Cationic Liposomes-Mediated Plasmid DNA Delivery in Murine Hepatitis Induced by Carbon Tetrachloride

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ABSTRACT

In order to elucidate the influence of hepatic disease-stage on cationic liposomes-mediated gene delivery, we investigated the cationic liposomes-mediated plasmid DNA delivery with time in murine hepatitis induced by subcutaneous injection of CCl_4.   Liver injury after injection of CCl_4 was confirmed by determination of serum AST and ALT activities.   Two kinds of liposomes constructed with N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethlylammoniumchloride and dioleylphosphatidylethanolamine (DOTMA-DOPE) or DOTMA and cholesterol (DOTMA-CHOL) were used for gene delivery vector.   We determined luciferase activities in various organs after intravenous administration of the lipoplexes.   The CCl_4-treated mice administered with DOTMA-DOPE lipoplexes showed the significant decreases of transgene expression in the liver and spleen at 18 h after CCl_4 injection.   On the other hand, the CCl_4-treated mice administered with DOTMA-CHOL lipoplexes showed a significant increase in the liver at 48 h.

In conclusion, our findings demonstrate that murine hepatitis induced by CCl_4 can influence cationic liposomes-mediated plasmid DNA delivery.   The extent of influences was also affected by lipid contents.   These results indicate the necessity of considering the timing and the formulation for gene therapy according to the disease stage.

KEY WORDS: gene delivery; cationic liposome; murine hepatitis; non-viral vector

1. INTRODUCTION

Gene therapy is expected as a drastic method for curing inborn and acquired diseases such as cancer, cardiovascular disease and rheumatoid arthritis (Frey, 1999; Gautam, 2002). Nucleic acids, however, are rapidly degraded by nucleases and exhibit poor cellular uptake when delivered in aqueous solutions. The success of gene therapy highly depends on the development of effective and secure delivery vectors. The gene delivery vectors are categorized into viral and nonviral vectors. Non-viral vectors have several advantages including non-immunogenicity, low acute toxicity, and flexibility to design a vehicle with well-defined structural and chemical properties to yield mass production (Tang, 1997; Mario, 1998). Two major form of non-viral vector are polyplexes (complexes of DNA and cationic polymer) and lipoplexes (complexes of DNA and cationic liposomes). These are independently effective for gene delivery in several cell lines and *in vivo* experiments.

One of the target internal organs of gene therapy is the liver for treatment of hepatic cancer, hepatic cirrhosis, and fulminant hepatitis. Successful gene delivery requires efficient uptake of DNA complexes into target cells, releasing of DNA into the cytoplasm, and adequate gene expression in the hepatocyte. These steps for gene delivery are considered to be affected by not only pharmaceutical factors of DNA complexes but also biological factors such as extent and stage of hepatic diseases. However, we have few reports in systematic study for the effect of hepatic disease-stage on gene delivery (Sasaki, 2006; Tada, 2006). In the previous study, we investigated the influence of murine hepatitis induced by carbon tetrachloride (CCl_4), which has been extensively used as an experimental model of liver disease such as hepatic cirrhosis and drug-induced hepatopathy, on gene expression of polyplex (Salgado, 2000; Ha, 2003; Sasaki, 2006). As the results, the transgene expression induced by the polyplex in the liver of

CCl$_4$-treated mice significantly decreased at 18 h after CCl$_4$ injection compared to the control mice.   This result indicated the necessity of considering the timing and dose for gene therapy according to the disease stage.   On the other hand, the extent of the influences must be affected by pharmaceutical formulations of the vectors.   The lipoplex is another useful vector for plasmid DNA delivery.   However, there have been no reports about the influence of pharmaceutical formulations on the fluctuation of gene expression in the hepatitis.

In the present study, we investigated the influence of the hepatitis induced by CCl$_4$ on lipoplexes-medicated plasmid DNA delivery.   Plasmid DNA encoding firefly luciferase was used as the model reporter gene.   Two types of lipoplexes such as N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethlylammonium chloride and dioleylphosphatidylethanolamine (DOTMA-DOPE) and DOTMA and cholesterol (DOTMA-CHOL) were used because of their high gene expression expression and low toxicity which has been reported previously (Farhood, 1995; Hong, 1997; Lasic, 1997; Koltover, 1998).

2. MATERIALS AND METHODS

2.1. Materials

DOTMA was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). DOPE was purchased from Nippon Oil and Fats Co. (Tokyo, Japan). CHOL was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). CCl_4 was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were obtained commercially as reagent-grade products.

