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In Vivo Comparative Study of Lipid/DNA Complexes With Different *In Vitro* Serum Stability: Effects on Biodistribution and Tumor Accumulation

YE ZHANG,[1] ERICA L. BRADSHAW-PIERCE,[1] ALEXANDRA DELILLE,[2] DANIEL L. GUSTAFSON,[1] THOMAS J. ANCHORDOQUY[1]

[1]Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Sciences Center, Denver, Colorado 80262

[2]College of Veterinary Medicine Animal Cancer Center, Colorado State University, Fort Collins, Colorado 80523

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ABSTRACT: To evaluate the *in vivo* biodistribution and expression of DOTAP-Chol/DNA complexes (lipoplexes) with different *in vitro* serum stability, quantitative real-time PCR, *in vitro* luciferase expression and whole body luminescence imaging were used. In general, less tissue biodistribution, lower luciferase expression and whole body luminescence were observed for DOTAP:Chol (mol/mol 1:4)/DNA lipoplexes which had higher *in vitro* serum stability as compared to DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes. Plasmid DNA biodistribution and expression were mainly confined to the lungs, and the results suggest that *in vitro* serum stability may serve as a predictor of transfection in the lung. No correlation between plasmid DNA tissue biodistribution and gene expression was observed by simultaneous determination of the level of plasmid DNA tissue biodistribution and gene expression. While high doses of the formulation possessing increased *in vitro* serum stability did exhibit reduced entrapment in the lung, no corresponding increase in the plasmid levels of other tissues was observed. However, this formulation did show increased accumulation in tumors that was not further enhanced by PEGylation. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:237–250, 2008

Keywords: cationic lipids; cholesterol; serum stability; transfection; gene delivery; PCR; pharmacokinetics/pharmacodynamics; in vivo bioluminescence imaging

INTRODUCTION

It has been found that the circulation lifetime and clearance of nonviral vectors are related directly to serum destabilization and opsonization.[1–6] For systemic administration, it has been reported that cationic lipoplexes were taken up extensively by the lung, followed by spleen, liver, heart and kidney.[7–13] A generally accepted explanation for this phenomenon is that lipoplexes form aggregates with blood components and the aggregated particles are entrapped by the lung capillary bed.[14–21] In order to target different tissues or distant tumors, it would be preferable if lipoplexes possessed improved serum stability and long circulation times. While many researchers have employed PEGylated components to increase circulation times, studies have shown that this approach also reduces transfection rates.[22–26] The

Abbreviations: DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; Chol, cholesterol; $DOTAP^+/DNA^-$, molar charge ratio of DOTAP to negatively charged phosphates in the DNA; DSPE-PEG 2000, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(amino(polyethylene glycol) 2000); VEGF, vascular endothelial growth factor.

Ye Zhang's present address is Sirna Therapeutics, Inc., 2950 Wilderness Place, Boulder, CO 80301.

Correspondence to: Ye Zhang (Telephone: +1-303-449-6500; Fax: +1-303-546-8152; E-mail: Zhangy@sirna.com)

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presence of at least 25 mol% of cholesterol in liposomes has been shown to increase significantly both their stability and retention within the circulation.[21,27–30] Our previous *in vitro* serum stability study investigated higher cholesterol contents and demonstrated that DOTAP:Chol (mol/mol 1:4)/DNA lipoplex ($DOTAP^+/DNA^- = 4$) is much more stable in serum than DOTAP:Chol (mol/mol 1:1)/DNA lipoplexes when assessed for both biological and biophysical characteristics. [31] Recent studies in our laboratory (L. Xu, unpublished work) have demonstrated that DOTAP:Chol formulations are similar to that described for phosphatidylcholine:Chol liposomes in which 80 mol% cholesterol can be achieved.[32–34] It should be noted that cholesterol can form highly symmetrical domains at high mole fraction, and this may contribute to the enhanced serum stability of such formulations.[32–34] Therefore, we proposed that DOTAP:Chol (mol/mol 1:4)/DNA lipoplexes would have extended circulation life and broader biodistribution as compared to DOTAP:Chol (mol/mol 1:1).

Despite the fact that many *in vivo* studies have been performed, there are very few pharmacokinetic/pharmacodynamic (PK/PD) studies of cationic lipid/DNA complexes (lipoplexes) in the literature. Most studies involving the intravenous administration of lipoplexes have focused exclusively on gene expression[8,27,28,35,36] or biodistribution at only a few time points.[8,28,37,38] In the present study, biodistribution (as determined by real-time PCR) and expression (evaluated by both *in vivo* bioluminescence imaging and an *in vitro* luciferase assay) were monitored at a series time points (0.25, 0.5, 1, 4, 8, 24, 48, and 96 h) to provide a more extensive evaluation of the PK/PD of cationic lipid/DNA lipoplexes. Furthermore, as described above, the employment of two formulations possessing substantial differences in *in vitro* serum stability allows us to assess the utility of such measurements in predicting *in vivo* PK/PD parameters.

MATERIALS AND METHODS

Materials

Luciferase plasmid DNA (5.9 kb) and vascular endothelial growth factor (VEGF) plasmid DNA (5 kb) were generously provided by Valentis, Inc. (Burlingame, CA). *N*-(1-(2,3-Dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(amino(polyethylene glycol)2000) (DSPE-PEG 2000) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Sucrose was purchased from Pfanstiehl Laboratories (Waukegan, IL). The luciferase assay kit, and purified firefly luciferase were obtained from Promega (Madison, WI). D-firefly luciferin was acquired from Xenogen Corp. (Alameda, CA). Isoflurane was purchased from Sigma Chemical Company (St. Louis, MO). All chemicals were of reagent grade or higher quality.

Preparation of Liposomes

DOTAP, combined with cholesterol in either a 1:1 or 1:4 (DOTAP:cholesterol) molar ratio, and with/without DSPE-PEG 2000 were mixed in chloroform. The lipid mixture was dried under a stream of nitrogen gas and placed under vacuum (100 mTorr) for 2 h to remove residual chloroform, and dried lipids were subsequently resuspended in autoclaved, distilled water. Cationic liposomes were prepared the day before the experiment, stored overnight at 4°C, and sonicated in sterile 10% sucrose immediately before use.

