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Stabilized plasmid–lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression

E. Ambegia[a], S. Ansell, P. Cullis[b], J. Heyes[a], L. Palmer[a], I. MacLachlan[a,*]

[a]*Protiva Biotherapeutics Inc., 100-3480 Gilmore Way, Burnaby, BC, Canada V5G 4Y1*
[b]*Department of Biochemistry and Molecular Biology, University of British Columbia 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3*

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Abstract

Stabilized plasmid lipid particles (SPLP) consist of a single copy of DNA surrounded by a lipid bilayer. The particles are small (~100 nm), stable, monodisperse and have a low surface charge. A diffusible polyethylene glycol (PEG) coating attached to a lipid anchor is critical to the SPLP's functionality. The PEG–lipid exchanges out of the bilayer at a rate determined by the size of the lipid anchor. Here we show that SPLP can be prepared using a series of PEG–diacylglycerol lipids (PEG–S-DAGs). SPLP were prepared incorporating PEG–dimyristoylglycerol (C_{14}), PEG–dipalmitoylglycerol (C_{16}) or PEG–distearoylglycerol (C_{18}) and the rate of PEG–lipid diffusion from the bi-layer determined using a FRET assay. SPLP pharmacokinetics confirm a correlation between the stability of the PEG–lipid component and circulation lifetime. PEG–S-DAGs with longer lipid anchors yield more stable SPLP particles with longer circulation half-lives yielding an increase in tumor delivery and gene expression. PEG–distearoylglycerol (C_{18}) containing SPLP bypass so-called 'first pass' organs, including the lung, and elicit levels of gene expression in distal tumor tissue 100- to 1000-fold greater than that observed in any other tissue. The incorporation of PEG–S-DAG in SPLP confirms that small size, low surface charge and extended circulation lifetimes are prerequisite to the accumulation and tumor selective expression of plasmid DNA following systemic administration.

Keywords: Gene therapy; Stabilized plasmid–lipid particle; Systemic drug delivery; Poly(ethylene glycol)–lipid conjugate; Lipid exchange

1. Introduction

Nucleic acid based therapeutics are of increasing interest for the treatment of diseases such as cancer and inflammation, disseminated diseases that will require the use of vector systems that can be administered systemically. While replication deficient viruses have been explored for use as vectors, several factors compromise their systemic administration. Since the expression of viral proteins often leads to an immune response against the infected cell, sustained protein expression cannot be achieved with most viral systems and the efficiency of subsequent treatments is compromised [1,2]. Furthermore, many viruses are limited by rapid clearance from the circulation, resulting in the majority of expression being seen in 'first pass' organs such as the liver [3].

Of the synthetic alternatives to viral vectors, DNA–cationic lipid complexes or 'lipoplexes' have received the most attention [4–7]. Lipid-based systems possess fewer safety or immunogenicity concerns than do viral systems and a far greater degree of control can be exercised over their structure on a molecular level. Lipoplexes and their constituent lipids are characterized and purified without difficulty and are easier and less expensive to produce on a large scale. Nonetheless, these vectors also possess their own inherent problems. They are heterogeneous, often aggregating and exhibiting transfection efficiencies that vary from sample to sample. Furthermore, despite demonstrating reasonable transfection efficiency in vitro, their large size and overall positive charge leads to rapid clearance in vivo, and thus the highest levels of transfection

* Corresponding author. Tel.: +1 604 630 5064; fax: +1 604 630 5103.
E-mail address: ian@protivabio.com (I. MacLachlan).

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are seen in first pass organs such as the lung, liver and spleen [8–12].

In an effort to overcome these problems, the development of SPLP using a detergent dialysis technique has been reported [13–17]. SPLP consist of a unilamellar lipid bilayer containing the cationic lipid DODAC, the neutral helper lipid DOPE, and a diffusible PEG–lipid conjugate, surrounding a single copy of plasmid DNA. The PEG–lipid conjugate provides the particle with a PEG coating that both stabilizes the particle and shields the surface positive charge, preventing rapid systemic clearance. Following administration, the PEG conjugate dissociates from the SPLP, revealing the positive charge and transforming the particle into a transfection-competent entity. The length of the ceramide lipid anchor determines the time that the PEG conjugate remains associated with the bilayer. PEGs with shorter lipid anchors, such as ceramideC_8, dissociate more quickly from the bilayer, quickly 'activating' the SPLP into which they are incorporated. As a result such particles show higher transfection potency in vitro than those containing PEG–lipid conjugates with longer lipid anchors (e.g. ceramideC_{20}) [15]. However, when injected systemically, conjugates with a larger, more securely fastened anchor will confer greater stability and extended circulation lifetimes [14]. Longer circulating SPLP are able to take advantage of 'passive targeting', whereby fenestrations in the tumor vasculature lead to greater particle accumulation at the tumor site [13].

