOPE, DODAC and PEG-Cer that are formed as intermediates during the dialysis process, to produce SPLP. The formation of structures such as cylindrical micelles and lamellar sheets during the formation of lipid vesicles by detergent dialysis is well established.[24–26] The results presented here indicate that by using the appropriate amount of citrate to shield the positive charge on the lipid structures containing higher amounts of cationic lipid, the affinity of the plasmid for these intermediates can be reduced to levels compatible with good entrapment. Within this model citrate concentrations below the optimum range do not result in adequate shielding of the positively charged lipid structures formed during dialysis, resulting in 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 previously.[15]

A major finding of this study is that SPLP containing 24 mol% DODAC and stabilized by PEG-CerC_8 exhibit *in vitro* and *in vivo* transfection properties that are comparable to, or better than, those observed for corresponding plasmid DNA–cationic lipid complexes. There are two factors that contribute to this enhanced activity. The first, as previously discussed, is related to the rapid dissociation of the PEG coating. The second factor that clearly plays a major role is the elevated cationic lipid content. It is possible that higher levels of cationic lipid result in enhanced association with, and uptake into, nearby cells. In this regard it has been noted that cellular uptake of plasmid delivered by SPLP stabilized by PEG-CerC_8 and containing 6 mol% DODAC is less than 3% of that delivered by DODAC/DOPE (1:1) complexes.[27] Alternatively, it is possible that the higher levels of cationic lipid contribute to the endosomolytic activity required to facilitate intracellular delivery of the encapsulated plasmid. A feature that is of particular interest concerns the dramatically enhanced transfection activity of SPLP following isolation by density gradient centrifugation as compared with the SPLP that contain empty vesicles (Figure 6). These results suggest that cells have limited uptake capacity and that saturation of this uptake by empty vesicles as compared with SPLP containing plasmid seriously compromises transfection activity.

The final topic of discussion concerns the well-defined nature of the SPLP system. Aside from the differences arising from the cationic lipid content and the ability of the PEG-Cer to dissociate, the physical characteristics of the SPLP generated here are remarkably similar to the SPLP generated employing 6% DODAC and PEG-CerC_{20}. The high plasmid-to-lipid ratio of 65 μg/μmol lipid, which corresponds to one plasmid per SPLP, is maintained, as is the size of the SPLP at a diameter of approximately 65 nm. The additional finding that SPLP contain a trapped volume is consistent with previous work[15] indicating that the interior volume of an SPLP is considerably larger than the volume taken up by the entrapped plasmid. The measured trapped volumes of 2 μl/μmol lipid correspond to an SPLP diameter of 66 nm, assuming an area per lipid molecule of 0.6 mm^2. This is in good agreement with the SPLP diameter measured by freeze–fracture electron microscopy (62 ± 8 nm). The fact that the SPLP system has an appreciable interior trapped volume clearly offers interesting opportunities for co-encapsulating agents to improve transfection properties along with the plasmid DNA.

The well-defined structure and stability of SPLP contrasts strongly with the properties of cationic lipid–plasmid DNA complexes. The purified SPLP exhibit a uniform size of approximately 70 nm and contain one plasmid per particle, with the plasmid fully encapsulated within a lipid envelope. As emphasized previously, complexes usually exhibit sizes greater than 200 nm diameter, contain an indeterminate number of plasmids per complex and do not fully protect associated plasmids from the external environment. In addition, SPLP exhibit certain practical advantages with regard to manufacturing and stability. Due to their small size, SPLP can be readily sterilized by passage through a 0.22 micron filter. Further, SPLP can be concentrated to plasmid concentrations greater than 1 mg/ml[15] and are also highly stable during extended storage. The preliminary studies performed here indicate that SPLP exhibit no significant changes in size or plasmid encapsulation during storage at 4°C for at least 5 months. Again, it is well recognized that complexes exhibit increased instability at plasmid concentrations of 100 μg/ml or greater. In our hands, DODAC-containing complexes prepared at a charge ratio of 3:1 could not be prepared at plasmid concentrations greater than 375 μg/ml (L Akhong, unpublished results). It should be noted that complexes are generally so unstable during extended storage that most laboratory and clinical applications employ a 'two-vial' approach, where the plasmid and cationic lipid vesicles are mixed together immediately before administration.

In summary, the results presented in this investigation illustrate that the SPLP system produced by detergent dialysis represents a highly flexible platform technology for construction of non-viral gene delivery vectors. It is demonstrated that SPLP can be stabilized by PEG-Cer molecules that freely dissociate, thus reducing or eliminating the inhibitory effects of a PEG coating on cell association. It is also shown that SPLP can be constructed to contain high amounts of cationic lipid if the dialysis medium contains high levels of a citrate buffer. SPLP containing approximately 24 mol% of the cationic lipid DODAC, together with the dissociable PEG coating, give rise to gene transfer vectors that exhibit *in vitro* transfection potencies that are comparable to those achieved for cationic lipid–plasmid DNA complexes. Further, these SPLP give rise to superior tumor transfection levels in a regional tumor model.