Preparation of Lipoplexes

Lipoplexes with $DOTAP^+/DNA^- = 4$ were prepared by mixing different volumes of our stock lipid suspension (2 mg/mL) with plasmid DNA encoding luciferase (1 mg/mL). Lipoplexes were incubated for 1 h at room temperature. The concentration of DNA in the lipoplex suspension was 65 μg/mL. To increase the amount of luciferase DNA delivered into the mice while keeping the injection volume low to avoid hydrodynamic effects, the lipoplexes were concentrated five times (to 325 μg DNA/mL) with Millipore ultrafree 100 K MW centrifugal filters (Bedford, MA). This protocol for concentrating the lipoplexes did not alter the particle size as detected by dynamic light scattering (data not shown).

Animal Use and *In Vivo* Treatment Protocols

All procedures were approved by the University of Colorado Health Sciences Center and Colorado State University Committee on Animal Research. Female BALB/c mice (Charles River laboratories, Inc., Wilmington, MA) weighing 20–25 g were

administered lipoplexes via a single i.v. bolus dose of 200 μL in the tail vein. Animals treated with cationic lipids complexed with plasmid DNA encoding VEGF were used as experimental controls. Following treatment, 3 mice in each formulation were sacrificed at 0.25, 0.5, 1, 4, 8, 24, 48, and 96 h. Blood, lung, liver, kidney, heart and spleen were collected from each animal after sacrifice by cardiac stick exsanguinations under isoflurane, quickly frozen in liquid nitrogen, and stored at −80°C until analysis. Tissues were analyzed for luciferase expression using a luciferase assay kit (Promega), and plasmid DNA levels were quantitated via real-time PCR. Another three mice in each formulation were used for *in vivo* bioluminescence imaging of luciferase activity.

Quantification of DNA in Tissues

Total DNA from each tissue was extracted with a Qiagen DNeasy tissue kit (Qiagen, Valencia, CA) following the protocol provided by the manufacturer. Briefly, tissue samples are first lysed using Proteinase K and the lysate is loaded onto the DNeasy mini columns. During centrifugation, DNA is selectively bound to a silica-gel membrane. DNA is then washed to remove impurities and total DNA is eluted and quantified by $A_{260/280}$ measurements in a Hitachi UV/Vis spectrophotometer. Furthermore, a QIAprep Spin Miniprep Kit (Qiagen) was used to extract plasmid DNA from total DNA following the protocol provided by the manufacturer because genomic DNA was found to interfere with real-time quantitative PCR. The plasmid DNA encoding luciferase was then amplified and quantified via real-time quantitative PCR in an ABI GeneAmp 5700 Sequence Detection System (PE Biosystems, Foster City, CA). Extracted DNA was added to a SYBR Green PCR master mix (Qiagen) and 0.3 μM of primers (Invitrogen, Carlsbad, CA). The primers were designed using PrimerExpress software (PE Biosystems). And the sequences of primers are: 5′-CTGGA GAGCA ACTGC ATAAG GC-3′ and 5′-CTCAG CGTAA GTGAT GTCCA CC-3′. In order to quantitate the amount of DNA present in a reaction tube, standard curves were generated and the amounts of plasmid in the samples were determined by interpolation. To account for potential differences in the efficiency of DNA extraction and purification, three known amounts of luciferase plasmid DNA (0.5, 5, and 50 pg) were spiked into extracts from untreated animals to determine extraction efficiencies for each tissue. The extraction efficiencies were $84.98 \pm 2.66\%$, $64.49 \pm 1.04\%$, $40.32 \pm 4.20\%$, $87.87 \pm 3.24\%$, $81.67 \pm 2.92\%$, and $77.36 \pm 1.80\%$ (mean ± one standard deviation of triplicate samples) in liver, lung, kidney, heart, spleen and blood, respectively. These efficiencies were found to be very reproducible within this range of plasmid DNA contents, and they were used to calculate total plasmid levels in each tissue.

Pharmacokinetic Analysis

The average of plasmid levels from tissues of three mice was used for pharmacokinetic analysis. Thus, no standard deviation is reported in the results (Tab. 1). Data were analyzed using WinNonlin Professional® (version 4.1). Blood and the tissue data were analyzed using a noncompartmental method. For each formulation, the area under the blood concentration versus time curve (AUC), and half-life time ($t_{1/2}$) were calculated.

In Vitro Firefly Luciferase Assay

Extraction of luciferase from mouse tissues was carried out as described.[39] Frozen tissues were homogenized in 4 mL/g tissue of Cell Lysis buffer (Promega). After freeze-thawing three times by alternating liquid nitrogen and 37°C water baths, the homogenates were centrifuged at 14000 rpm for 10 min. Luciferase activity was assessed using 20 μL supernatant with 100 μL Luciferase Assay Reagent (Promega) in a glass cuvette. Preliminary studies attempted to utilize Monolight 2010 Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) for the luciferase assay. However, no luminescence signal could be detected for the 13 μg plasmid administration in either formulation due to the inadequate sensitivity of this instrument. In contrast, an IVIS imaging system possessed sufficient sensitivity to measure total luminescence, and the relative signal intensities were quantified using LivingImage software. Total protein contents were determined with a Bio-Rad protein assay kit (Hercules, CA) according to the manufacturer's instructions. The absorbance was measured at 550 nm using a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA). A standard curve of luciferase expression in each organ was generated by spiking known amounts of purified firefly luciferase into tissues from

Table 1. Noncompartmental Pharmacokinetic Analysis of Lipoplexes after Intravenous Bolus Administration

	DOTAP:Chol (1:4)		DOTAP:Chol (1:1)	
	AUC (h ng/mL)	Half-life (h)	AUC (h ng/mL)	Half-life (h)
(a) 13 μg luciferase plasmid DNA				
Blood	19.32	20.43	35.37	13.78
Lung	14.16	15.82	51.74	11.31
Liver	38.53	15.15	51.83	15.10
Kidney	1.13	13.67	7.17	16.70
Heart	2.32	10.19	9.84	7.83
Spleen	141.07	11.94	56.20	43.41
(b) 65 μg luciferase plasmid DNA				
Blood	96.23	68.90	263.15	48.68
Lung	285.62	15.40	569.74	12.74
Liver	117.23	43.36	182.24	45.16
Kidney	52.63	22.30	106.24	77.73
Heart	150.08	36.17	124.75	21.38
Spleen	276.80	16.55	541.77	90.64

untreated mice. The luciferase content of samples was calculated using the corresponding standard curve and reported as picograms of luciferase per mg total tissue protein.