This work focuses on the use of an alternative type of PEG–lipid conjugate, PEG–diacylglycerols (PEG–S-DAGs). PEG–S-DAGs are more quickly and easily synthesized than their PEG–ceramide counterparts. They are also more easily purified and do not require the tangential flow purification step used to remove unwanted by-products from PEG–ceramides. Here we report results of both in vitro and in vivo experiments illustrating the pharmacokinetics, biodistribution and transfection properties of SPLP prepared with three PEG–S-DAG analogues. The PEG–S-DAGs possess hydrophobic domains of varying size, with two alkyl chains, either 14 (PEG–S-DMG), 16 (PEG–S-DPG) or 18 (PEG–S-DSG) carbon units in length. These are connected via a succinic acid linker to a PEG with a molecular weight of approximately 2000 Da (Fig. 1). We provide evidence for the mode of action of PEG–lipids using an in vitro lipid exchange assay. By measuring the fusion of dye-labeled vesicles, we confirm that the use of shorter acyl chain PEG–lipids results in the production of less stable particles, which exhibit a much higher degree of fusogenicity over time. We also fully characterize the behavior of these SPLP in vivo, showing their pharmacokinetics and the biodistribution of gene expression following systemic administration. If previous hypotheses are correct, SPLP containing PEG–S-DAGs should function in a manner analogous to SPLP containing the PEG–ceramides. SPLP containing the shorter chain PEG–S-DAGs would be expected to show higher transfection efficiencies in vitro. Those possessing the longer chain PEG–S-DAGs should be more stable, exhibiting longer circulation times, the ability to bypass the 'first pass' organs and a greater tendency to accumulate at the tumor site. We demonstrate that this is the case.

2. Materials and methods

2.1. Lipids and plasmid

The cationic lipid DODAC and the PEG–CerC_{20} were synthesized as described previously [14,18]. DOPE was obtained from Northern Lipids (Vancouver, BC, Canada). The detergent octyl glucopyranoside (OGP) was obtained from Sigma-Aldrich Co. (Oakville, ON, Canada). ^{3}H-labelled CHE was obtained from Mandel NEN Products (Guelph, ON, Canada). The pCMVluc plasmid, encoding the luciferase reporter gene under the control of the cytomegalovirus promoter, was propagated in *E. coli* strain DH5α and purified by standard alkaline lysis/caesium chloride density gradient centrifugation.

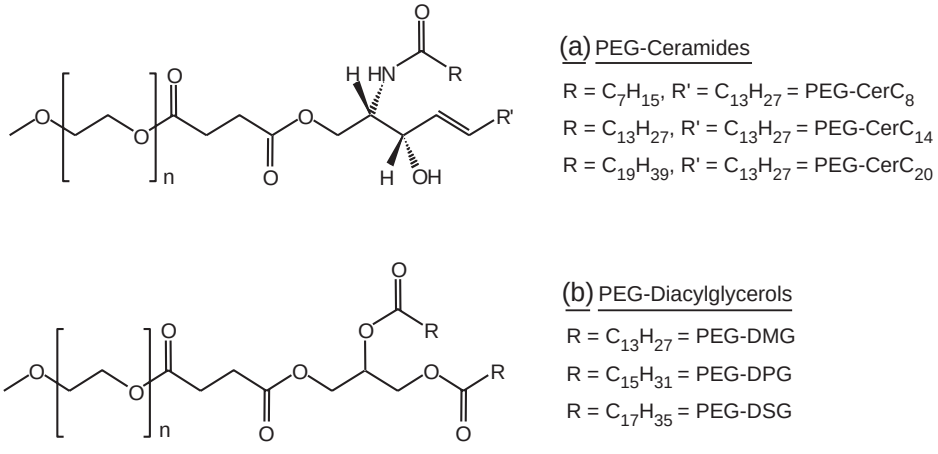

Fig. 1. The chemical structures of the PEG–lipids incorporated into SPLP (a) PEG–ceramides (b) PEG–*S*-diacylglycerols. DMG=Dimyristoylglycerol, DPG=Dipalmitoylglycerol, DSG=Distearoylglycerol.

2.2. Poly(ethylene glycol)–diacylglycerol conjugate synthesis

The poly(ethyleneglycol)–diacylglycerol conjugates (PEG–S-DAGs) were synthesized in-house. Briefly, succinic anhydride in 5-fold excess was stirred with 2000-weight monomethoxypolyethylene glycol in pyridine. Following purification by flash column chromatography, the free carboxylic acid was converted to the acyl chloride with a 10-fold excess of oxalyl chloride. Subsequent reaction with the relevant 1,2-diacylglycerol in the presence of triethylamine gave the PEG–S-DAGs in approximate 80% overall yield.

2.3. SPLP formulation

SPLP were prepared as described elsewhere [16]. Briefly, DOPE, DODAC and PEG–$CerC_{20}$ or PEG–S-DAG at a molar ratio of 82.5:7.5:10 were dissolved in aqueous solutions of OGP with or without [^{3}H]cholesteryl hexadecyl ether (1 μCi per mg of lipid). pCMVluc plasmid solution (400 μg for 10 mg of lipid) was added to a final lipid and detergent concentration of 10 mM and 200 mM respectively. The solution was dialyzed for 48 h and unencapsulated DNA removed by anion exchange chromatography (DEAE Sepharose CL6B). Empty vesicles were then removed by one-step sucrose density ultracentrifugation. Fractions containing SPLP were consolidated and dialyzed with HBS to remove sucrose. The final sample was then concentrated down by Amicon ultrafiltration to a final DNA concentration of 0.5 mg/ml. All samples were filter sterilized (0.22 μm) prior to injection into tumor bearing mice.