Materials and methods

Materials

Dioleoyldimethylammonium chloride (DODAC) was kindly provided by Dr S Ansell (Inex Pharmaceuticals, Burnaby, BC, Canada) and 1-O-[2′-(ω-methoxypolyethyleneglycol) succinoyl]-2-N-octanoylsphingosine (PEG-CerC_8) was synthesized as described elsewhere.[28] Dioleylphosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada). Dialysis tubing (SpectraPor 5, 12 000–14 000 MWCO) was obtained from Spectrum Medical Industries (Laguna Hills, CA, USA). The PicoGreen reagent was purchased from Molecular Probes (Eugene, OR, USA). Mouse serum was obtained from CelarLane (Mississauga, ON, Canada). ^{14}C-cholesteryl hexadecyl ether (^{14}C-CHE) was obtained from Mandel Scientific (Guelph, ON, Canada). Triton X-100, DEAE-Sepharose CL-6B and octyl-β-d-glucopyranoside (OGP) was obtained from Sigma (St Louis, MO, USA). ^{14}C-sucrose was obtained from NEN-Dupont (Markham, ON, Canada). The luciferase assay kit was obtained from Promega (Madison, WI, USA). The pCMVLuc plasmid (5650 bp, coding for the luciferase reporter gene) was provided by Dr P Tam (Inex Pharmaceuticals). The gene was under the control of the human CMV immediate–early promoter-enhancer element. The ^{3}H-pCMVLuc plasmid was produced as

previously described.[29,30] The cell lines COS-7 (African green monkey kidney, SV40 transformed, ATCC CRL-1651), HepG2 (human hepatocellular carcinoma, ATCC HB-8065), were obtained from the American Tissue Culture Collection. The B16BL-6 mouse melanoma cell line was obtained from Frederick Cancer Research Center (Frederick, MD, USA).

Encapsulation of plasmid DNA

Lipid dispersions were prepared from stock solutions of DODAC, DOPE and PEG-CerC_8 in chloroform / methanol (2 / 1; vol / vol). Appropriate amounts of lipid were transferred into a glass test tube and solvent was removed under a stream of N_2 gas followed by storage under vacuum for 3–5 h. An aqueous solution of OGP (0.2 ml of a 1 m solution for 10 mg lipid) was then added to the lipid film. The mixture was vortexed until the lipid film was dissolved and the solution became clear. An appropriate amount of pCMVLuc plasmid solution (typically 100–400 μg for 10 mg lipid) and citrate buffer was then added, to achieve a final lipid concentration of 10 mg / ml and a final OGP concentration of 200 mm. The solution was normally optically clear at this point; if not, 50–100 μl of the 1 m OGP solution was added. This mixture was incubated at room temperature for 1 h and then dialyzed for 2 days against 4 liter of citrate buffer containing the indicated concentration of sodium citrate as well as 5 mm HEPES and 150 mm NaCl (pH 7.2), with two changes of buffer daily. Non-encapsulated plasmid was removed by anion exchange chromatography on a DEAE-Sepharose column (1 × 5 cm).

Evaluation of plasmid encapsulation by the PicoGreen fluorescence assay

Plasmid encapsulation was evaluated by measuring the accessibility of the DNA-interchelating dye PicoGreen to plasmid at an excitation wavelength of 485 nm and emission wavelength of 525 nm (Aminco Bowman Series 2 Luminescence Spectrometer, SLM-Aminco, Urbana, IL, USA). Typically, 4 μl of PicoGreen was added to 1 ml of sample containing 0.2–0.8 μg plasmid. Plasmid encapsulation was calculated as $E(\%) = (I_o - I)/I_o \times 100$ where I and I_o refer to the fluorescence intensities before and after the addition of Triton X-100 (final concentration 0.4%, v / v). Fluorescence intensities in the absence of PicoGreen were used as background references. The plasmid contents of formulations after DEAE column chromatography and after isolation by sucrose density gradient centrifugation were determined either by the PicoGreen fluorescent assay and / or by using ^{3}H-labelled plasmid.

Isolation of SPLP by sucrose density gradient centrifugation

Isolation of SPLP from empty vesicles was carried out by sucrose density gradient centrifugation. The gradient was formed with 2 ml of 10% sucrose, 6 ml of 2.5% sucrose (for formulations containing 22–30 mol% DODAC) or 5.0% sucrose (for formulations containing 7–22 mol% DODAC) and 1 ml of 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 160 000 *g* for 12–20 h. The lipid-encapsulated plasmid banded tightly at the lower interface of the gradient, and the empty vesicles formed a broad band near the top of the gradient. The gradient was either separated into aliquots (1 ml) or the band containing SPLP was carefully isolated by pipetting, depending on the purpose of the experiment. The lipid and plasmid concentrations of the fractions were evaluated by HPLC analysis and the PicoGreen fluorescent assay, respectively, or by scintillation counting when ^{14}C-CHE and / or ^{3}H-plasmid was used.