In Vivo Bioluminescence Imaging of Luciferase Activity

The mice received an intraperitoneal injection of 100 μL aqueous solution of D-firefly luciferin substrate (150 mg/kg). Ten minutes later, mice were anesthetized with 2.5% isoflurane in 5 L O_2/min. Afterwards, mice were placed in a light-tight chamber and imaging was performed using an IVIS imaging system (Xenogen Corp.). Photons were quantified using LivingImage software (Xenogen Corp.).

Tumor Inoculation

MCF-7/ADR breast cancer cells (1×10^7 cells/mL) in a volume of 0.1 mL were s.c. injected into 15 female BALB/c nude mice 6–8 weeks old (Charles River Laboratories, Inc.). Animals with tumors were treated with lipoplexes 4 weeks after inoculation.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to determine statistical significance ($p < 0.005$) among the mean values for tumor accumulation (Fig. 6). A Tukey's multiple comparison test was used to determine statistical significance ($p < 0.05$) between formulations.

RESULTS

Quantification of Luciferase Plasmid DNA Using Real-Time PCR

Figure 1 shows a representative luciferase plasmid DNA standard curve. Using this method, plasmid DNA levels as low as 0.0001 pg (~20 molecules) could be reproducibly detected. No significant difference in amplification efficiency was found between linear and supercoiled luciferase plasmid DNA (data not shown), suggesting the PCR products were not affected by the

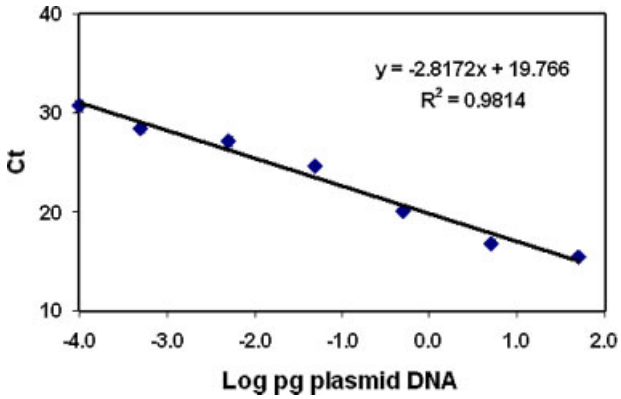

Figure 1. The standard curve of quantification of plasmid DNA with real time PCR. Symbols and error bars represent the mean ± one standard deviation of triplicate samples. C_t: threshold cycle.

supercoiled conformation of the plasmid. Thus, alterations in the tertiary structure that may occur *in vivo* will not affect our results.

Whole Blood Analysis

The kinetics of DOTAP:Chol (mol/mol 1:1 and 1:4)/DNA lipoplexes in the blood is shown in Figure 2. For the 13 μg plasmid injection, the two formulations were eliminated from the blood at comparable rates up to 96 h. For the 65 μg plasmid DNA treatment, there were high amounts of DNA found in blood for both lipoplex formulations up to 4 h after injection. A rapid decrease of DNA from 4 to 8 h, and then, no substantial changes in DNA content of blood were observed up to 96 h. This prolonged period in which the plasmid remains in the blood suggests that lipoplexes may become associated with platelets and/or leukocytes as shown in previous studies.[40]

The AUC value in blood of each formulation at both doses is shown in Table 1. The 1:1 lipoplexes exhibited higher AUCs than the 1:4 lipoplexes at both doses. However, the calculated half-life was slightly slower for 1:4 ($t_{1/2} = 20.43$ and 68.90 h) compared to 1:1 lipoplexes ($t_{1/2} = 13.78$ and 48.68 h) (Tab. 1a and b). These half-lives are consistent with the relative *in vitro* serum stabilities exhibited by these formulations,[31] but it is difficult to reach any definitive conclusions regarding a relationship between *in vitro* serum stability and *in vivo* circulation life time.

Tissue Distribution and Pharmacokinetics of Lipoplexes

A different biodistribution for the two lipoplexes was observed in the study. Mice receiving high doses of plasmid DNA generally had higher DNA distribution in tissues compared to mice receiving the low dose of DNA (Fig. 3A–E). For the low dose, more DNA in 1:1 lipoplexes distributed to lung, liver and kidney compared to that in 1:4 lipoplexes (Fig. 3A–C). The same trend was observed at the higher dose, but the differences were not as marked. Delivery to the heart and spleen did not exhibit consistent trends between lipoplex formulations (Fig. 3D and E).

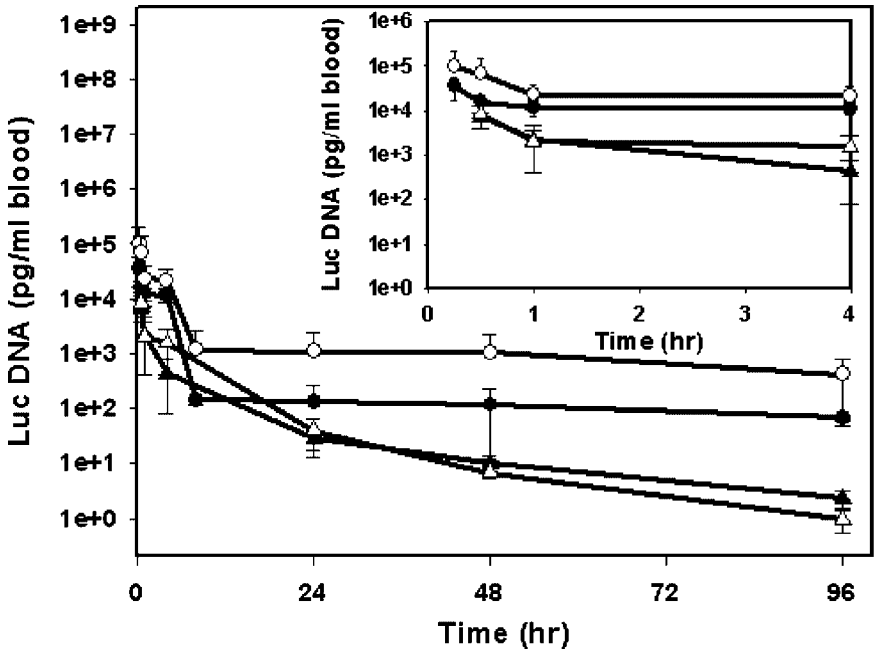

Figure 2. Blood clearance kinetics of plasmid DNA in mice. Closed circle, DOTAP:Chol (mol/mol 1:4)/DNA (65 μg) lipoplexes; open circle, DOTAP:Chol (mol/mol 1:1)/DNA (65 μg) lipoplexes; closed triangle, DOTAP:Chol (mol/mol 1:4)/DNA (13 μg) lipoplexes; open triangle, DOTAP:Chol (mol/mol 1:1)/DNA (13 μg) lipoplexes. Inset: Expanded *x*-axis of the first 4 h. Symbols and error bars represent the mean ± one standard deviation of samples from three mice.