2.4. Exchange assay

Five sets of liposomes were prepared for the PEG–lipid exchange assay. These liposomal systems included one for each of the three PEG–lipids, DOPE:DOPS:PEG–lipid:Rh-PE:NBD-PE (48:48:2:1:1), DOPE:DOPS (50:50) (for background count), and POPC LUVs (the PEG–lipid sink). These liposomes were prepared by freeze/thaw followed by extrusion through two 0.1 μm polycarbonate filters using an Extruder from Lipex Biomembranes (Vancouver, BC). For the assay, all formulations were pre-warmed to 37 °C. Each PEG–lipid sample (performed in triplicate) would contain 25 μM of the labelled LUVs containing the PEG–lipid, 75 μM (3×) of the unlabelled DOPE:DOPS LUVs, and 250 μM (10×) of the POPC lipid sink. At t=0, the liposomal formulations for each sample were mixed and the NBD fluorescence was measured using a Cary Eclipse Fluorescence Spectrophotometer (Varian Corp, Mississauga, ON) with settings of λ_{ex}=465 nm, λ_{em}=517 nm, Ex and Em slit widths=5 nm, and PMT voltage=750 V. The fluorescence at time 0 is F_0. Background blank samples were prepared in duplicate containing 100 μM (4×) of the unlabelled LUVs and 250 μM (10×) of the POPC lipid sink. The fluorescence of the background blank samples at time 0 is B_0. Following the measurements at time 0, 5 mM of $CaCl_2$ was added to the samples (both PEG–lipid and background blanks) and the incubation at 37 °C continued. At various time points following the addition of the $CaCl_2$, the fluorescence of the samples and blanks was measured (F_t and B_t, respectively). At the end of the experiment (t=23 h), Triton X-100 was added to 0.33% causing complete lysis of the liposomes (giving the 100% fluorescence value for the labeled samples) and measurements were made on the samples and blanks (F_T and B_T, respectively). From the results, the %Fusion at each time point for the various PEG–lipid systems was determined using the following equation:

$$\%\text{Fusion} = \frac{(F_t - B_t) - (F_0 - B_0)}{(F_T - B_T) - (F_0 - B_0)} \times 100\% .$$

2.5. In vitro transfection assay

Neuro-2a cells were cultured in Minimum Essential Medium (MEM; Invitrogen, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen, ON, Canada) at 37 °C with 5% CO_2. Cells were dispensed into 24-well plates, with each well receiving 5×10^4 cells and 1 ml of growth medium, and incubated overnight. 500 μl of transfection media (2.5 μg/well) was added to each well and the plates incubated for the stated time points. Media was replaced and the cells cultured for a further 24 h. The cells were washed twice with phosphate buffered saline (PBS) and stored at −70 °C until analysis. Cells were treated with 150 μl of Cell Lysis Reagent (Promega, WI, USA), and 20 μl of the lysate assayed for luciferase activity.

2.6. Biodistribution, clearance and in vivo transfection assay

10 days prior to SPLP treatment, 5-week-old male A/J mice (Harlan, IN, USA) were inoculated subcutaneously in the hind flank with 1.5×10^6 Neuro-2a neuroblastoma cells. Materials (SPLP — 200 μl total volume containing 2 mg total lipid and 100 μg total DNA) were administered by lateral tail vein injection. At appropriate time points, mice were anaesthetized and blood collected by cardiac puncture into microtainer tubes. Plasma was separated from red blood cells via centrifugation and analyzed for ^{3}H-CHE by liquid scintillation counting using Picofluor 20 and a Beckman LS6500 (Beckman Instruments, CA, USA). Organs were harvested at the specified times and homogenized in lysing matrix tubes containing 500 μl of distilled water. 100 μl of liver lysate and 200 μl of all other lysates were assayed for radioactivity by liquid scintillation counting with Picofluor 40. For gene expression studies 20 μl of the lysate was assayed for luciferase activity.

2.7. Luciferase assay

Luciferase assays were performed using the Promega Luciferase Assay reagent kit (Promega E1501) according to the manufacturers instructions. Cell lysates were assayed for luciferase activity using a 96-well microplate luminometer. A curve obtained from firefly luciferase (Roche, QC, Canada) standard solutions was used to calibrate luminescence readings.

3. Results

3.1. In vitro lipid exchange assay

To provide additional evidence for the hypothesis that increased PEG–lipid acyl chain length correlates with increased particle stability, a lipid exchange assay was developed using fluorescent resonance energy transfer (FRET) [19]. PEG–lipid-containing LUVs incorporating FRET labels were mixed with unlabelled LUV in the presence of a PEG–lipid sink at 37 °C. As the PEG–lipids exchange out of the LUV and are incorporated in the lipid sink the LUV are rendered increasingly fusogenic and fuse with the unlabelled LUV. A dilution of the FRET labels thus occurs and the proximity of energy donors and acceptors decreases. An increase in fluorescence is observed due to the reduced ability of the energy acceptors to quench the donors. Greater PEG–lipid diffusion results in greater fusion between LUV and an increase in fluorescence. Results are reported as a function of time over 25 h, and entitled 'percentage of total fusion'. The value for 'total fusion' is obtained at the end of the experiment by adding detergent to the sample (causing complete lysis of all vesicles) and measuring the final fluorescence signal. It can be seen from Fig. 2 that the fusion properties of SPLP containing PEG–S-DMG are vastly different from those with PEG–S-DPG and PEG–S-DSG. The final degree of fusion obtained (72%) is at least double that observed for the two longer PEG–lipids. Results were found to be significant, according to *t*-test ($P<0.01$).