Measurement of size distribution

The size distributions were measured by quasi-elastic light scattering (QELS) and freeze–fracture electron microscopy. QELS measurements employed a Nicomp 370 Sub-Micron particle sizer (Santa Barbara, CA, USA) operated in the volume-weighted vesicle mode. Sample polydispersity was evaluated by the goodness-of-fit parameter χ^2, where values of $\chi^2 < 3$ indicate monodisperse formulations (according to specification by the manufacturer). Freeze–fracture electron microscopy was performed employing a Balzers Freeze-Etching system, BAF 400D (Balzers, Liechtenstein). Samples were cryofixed in the presence of 25% glycerol by plunging them into liquid freon 22. The fractured surface was shadowed unidirectionally with platinum / carbon (45°) and coated with carbon (90°) immediately after fracturing. Replicas were analyzed using a JEOL Model JEM 1200 EX electron microscope (Soquelec, Montreal, QC, Canada).

HPLC analysis of lipid composition

Lipid compositions were analyzed by HPLC (Beckman System Gold 128) using an Ultrasphere cyanopropyl column (2 × 15 mm, 5 μm). A ternary mobile phase consisting of two solvents (solvent A: 99.95% $CHCl_3$ 0.05% TFA; solvent B: 90% isopropanol, 9.95% water, 0.05% TFA) was employed. Separation was performed at a flow rate of 0.3 ml / min, and a gradient of 0–90% solvent B was applied in 9 min. Elution with 90% solvent B was maintained for 1 min and then the mobile phase was cycled back to 100% solvent A.

Trapped volume measurements

The trapped volume of isolated SPLP was determined using ^{14}C-sucrose as a marker for the internal volume.[21,22] A first step was to examine sucrose retention in vesicles with the SPLP lipid composition. This study employed 100 nm diameter LUV prepared by the freeze–thaw extrusion method.[19] Briefly, 10 mg of the appropriate lipid mixture was hydrated in 1 ml of citrate buffer containing 10 μCi of ^{14}C-sucrose and 5% (w / v) sucrose. After five freeze–thaw cycles, the sample was extruded 10 times through a filter of 100 nm pore size using an Extruder (Lipex, Vancouver, BC, Canada). Untrapped ^{14}C-sucrose was removed by passing the sample through a Sephadex G-50 spin column. The sample was then dialyzed (12–14 000 MWCO) against an HBS solution containing 5% (w / v) sucrose. Aliquots were removed at various time-points and the specific activity determined. The SPLP formulations used for trapped volume determinations were prepared as described above, except that 20 μCi of ^{14}C-sucrose was included in the detergent dialysis solution. The solution was then dialyzed in 150 ml of citrate buffer in the presence of 20 g SM-2 Bio-Beads (BioRad, Hercutes, CA, USA). The Bio-Beads were replaced with fresh ones after 12 h and the sample was dialyzed for an additional 12 h. The lipid-plasmid formulations were then purified and isolated using DEAE column chromatography and sucrose density gradient procedures as described above.

Assay for serum stability of SPLP

A 50 μl aliquot of SPLP containing ^{3}H-plasmid and ^{14}C-CHE was mixed with 450 μl of normal mouse serum. After incubation at 37°C for 2 h the mixture was loaded on to a Sepharose CL-4B gel filtration column and eluted with HBS (pH 7.2). Fractions of 0.5 ml were collected and the lipid and plasmid concentrations assayed by scintillation counting.

In vitro and in vivo transfection

One day before transfection, COS-7 cells were plated in 24-well plates at 45 000 cells per well. At the time of transfection, cells were 70% confluent. Formulations of up to 50 μl containing 0.2–1.0 μg plasmid were added to each well (in triplicate). After 24 h incubation the cells were lysed in 200 μl 0.1% Triton X-100, 250 mm NaH_2PO_4 pH 7.4 and 20 μl of the lysate was assayed for luciferase expression as previously described.[15] *In vivo* transfection was measured using an intraperitoneal B16BL-6 tumor model. Female C57BL/6 mice were injected intraperitoneally with 100 000 B16BL-6 tumor cells. Seven days following tumor seeding, SPLP formulations containing 30 μg plasmid in 500 μl were injected into the peritoneal cavity. Tumors were collected after 24 h, fast-frozen in liquid nitrogen and stored at –70°C before being assayed for luciferase. Tissue homogenization was performed with a FastPrep instrument using supplied tubes and beads. Tissues were homogenized in Cell Culture Lysis Reagent (CCLR; Promega), supplemented with BSA (1 mg/ml). Samples were then centrifuged for 2 min at 10 000 *g* to remove debris.

Luciferase assay

Luciferase assays were performed using the Promega Luciferase Assay reagent kit (Promega E1501) according to the manufacturer's instructions. Cell lysates were assayed for luciferase activity using a 96-well microplate luminometer (Dynex Technologies ML 3000). Luciferase (firefly luciferase, Sigma) standard solutions were prepared by serially diluting 1 μg/μl luciferase in CCLR supplemented with BSA (1 mg/ml). To determine the luciferase activity in tissue, the standard curve employed was obtained by diluting luciferase standard in control tissue homogenate to compensate for quenching.

Assay for aspartate aminotransferase

Plasma from mice was recovered 24 h following injection of SPLP into the peritoneal cavity and assayed immediately for aspartate aminotransferase activity using commercially available kits (Sigma).

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