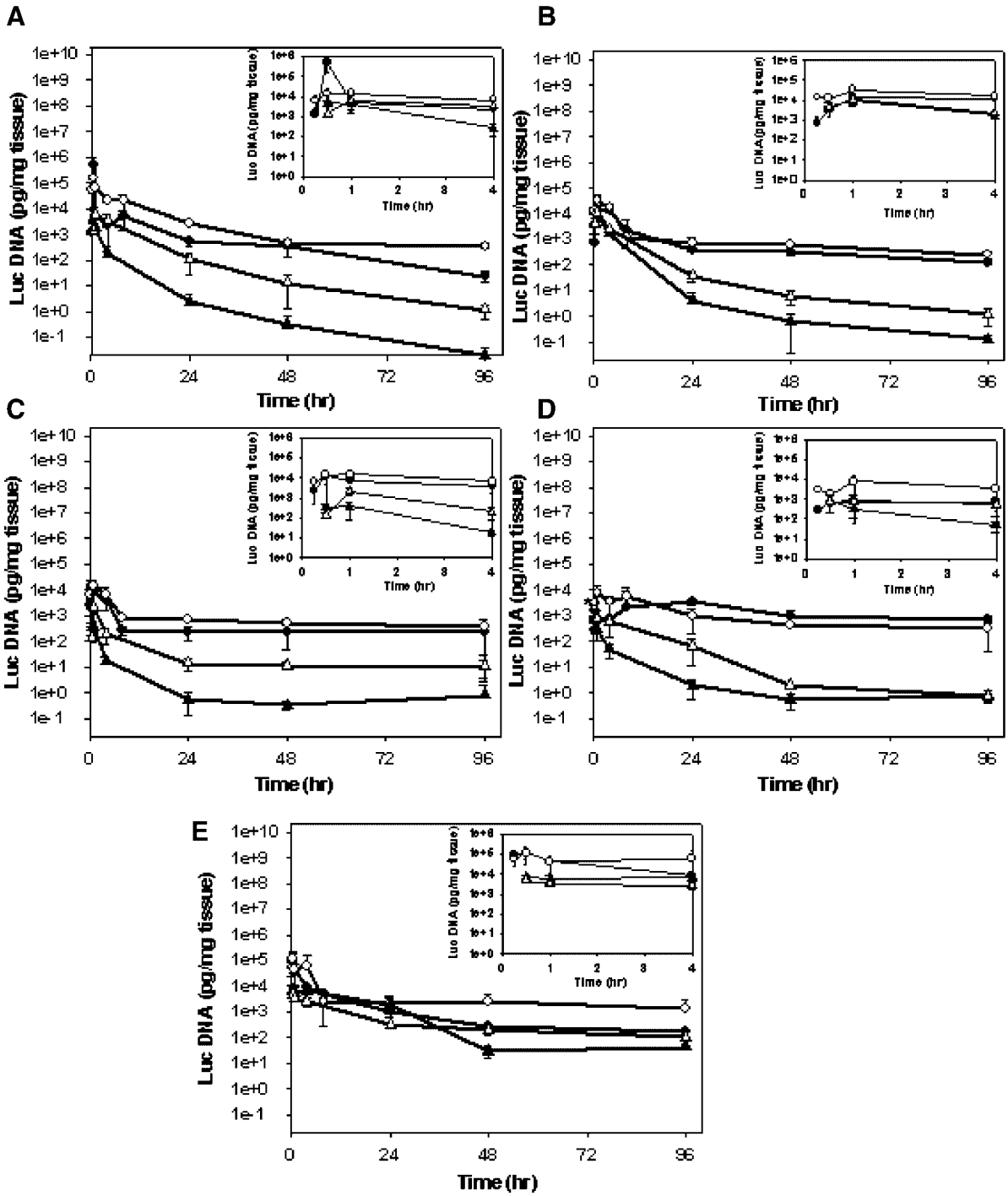

Figure 3. Tissue distribution of plasmid DNA in mice. (A) Lung; (B) liver; (C) kidney; (D) heart; (E) spleen. Closed circle, DOTAP:Chol (mol/mol 1:4)/DNA (65 μg) lipoplexes; open circle, DOTAP:Chol (mol/mol 1:1)/DNA (65 μg) lipoplexes; closed triangle, DOTAP:Chol (mol/mol 1:4)/DNA (13 μg) lipoplexes; open triangle, DOTAP:Chol (mol/mol 1:1)/DNA (13 μg) lipoplexes. Inset: Expanded *x*-axis of the first 4 h. Symbols and error bars represent the mean ± one standard deviation of samples from three mice.

Pharmacokinetic analysis showed that the greatest amounts of plasmid DNA in 1:1 lipoplexes distributed to lung and spleen, followed by liver and heart (Tab. 1). This distribution pattern was also observed with 1:4 lipoplexes at high dose, but low doses of the 1:4 lipoplexes distributed DNA primarily to the liver and spleen. DNA in the 1:1 formulation had a prolonged half-life in the spleen and kidney as compared to other tissues. In contrast, the 1:4 formulation exhibited longer half-lives in the liver and heart at the high dose, and no dramatic differences among tissues was observed at the low dose (Tab. 1).

The plasmid DNA amount in each tissue was estimated by determining the plasmid DNA concentration in a tissue sample at each time point, and multiplying by the tissue weight (Tab. 2). A progressive decrease of plasmid DNA in blood over time was found in all cases. Similarly, a gradual decrease in the total amount of plasmid DNA was detected at progressively later time points. However, the measured value for total DNA for the high-dose 1:4 lipoplexes at the 0.5 h time point (76.44 ± 22.68 μg) was higher than the dose value (65 μg); likely due to the large variation in plasmid DNA amount found in the lung (39.64 ± 28.39 μg) at this time point. Among all the tissues tested, liver generally had the highest plasmid content. Although the high dose exhibited greater plasmid accumulation in lung at 0.5 and 8 h, liver had a higher accumulation at other time points (Tab. 2). No luciferase plasmid DNA was detected in blood or tissues of control mice injected with cationic lipids complexed with plasmid DNA encoding VEGF.