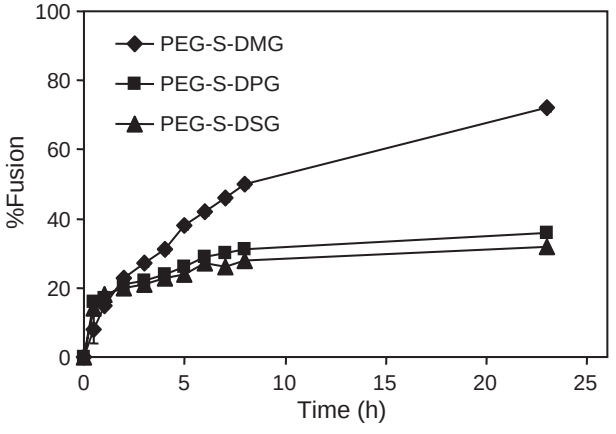

Fig. 2. Exchange assay examining the rate of diffusion of the different PEG–lipids from LUV by measuring respective rates of fusion in the presence of a PEG–lipid sink. Fluorescence resonance energy transfer labels were incorporated at a concentration of 1 mol%. Excitation and emission wavelengths are λ_{ex}=465 nm and λ_{em}=517 nm respectively. Error bars represent standard deviation, n=3.

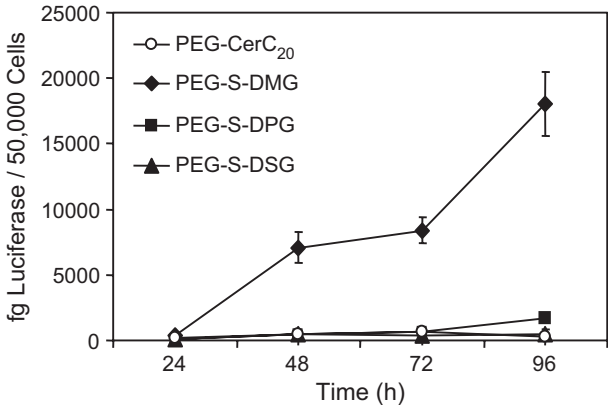

Fig. 3. The effect of replacing PEG–CeramideC_{20} with PEG–diacylglycerols on the in vitro transfection potency of SPLP. Neuro-2a cells were treated with SPLP containing plasmids encoding the luciferase gene, under the control of the cytomegalovirus (CMV) promoter. The cells were subsequently lysed and luciferase concentrations determined. Error bars represent standard deviation, n=3.

3.2. In vitro transfection potency of SPLP containing PEG–S-DAGs

SPLP containing the short anchor PEG–S-DAGs would be expected to behave similarly to their PEG–ceramide counterparts. The PEG coating inhibits association/fusion with cell membranes [20,21], therefore transfection efficiency will be higher in systems in which it is removed more quickly/completely. To evaluate this hypothesis SPLP were prepared containing (i) DODAC, (ii) DOPE and (iii) one of the three PEG–S-DAGs or PEG–$CerC_{20}$ in a molar ratio of 7.5:82.5:10 and used to transfect Neuro-2a cells. Luciferase gene expression was determined over 96 h, as shown in Fig. 3. SPLP containing the PEG–S-DAG with the shortest acyl chain, PEG–S-DMG (C_{14}), yielded the highest levels of gene expression. SPLP containing the PEG–S-DPG (C_{16}) and PEG–S-DSG (C_{18}) perform similarly to those containing the PEG–$CerC_{20}$. Results were found to be significant by *t*-test ($P<0.01$).

3.3. Pharmacokinetics and biodistribution of SPLP containing PEG–S-DAGs

It was necessary to determine the clearance and biodistribution of SPLP containing the PEG–S-DAGs. SPLP were prepared with DODAC, DOPE, PEG–lipid (7.5:82.5:10 molar ratio), ^{3}H-CHE marker and a plasmid containing the luciferase reporter gene. SPLP were administered by a single i.v. injection in the tail vein and the percentage of injected dose remaining in the plasma

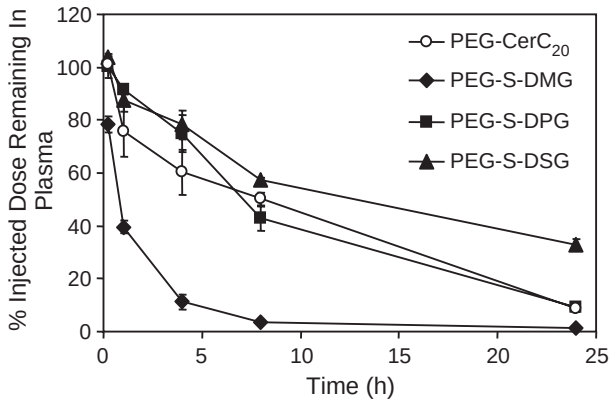

Fig. 4. Percent pharmacokinetics of SPLP containing PEG–$CerC_{20}$, PEG–S-DMG, PEG–S-DPG or PEG–S-DSG. The percentage of injected dose remaining in the plasma of male A/J mice following a single intravenous administration is displayed. SPLP were labeled with [^{3}H]cholesteryl hexadecyl ether (1 μCi/mg of lipid). Error bars represent the standard error of the mean (S.E.), n=4.

determined at various time points. The percentage of injected dose remaining in circulation is displayed as a function of time in Fig. 4. SPLP containing the PEG–S-DMG were cleared most rapidly from the blood, with a $t_{1/2}$ of 1 h. Formulations containing the PEG–S-DPG and PEG–$CerC_{20}$ remained in the blood longer with $t_{1/2}$ of 6 and 7 h respectively. The PEG–S-DSG formulation exhibited the longest circulation lifetime with a $t_{1/2}$ of 15 h. These results are in good agreement with the results of Monck et al., who found that SPLP containing the short chain PEG$CerC_{14}$ had a much shorter half-life in the blood than those containing the long chain PEG$CerC_{20}$ [14].

It was also of interest to confirm the ability of SPLP containing PEG–S-DAGs to bypass the first-pass organs and accumulate at the tumor site [8,9,11,12]. SPLP were administered to mice bearing subcutaneous Neuro-2A tumors. The accumulation of the SPLP in the liver, lung, spleen and tumor is shown in Fig. 5. SPLP containing the longer PEG–S-DPG and PEG–S-DSG behave similarly to those containing PEG–$CerC_{20}$. As expected, the PEG–S-DMG SPLP showed signs of losing their charge-shielding PEG coating more quickly. They accumulated to a greater extent in organs of the reticulo–endothelial system (RES), particularly the liver. However, all SPLP demonstrated very low levels of accumulation in the lung. SPLP with longer-chain PEG–lipids (PEG–S-DPG, PEG–S-DSG, PEG–$CerC_{20}$) demonstrated increased levels of tumor accumulation when compared with PEG–S-DMG SPLP, presumably due to less accumulation in first pass organs (P<0.01).