Table 2. Amount of Plasmid DNA in Tissues at Different Time Points after Intravenous Bolus Administration

Time (h)	Blood (μg)	Lung (μg)	Liver (μg)	Kidney (μg)	Heart (μg)	Spleen (μg)	Total (μg)
(a) DOTAP:Chol (mol/mol 1:4)/DNA (13 μg) lipoplexes							
0.5	6.81 ± 0.33	0.37 ± 0.13	3.26 ± 1.35	0.07 ± 0.03	0.09 ± 0.07	0.78 ± 0.26	11.39 ± 5.19
1	1.67 ± 2.42	0.31 ± 0.13	5.82 ± 2.22	0.08 ± 0.06	0.03 ± 0.02	0.65 ± 0.32	8.55 ± 1.60
4	0.48 ± 0.39	0.02 ± 0.01	1.11 ± 0.19	0	0	0.79 ± 0.09	2.41 ± 0.85
24	0.03 ± 0.01	0	0	0	0	0.23 ± 0.17	0.27 ± 0.18
48	0.01 ± 0.00	0	0	0	0	0.01 ± 0.00	0.02 ± 0.00
96	0	0	0	0	0	0.01 ± 0.00	0.01 ± 0.00
(b) DOTAP:Chol (mol/mol 1:1)/DNA (13 μg) lipoplexes							
0.5	6.32 ± 0.83	0.11 ± 0.12	2.70 ± 1.20	0.03 ± 0.03	0.07 ± 0.10	0.55 ± 0.02	9.77 ± 4.67
1	2.24 ± 1.78	0.45 ± 0.34	7.40 ± 2.64	0.47 ± 0.20	0.08 ± 0.08	0.36 ± 0.06	11.01 ± 5.33
4	1.03 ± 0.60	0.25 ± 0.23	1.62 ± 0.15	0.04 ± 0.03	0.05 ± 0.04	0.30 ± 0.11	3.30 ± 0.28
24	0.04 ± 0.03	0.01 ± 0.01	0.02 ± 0.01	0	0.01 ± 0.01	0.04 ± 0.06	0.12 ± 0.06
48	0.01 ± 0.00	0	0	0	0	0.02 ± 0.01	0.04 ± 0.02
96	0	0	0	0	0	0.01 ± 0.00	0.01 ± 0.01
(c) DOTAP:Chol (mol/mol 1:4)/DNA (65 μg) lipoplexes							
0.25	39.44 ± 12.32	0.09 ± 0.11	0.5 ± 0.53	0.52 ± 0.41	0.03 ± 0.01	10.08 ± 1.10	50.67 ± 11.63
0.5	17.43 ± 4.79	39.64 ± 28.39	2.28 ± 1.20	2.71 ± 2.63	0.06 ± 0.07	14.31 ± 8.09	76.44 ± 22.68
1	13.29 ± 3.67	0.40 ± 0.26	9.79 ± 1.83	1.60 ± 1.79	0.07 ± 0.06	5.25 ± 0.64	30.39 ± 6.62
4	12.62 ± 3.63	0.16 ± 0.16	8.39 ± 0.61	0.84 ± 1.02	0.07 ± 0.05	1.00 ± 0.09	23.09 ± 4.33
8	0.15 ± 0.02	0.38 ± 0.26	1.53 ± 2.00	0.06 ± 0.02	0.21 ± 0.19	0.60 ± 0.01	2.93 ± 2.17
24	0.15 ± 0.15	0.04 ± 0.02	0.25 ± 0.02	0.05 ± 0.03	0.32 ± 0.33	0.12 ± 0.01	0.94 ± 0.46
48	0.13 ± 0.11	0.03 ± 0.02	0.22 ± 0.06	0.05 ± 0.04	0.10 ± 0.09	0.03 ± 0.01	0.55 ± 0.16
96	0.07 ± 0.01	0	0.08 ± 0.01	0.05 ± 0.04	0.07 ± 0.06	0.02 ± 0.01	0.27 ± 0.10
(d) DOTAP:Chol (mol/mol 1:1)/DNA (65 μg) lipoplexes							
0.25	48.83 ± 18.02	3.90 ± 1.01	9.22 ± 2.00	1.35 ± 0.92	0.27 ± 0.04	6.29 ± 3.70	57.12 ± 26.25
0.5	17.95 ± 11.99	11.74 ± 0.40	8.48 ± 1.82	3.05 ± 2.01	0.16 ± 0.03	13.87 ± 10.4	55.27 ± 22.19
1	17.17 ± 6.58	5.17 ± 0.04	21.92 ± 12.1	3.21 ± 1.73	0.72 ± 0.63	5.00 ± 3.81	51.19 ± 9.29
4	13.40 ± 14.43	1.60 ± 0.29	11.21 ± 3.08	1.45 ± 1.09	0.31 ± 0.24	6.94 ± 10.56	34.91 ± 8.3
8	1.27 ± 1.52	1.68 ± 1.06	0.84 ± 0.55	0.17 ± 0.02	0.50 ± 0.69	0.30 ± 0.27	4.77 ± 3.87
24	1.22 ± 1.48	0.20 ± 0.04	0.50 ± 0.29	0.15 ± 0.03	0.10 ± 0.08	0.25 ± 0.26	2.42 ± 2.13
48	1.16 ± 1.31	0.04 ± 0.01	0.39 ± 0.01	0.12 ± 0.03	0.04 ± 0.06	0.28 ± 0.30	2.02 ± 1.38
96	0.46 ± 0.41	0.02 ± 0.01	0.17 ± 0.06	0.08 ± 0.08	0.03 ± 0.03	0.15 ± 0.21	0.91 ± 0.44

The mean and the standard deviation of tissue samples from three mice are shown.

Luciferase Expression in Tissues

Luciferase expression was detected only in kidney 4 and 24 h after low-dose administration of 1:4 lipoplexes, whereas 1:1 lipoplexes induced measurable expression in all tissues, albeit not at all time points (Fig. 4). No expression was observed 96 h after administration in any tissue with either lipoplex. Expression was consistently greater in the 1:1 lipoplexes as compared to 1:4, and lung had the highest luciferase expression at all time points. Although these results are consistent with the greater distribution of the 1:1 lipoplexes to all tissues (Fig. 3), higher luciferase expression did

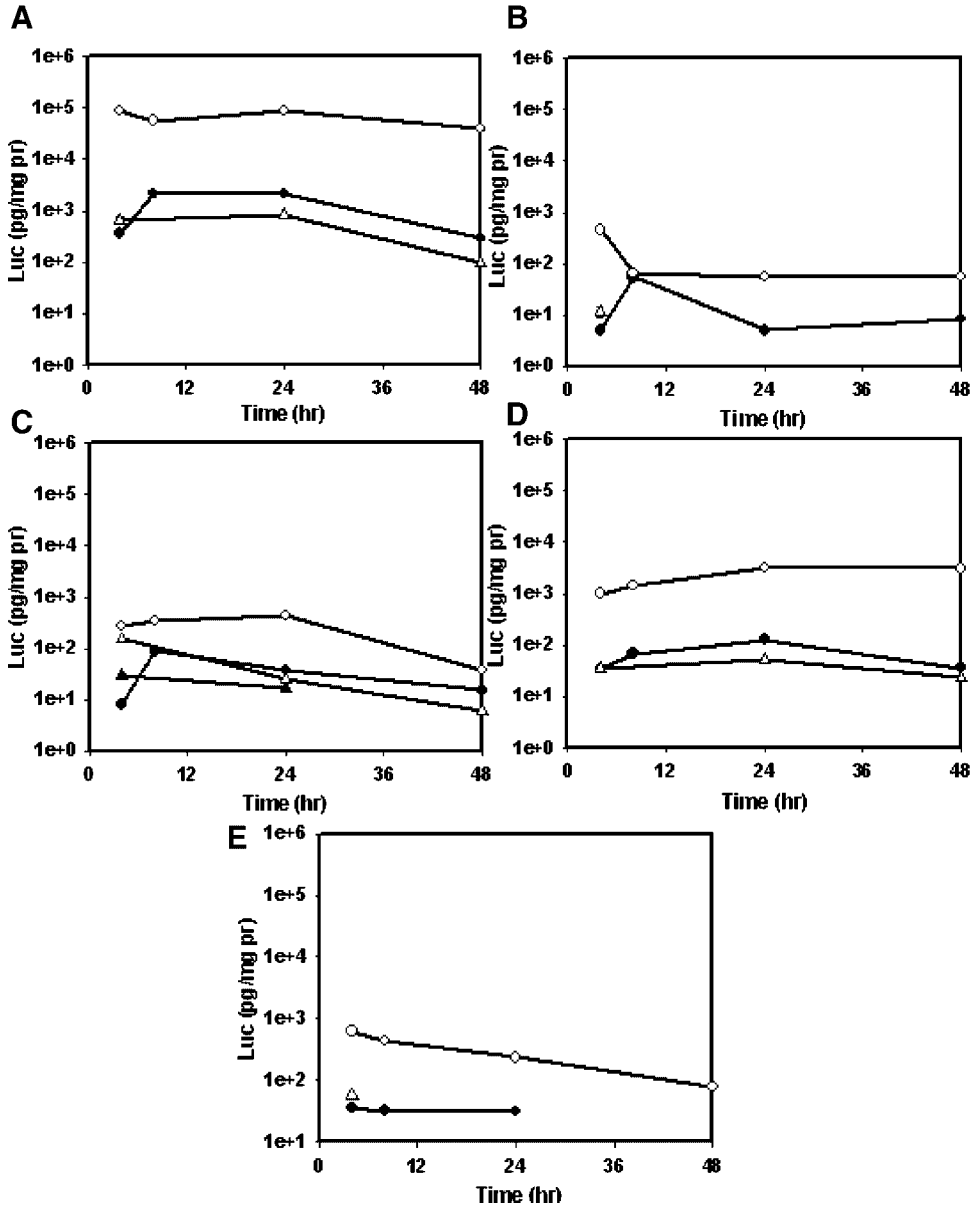

Figure 4. Luciferase expression in mouse tissues. (A) Lung; (B) liver; (C) kidney; (D) heart; (E) spleen. Closed circle: DOTAP:Chol (mol/mol 1:4)/DNA (65 μg) lipoplexes; open circle, DOTAP:Chol (mol/mol 1:1)/DNA (65 μg) lipoplexes; closed triangle, DOTAP:Chol (mol/mol 1:4)/DNA (13 μg) lipoplexes; open triangle, DOTAP:Chol (mol/mol 1:1)/DNA (13 μg) lipoplexes. Symbols represent the mean of samples from three mice; standard deviations are within the symbol size. Expression was not detected at 96 h, and time points lacking symbols indicate that expression was undetectable at that time point.

not correlate with a greater accumulation of plasmid DNA. For example, distribution was generally highest to the liver and spleen at 4 h (Fig. 3A, B, and E), but these organs exhibited much lower luciferase expression than lung (Fig. 4A, B, and E). No luciferase expression was detected in tissues of control mice injected with cationic lipids complexed with plasmid DNA encoding VEGF.

In Vivo Whole Body Bioluminescence Monitoring

Three mice administered each lipoplex formulation were followed to assess the temporal and spatial dynamics of luciferase expression using an IVIS imaging system. Expression was not detectable at the low dose by this technique and representative images for the high dose at different time points are shown in Figure 5. For 1:4 lipoplexes, luciferase expression was observed 4 h after administration and the distribution was mainly confined to the lungs (Fig. 5A), consistent with the luciferase expression measured in excised tissues (Fig. 4). The expression peak was observed at 8 h, followed by a decrease of expression at 24 and 48 h, and no expression was detected at 96 h. For 1:1 lipoplexes 4 h after administration, a much stronger luciferase expression was observed in the lungs as compared to 1:4 lipoplexes, and expression was detectable at 96 h (Fig. 5B). The expression peak was found at 8 h, and other organs exhibited expression at 8, 24, and 48 h. Consistent with tissue expression as quantified by a standard luciferase assay, 1:1 lipoplexes had higher tissue luciferase expression compared to the 1:4 lipoplexes; and the luciferase is mainly expressed in the lung for both formulations.