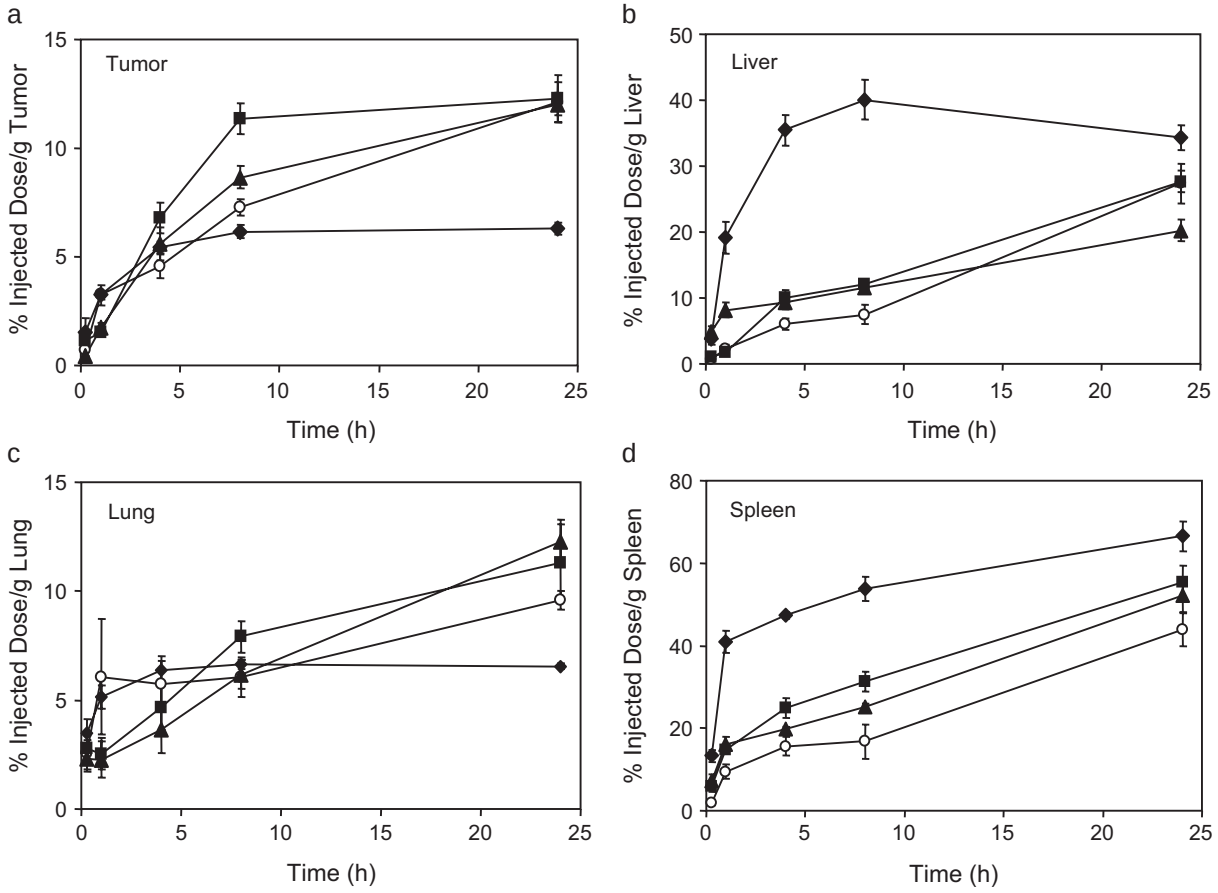

Fig. 5. Biodistribution of SPLP formulations containing the different PEG–lipids (♦=PEG–S-DMG, ■=PEG–S-DPG, ▲=PEG–S-DSG, ○=PEG–C_{20}Ceramide). Following a single intravenous administration of ^{3}H-CHE-labeled SPLP in Neuro-2A tumor-bearing male AJ mice, measurements were taken in the tumor (a), liver (b), lung (c) and spleen (d). Error bars represent the S.E., n=4. Tumors were 420 mg, ±30 mg (S.E.) at the time of harvest.

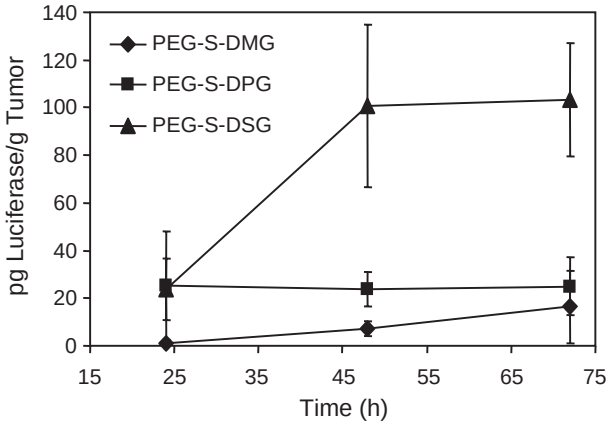

Fig. 6. Time course experiment showing luciferase gene expression in the tumor of male A/J mice following a single intravenous administration of SPLP containing PEG–diacylglycerols. Injected dose was 200 μl total volume, containing 2 mg total lipid and 100 μg total DNA. Error bars represent the S.E., n=4. Tumors were 158 mg, ±60 mg (S.E.) at the time of harvest.

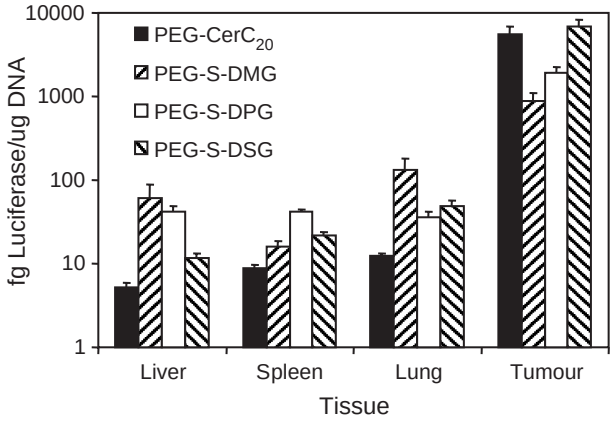

Fig. 8. Biodistribution of luciferase expression, represented as a function of DNA accumulation in Neuro-2a tumor-bearing male A/J mice. Time point was 48 h after a single intravenous administration of SPLP containing PEG–CeramideC$_{20}$ or PEG–S-DAGs. The considerable impact of tissue type on gene expression can be seen. Tumors were 158 mg, ±60 mg (S.E.) at the time of harvest.

The PEG–S-DAG SPLP clearly have sufficient circulation lifetimes to facilitate passive disease site targeting.

3.4. Protein expression following systemic administration of SPLP containing PEG–S-DAGs

It was of obvious interest to evaluate the efficiency of protein expression both in the tumor and first pass organs. SPLP containing PEG–CerC$_{20}$ are known to passively target distal tumor sites and elicit transgene expression following systemic administration [13]. SPLP that are more rapidly cleared from the circulation have less time to accumulate at the tumor site and are expected to yield lower levels of gene expression. The time course of luciferase gene expression in the tumor resulting from administration of PEG–S-DAG SPLP is shown in Fig. 6. Gene expression would appear to increase over the 72 h time period post-injection. Of the PEG–S-DAG SPLP formulations, those containing PEG–S-DSG yield the highest luciferase gene expression in the tumor. The amount observed is very similar to that of the PEG–CerC$_{20}$ SPLP (P<0.05).