Tumor Accumulation in Nude Mice

Our two formulations, DOTAP:Chol (mol/mol 1:4)/DNA and DOTAP:Chol (mol/mol 1:1)/DNA, were fortified with polyethylene glycol (PEG)-lipid conjugates at a constant mole ratio of PEG to cationic lipid of 0.105. This ratio results in 5% PEG by mole in the 1:1 formulation; a level that has been shown to increase circulation times and tumor accumulation in other studies.[41,42] To enable a direct comparison, the total amount of

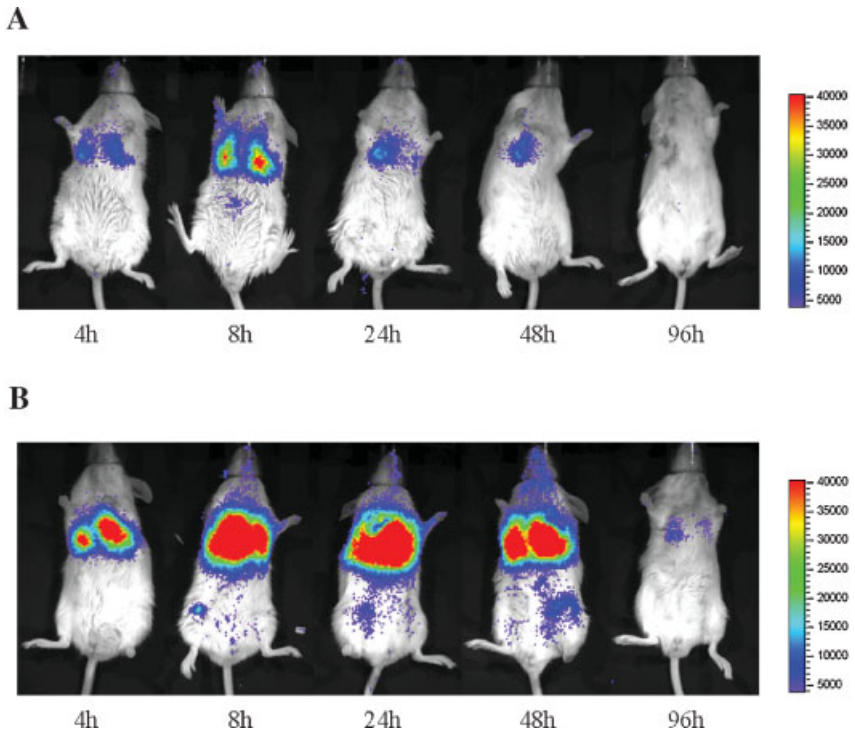

Figure 5. The dynamic change of luciferase expression in mice (4–96 h). (A) DOTAP:Chol (mol/mol 1:4)/DNA (65 μg) lipoplexes. (B) DOTAP:Chol (mol/mol 1:1)/DNA (65 μg) lipoplexes.

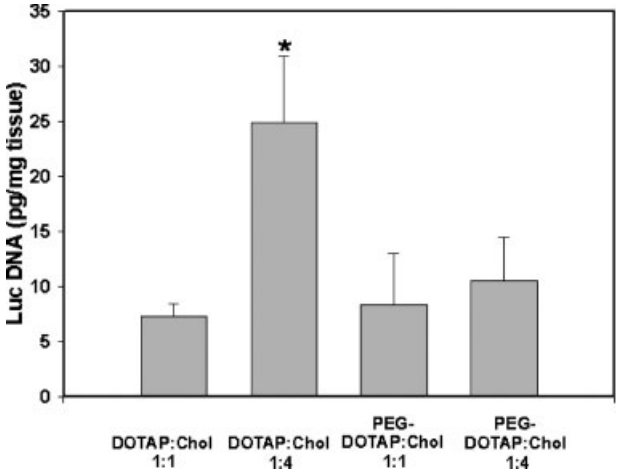

Figure 6. Tumor distribution of plasmid DNA in nude mice. Symbols and error bars represent the mean ± one standard deviation of samples from three mice. The asterisk denotes that significantly greater levels of plasmid were observed in mice treated with the 1:4 formulation (non-PEGylated) as compared to the 1:1 formulation.

PEG was held constant in both formulations DSPE-PEG:DOTAP:Chol (mol/mol/mol 0.1:0.95:3.8)/DNA and DSPE-PEG:DOTAP:Chol (mol/mol/mol 0.1:0.95:0.95)/DNA]. These liposomes were used to i.v. administer 65 μg plasmid DNA into female BALB/c nude mice with breast tumors. Four hours after administration, *in vivo* bioluminescence imaging of luciferase activity was measured and no signal was detected with any formulation (data not shown). The mice were then sacrificed and plasmid DNA levels in tumors were 7.33 ± 1.11, 24.85 ± 6.10, 8.32 ± 4.66, and 10.47 ± 3.96 pg/mg tissue for DOTAP:Chol (mol/mol 1:1)/DNA, DOTAP:Chol (mol/mol 1:4)/DNA, and DSPE-PEG:DOTAP:Chol (mol/mol/mol 0.1:0.95:0.95)/DNA DSPE-PEG:DOTAP:Chol (mol/mol/mol 0.1:0.95:3.8)/DNA lipoplexes, respectively (Fig. 6). It is noteworthy that a significantly greater level of plasmid was observed in tumors of mice administered the 1:4 formulation (non-PEGylated) as compared to the 1:1 formulation ($p < 0.01$). It should also be noted that tumor-bearing animals exhibited significantly greater ($p < 0.001$) lung accumulation of the 1:1 formulation (non-PEGylated) as compared to the 1:4 formulation (data not shown).

DISCUSSION

In the present study, the DOTAP:Chol (mol/mol 1:1)/DNA lipoplex formulation generally had higher levels of plasmid DNA in most of the tissues, and higher luciferase expression in all the tissues tested (Figs. 3 and 4). Consistent with other studies employing cationic lipid-based formulations, the highest luciferase expression was found in the lungs for both formulations (Figs. 4 and 5).[38,43] Considering that higher lung deposition is generally related to lipoplex aggregation in blood and lung entrapment,[14–21] our serum stability studies demonstrating the ability of the 1:4 formulation to resist aggregation would suggest that this formulation should exhibit reduced accumulation in the lung. A greater accumulation of plasmid and higher expression in lung was observed for 1:1 lipoplexes; consistent with the lower *in vitro* serum stability of the formulation (Figs. 3A and 4A). However, no broader distribution of the DOTAP:Chol (mol/mol 1:4)/DNA lipoplex formulation was observed, suggesting the *in vitro* serum stability is primarily related to lung transfection, but not correlated to *in vivo* biodistribution and protein expression in other tissues. The high lung accumulation of lipoplexes might also be due to the activation of receptors on endothelial cells by the lipoplexes, leading to leukocyte and platelet adhesion, and the consequent improved endothelial cell transfection of lipoplexes bound to leukocytes and/or platelets.[40]

In the experiments employing whole body bioluminescence, expression was primarily confined to the lungs. However, expression in other tissues (liver, kidney, heart, spleen) was detected by the *in vitro* luciferase assay (Fig. 4). We suggest three potential factors that may contribute to these observations: (1) The strong luminescence signals from lung make it difficult to resolve expression in other tissues via imaging; (2) the decreased detection sensitivity of whole body bioluminescence method compared to *in vitro* studies, that is, the net reduction of bioluminescent signal is approximately 10-fold for every centimeter of tissue depth;[44] and (3) organs with a high vascular content such as liver and spleen have the lowest transmission due to absorption of light by oxyhemoglobin and deoxyhemoglobin.[45]

No correlation between plasmid tissue biodistribution and gene expression was observed in the study (Figs. 3 and 4). For example, no significant differences were found for plasmid DNA distribution in lung and spleen for the high dose administration (Figs. 3A and E), but luciferase expression was approximately two orders of magnitude higher in lungs as compared to that in

spleen (Fig. 4A and E). Similarly, a substantial decrease of tissue expression of luciferase protein was observed in mice (high dose) from 48 to 96 h (Fig. 4; undetectable at 96 h), but minimal changes in plasmid DNA levels were found during this period (Fig. 3). The lack of correlation despite the simultaneous determination of the level of plasmid DNA and gene expression in tissues suggests that factors other than DNA delivery play a major role in determining the efficiency of *in vivo* gene transfection, consistent with earlier reports.[11,28,43] These other factors may include: (1) Specific transactivating elements that enhance transcription from the CMV promoter which are present to a greater extent in the lung than other tissues, resulting in more efficient lung expression compared to other tissues;[28] (2) The different activity of "transfection-controlling" cellular genes or gene products in tissues, which have been found to be rate-limiting for expressing transfected genes;[46] (3) The different cell types in organs that might not be as readily transfected as others.[47] Previous studies have shown that lipoplex accumulation in lung predominantly occurs in endothelial cells, whereas the main site of uptake in spleen and liver is in macrophages.[40] Therefore, it seems likely that macrophages might process the lipoplexes differently than endothelial cells due to their specialized intracellular mechanisms for degrading phagocytosed materials;[40] and (4) Potential silencing of CMV promoter within mammalian cells after a couple of days due to cytosine methylation at the GATC motif contained in this promoter.[48–50]

To deliver genes into organs other than the lung, it is necessary for delivery systems to be stable in blood such that aggregation does not cause entrapment by the lung capillary bed. PEG-lipid conjugates can provide a steric barrier at the liposomal surface, and have been extensively used to increase circulation times. This approach masks the positive charge on lipoplexes and reduces interactions with serum components, thereby extending the residence time in the blood and allowing accumulation at distal sites.[22,51–53] It has been reported that liposome formulations incorporating PEG are associated with prolonged circulation times in blood, a marked decrease in uptake by tissues such as lung, liver and spleen, and a corresponding increased accumulation in implanted tumors.[24,41,42,54–59] Alternatively, serum stability can be enhanced by incorporating higher cholesterol contents.[21,28,29,31,60] Our results with tumor-bearing mice show that 1:4 lipoplexes exhibited significantly higher tumor accumulation than the 1:1 lipoplex formulation (24.9 ± 6.1 pg/mg vs. 7.3 ± 1.1 pg/mg) ($p < 0.01$), consistent with the greater *in vitro* serum stability demonstrated by the former formulation.[31] Surprisingly, no enhancement of tumor accumulation was seen with PEGylated lipoplexes. Although enhanced accumulation might have been evident at higher PEGylation levels, previous studies have shown that 5% PEG is sufficient to increase circulation times and tumor accumulation.[42,59] Also, no expression was observed in the nude mice used for the tumor accumulation experiments, and these mice also exhibited low levels of plasmid distribution to the lungs (55.0 ± 27.3 and 258.2 ± 22.4 pg/mg for the non-PEGylated 1:4 and 1:1 lipoplexes, respectively). The levels of plasmid observed in the lungs of tumor-bearing nude mice are dramatically lower than that observed in BALB/c mice lacking tumors (2111.5 ± 250.2 and 21097.1 ± 3799.1 pg/mg for non-PEGylated 1:4 and 1:1 lipoplexes, respectively). These observations suggest that the pharmacodynamics and pharmacokinetics are dramatically altered in nude mice bearing tumors, and that the serum stable 1:4 formulation accumulates in tumors to levels approximately threefold higher than the less stable formulation. Furthermore, these results show that enhanced tumor accumulation may be achieved by formulating lipoplexes with higher cholesterol contents in the absence of PEGylated components.

The greater tumor accumulation of the 1:4 formulation is consistent with the greater half-life in the blood and the tendency of long circulating formulations to accumulate in tumors. However, this appears to contradict the fact that this formulation exhibited lower tissue accumulation in mice lacking tumors. Considering the increased "leakiness" of the abnormal tumor vasculature,[61,62] it should not be surprising that serum stable formulations accumulate in tumors despite exhibiting reduced distribution to normal tissues. A similar observation of tumor accumulation was also reported in viral vectors.[63] We suggest that this prolonged lifetime of the serum stable formulation in the blood causes greater exposure of the 1:4 formulation to blood nucleases, that ultimately result in degradation of the DNA prior to tissue uptake. This suggestion is consistent with our data showing that tissue uptake is not enhanced in mice lacking tumors despite increased circulation half-life. It follows that efficient delivery to tumors will require serum

stable formulations to incorporate specific ligands that target tumor cells and enhance uptake in order to prevent prolonged exposure to blood nucleases.

In summary, serum-stable lipoplexes incorporating high cholesterol contents exhibited longer half-lives in the blood and less accumulation in lung and liver as compared to a standard 1:1 (DOTAP:Chol) formulation. However, the increased stability did not cause broader biodistribution to organs in mice lacking tumors. In contrast, when these lipoplexes were injected into tumor-bearing mice, a significantly greater accumulation of plasmid in tumors was observed to correlate with reduced entrapment in the lung. These data suggest that employment of high cholesterol contents may present an alternative to PEGylation as a strategy by which increased serum stability and tumor accumulation may be achieved. Given the relative ease by which non-PEGylated vectors can be targeted, future studies will investigate whether tumor accumulation of high cholesterol lipoplexes can be further enhanced by incorporating targeting ligands (e.g., tumor-homing peptides).

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