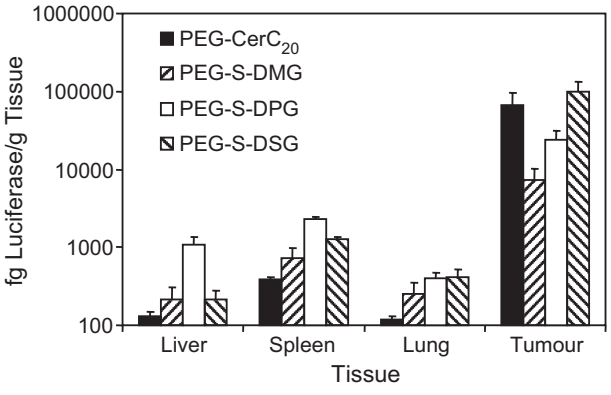

Fig. 7. Biodistribution of luciferase gene expression in Neuro-2a tumor-bearing male A/J mice. Time point was 48 h after a single intravenous administration of SPLP containing PEG–CeramideC$_{20}$ or PEG–S-DAGs. Error bars represent the S.E., n=4. Tumors were 158 mg, ±60 mg (S.E.) at the time of harvest. It should be noted that the y-axis is a log scale, unlike previous figures.

Given the pattern of SPLP biodistribution presented in Fig. 5, it was of interest to examine the resulting gene expression. Transgene expression in the tumor, lung, liver and spleen 48 h after SPLP administration is shown in Fig. 7. Clearly, the levels of gene expression in the tumor are far greater than in first pass organs for all four types of SPLP. The PEG–S-DSG and PEG–CerC$_{20}$ SPLP in particular exhibit large differentials that represent from 100- to almost 1000-fold increases over the other tissues. It would be expected that for each tissue type, biodistribution would be reflected in gene expression. For example, SPLP containing PEG–S-DSG and PEG–CerC$_{20}$ exhibit similar biodistribution profiles and accumulate at the tumor site in similar amounts. Thus, the resulting tumor transfection may be expected to be comparable. This is indeed the case.

A measure of the relative potency of SPLP in the different tissue types can be obtained by evaluating gene expression as a function of the amount of SPLP accumulation. Fig. 8 illustrates this relationship. The liver and spleen, despite accumulating large concentrations of SPLP, demonstrate very modest transgene expression. Intriguingly, this analysis shows that SPLP are up to 1000-fold more potent when transfecting tumor tissue than when transfecting cells of the lung, liver and spleen.

4. Discussion

This study demonstrates that PEG–S-DAGs can be successfully incorporated in SPLP and the resulting particles behave in a manner similar to those containing PEG–ceramides. PEG–S-DAGs are more easily and less

expensively synthesized than their ceramide counterparts and are easier to purify. PEG–S-DAGs with three different lipid domains of varying lengths were synthesized and incorporated into SPLP. In this study the rate and extent to which the different PEG molecules dissociate from the bilayer was modeled using an in vitro exchange assay, providing evidence for the proposed mode of action of these compounds. The in vitro and in vivo transfection efficiency, biodistribution and serum clearance of SPLP containing PEG–lipid conjugates of different lengths were evaluated.

In the exchange assay, vesicles containing PEG–S-DMG were clearly shown to become more fusogenic over time as the PEG–lipid dissociated from the particle. This is due to the uncovering of the fusogenic lipid bilayer as the outer PEG coating is removed. After 23 h at 37 °C, the degree of fusion in the sample had reached 72%. The PEG–S-DPG and PEG–S-DSG exhibited much reduced fusion profiles, with these samples achieving 36% and 32% fusion by the end of the experiment. We suggest that this is good evidence of the speed and extent to which the different PEG–lipids are exchanging from the bilayer. It is thought that lipidic compounds are held in the bilayer of lipid vesicles (such as SPLP) predominately by hydrophobic interactions between their hydrophobic domains [22]. Thus, a longer PEG–S-DAG acyl chain will have stronger forces securing it to the bilayer, and the molecule will remain bound for a greater period of time. It is true that other factors may influence the rate of diffusion, such as lipid head group chemistry [23], or the presence (or absence) of specific proteins [24]. However the exchange assay result correlates well with investigations into criteria that are most likely to be controlled by PEG dissociation — in vitro gene expression and in vivo circulation lifetime. In these experiments too, the PEG–S-DMG particles behave in a markedly different fashion to the PEG–S-DPG and PEG–S-DSG, as described below.

When examining gene expression in vitro, factors such as blood components and clearance by the liver and lung need not be considered. Since the dissociation of the PEG is required for the particle to become transfection competent, gene expression is related entirely to the rate at which the PEG-molecule is removed from the SPLP bilayer. As predicted, when examining the in vitro transfection efficiency of the different PEG–S-DAG containing SPLP the potency the short chain PEG–S-DMG formulation was found to be greatest. This result correlates with our exchange assay, as well as the findings of other authors [15,16], who have found SPLP containing shorter chain PEG–ceramides to be more transfection competent in vitro. SPLP containing the longer PEG–S-DPG and PEG–S-DSG perform similarly to those containing the PEG–$CerC_{20}$.

The behavior of SPLP in vivo is considerably more complex. PEG coatings conceal the positive charge of SPLP and prevent the interaction of the fusogenic DOPE with other lipid membranes (e.g. cellular membranes, other SPLP etc.) [20,21]. This lends the particle characteristics that allow for an extended circulation lifetime in the blood. The longer the PEG coating remains intact on the surface of the SPLP, the longer the particle's systemic half-life. This observation has been verified by other researchers, using PEG–ceramides [14,25]. Even very short PEG ceramides, such as the C_8, substantially increase the circulation time of SPLP when compared to DNA:lipid complexes [13]. PEG–S-DAGs impart similar characteristics. PEG–S-DMG formulations are cleared with a half-life of less than an hour. Increasing the length of the acyl chains of the PEG–S-DAGs increases the length of time that the SPLP will remain in the blood compartment. As with the in vitro transfection experiment, it is the PEG–S-DSG that most closely resembles the PEG–$CerC_{20}$ control.

PEG association and circulation lifetime have a direct effect on the biodistribution of SPLP. Unlike lipoplex systems, the amount of PEG–S-DAG SPLP accumulation in the lung is extremely low, corresponding to approximately 1% of the overall injected dose. Accumulation in the liver and spleen is somewhat higher. Extrapolating the results of the exchange assay, one would expect PEG–S-DMG SPLP to lose the stabilizing PEG coating more quickly, resulting in greater accumulation in the organs of the RES, as is the case (Fig. 5). To take advantage of passive targeting, particles must bypass these organs and remain in the circulation long enough to encounter the fenestrations in the tumor vasculature. SPLP containing the longer chain PEG compounds are therefore most successful. In this regard, PEG–S-DSG SPLP once more give the most similar results to the PEG–$CerC_{20}$ control.

The gene expression profiles of the SPLP are significant. Systemic delivery and subsequent gene expression in distal tumors have already been reported [13,14]. MacLachlan et al. first demonstrated how the expression obtained with PEG–$CerC_{20}$ SPLP was highly selective for the tumor by comparison with the other organs [26]. Our results with the PEG–S-DAGs are similar to those obtained with the PEG–$CerC_{20}$. Large differentials between transfection of the tumor and other organs are seen, in some cases up to 1000-fold. Due to slightly reduced accumulation of PEG–S-DMG SPLP in the tumor, transfection would be expected to be somewhat lower, as is the case. Of greatest interest was the analysis of relative SPLP potency at each organ site. It can be seen that the high transfection efficiency at the tumor is not simply a result of greater accumulation at this site; in fact, SPLP accumulation at the tumor is only marginally greater than at the lung, and far less than the liver or spleen. Rather, it appears that the SPLP collecting at the tumor site are more efficient at transfecting this tissue. The explanation may incorporate several factors.

The mitotic index (i.e. the speed at which the cells proliferate) may play a role in the preferential transfection of tumor tissue. It is known that nuclear delivery of non-viral vectors is facilitated by the breakdown of the nuclear envelope, and occurs during the prometaphase at the

beginning of the cell's M phase [27]. In vitro experiments using lipoplexes and SPLP showed that transfection efficiency was reduced by a factor of 20 in a cell population arrested in the G1 phase. This has implications for the SPLP mediated transfection of quiescent tissue. Until the nuclear membrane is disrupted, the DNA, now stripped of the protective lipid shell, will remain in the cytoplasm where it is subject to degradation by cytoplasmic nucleases. Lechardeur et al. reported that the half-life of plasmid DNA in the cytoplasm of HeLa and Cos cells is 50–90 min [28]. Hence, in more quickly dividing cells, such as tumor cells, there is less chance of the plasmid being the subjected of enzymatic breakdown. With respect to gene expression, this would manifest itself as tumor cells being transfected far more efficiently than other, more quiescent tissue.

Tam et al. also reported that accumulation of SPLP in the liver does not yield significant gene expression [13]. Analysis indicated that, despite high concentrations of the ^{3}H-CHE marker, there was actually very little intact plasmid in the liver. They postulated that this might reflect relatively rapid breakdown of the SPLP and its associated plasmid following uptake into liver phagocytes (Kupffer cells). These cells are known to play a leading role in the clearance of liposomal systems from the circulation [29].

It is also true that perturbations in transcriptional efficiency may have an effect on gene expression, both directly and indirectly [30]. The altered phenotype of the tumor cell could simply result in a more efficient transcription of the transgene. Alternatively, disregulated expression of certain genes common in transformed tissue may lead to biophysical/biochemical changes in the cell, such as increased rates of endocytosis or over-expression of cell surface receptors. Both of these could lead to more of the SPLP actually being internalized and increase the chance of transgene expression.

5. Summary

In summary, the results presented here show that PEG–S-DAGs can be substituted for PEG–ceramides to produce SPLP using the detergent dialysis procedure. The resulting SPLP show the same relationship between the PEG anchor chain length and transfection/pharmacokinetic behavior. These results support the theory of diffusible PEG–lipids that provide serum stability and long circulation lifetimes. We provide further evidence for this argument by showing that shorter chain PEG–lipids exchange more completely from the bilayer in an in vitro exchange assay. PEG–S-DAGs are easier to synthesize and purify than PEG–ceramides and present an attractive alternative for the production of SPLP. We conclude that PEG–S-DSG is a functionally effective replacement for PEG–CerC_{20} in SPLP formulations for systemic tumor delivery and gene expression.

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