2.2. Construction of plasmid DNA

pCMV-luciferase was constructed by subcloning the *Hind* III / *Xba* I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, U.S.A.) into the polylinker of CMV early immediate promoter-driven pcDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.). Plasmid DNA was multiplied in the *Escherichia coli* strain XL1-blue, isolated, and purified using a EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). Plasmid DNA was dissolved in 5% dextrose solution and stored at -80 °C until analysis. The plasmid DNA concentration was measured at 260 nm and adjusted to 1 mg/mL.

2.3. Preparation of cationic liposomes

DOTMA-DOPE (1:1 molar ratio) and DOTMA-CHOL (1:1 molar ratio) liposomes were prepared according to a previous report (Kawakami, 2001). After a mixture of DOTMA (9.5 mg) and DOPE (7.9 mg) or DOTMA (9.5 mg) and CHOL (5.8 mg) in chloroform were placed in a round-bottom glass tube, chloroform was evaporated. The lipid film obtained was further dried *in vacuo* in a desiccator for 4 h. The film was resuspended in sterile 5% dextrose (5 mL) by vortexing followed by ultrasonic radiation at 100W for 3 min under nitrogen gas at 0 °C.

The resulting liposomes were extruded 11 times through doublestacked 100 nm polycarbonate membrane filters.

2.4. Preparation of lipoplexes

Cationic liposomes formulations were added to the stock solution of plasmid DNA (1 mg/mL) and 5% dextrose, and mixed thoroughly by pipetting and left for 30 min at room temperature to allow complex formation. The mixing ratio of liposomes and plasmid DNA was expressed as a +/− charge ratio, which is the molar ratio of cationic lipids to plasmid DNA phosphate residue.

2.5. Evaluation of physicochemical properties of liposomes

The particle size and the ζ-potential of liposomes were measured with Zetasizer Nano ZS (Malvern Instruments, Ltd., United Kingdom).

2.6. Gel Retardation Assay

Two micrograms of plasmid DNA and an appropriate amount of stock cationic liposomes solution were diluted with 5% dextrose, respectively. After dilution, the diluted cationic liposomes solution was added to the DNA solution and the resulting mixture was used for analysis. After 30 minutes at room temperature, 10 μL aliquots of the complex solution were mixed with 2 μL loading buffer (30% glycerol, 0.2% bromophenol blue) and loaded onto a 0.6% agarose gel. Electrophoresis (i-Mupid® J, Cosmo Bio, Tokyo, Japan) was carried out at 50 V in running buffer solution (40 mM Tris/HCl, 40 mM acetic acid, 1 mM EDTA). Retardation of the complexes was visualized by ethidium bromide staining.

2.7. *In vivo* gene expression experiments

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee. Male ddY mice (5-6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). After shipping, mice were acclimatized to the environment for one day at least before experiments were begun. CCl_4 was dissolved in olive oil at a concentration of 10% (v/v). After mice were fasted for 20 h, the mice of experimental group were injected subcutaneously with CCl_4 (5 mL/kg body weight) to induce liver injury. The mice of control group were injected subcutaneously with olive oil of the same quantity. Lipoplexes were prepared before every experiment. Individual mice were injected intravenously with the lipoplexes at charge ratio 2 (pDNA 40 μg in 300 μL/mouse) at 18, 48, and 168 h after CCl_4 injection. After 6 h following intravenous injection of complexes, mice were sacrificed, and the liver, kidney, spleen, heart, and lung were dissected. Each experiment was performed using three to seven mice.

2.8. Evaluation of luciferase expression

The tissues were washed twice with cold saline and homogenized with a lysis buffer. The lysis buffer consisted of 0.1 M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA. The lysis buffer was added in a weight ratio of 3 μL/mg for liver samples, 5 μL/mg for kidney samples, and 10 μL/mg for other organ samples. The homogenates were centrifuged at 15,000 rpm (Kubota 3700, Kubota, Tokyo, Japan) for 5 min. The supernatants were used for luciferase assays. The 10 μL of supernatant was mixed with 50 μL of luciferase

assay buffer (Picagene, Toyo Ink, Tokyo, Japan) and the light produced was immediately

measured using a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany).

The luciferase activity is indicated as relative light units (RLU) per gram of tissue.

2.9. Statistical analysis

Statistical analysis was performed using Student's t-test. A p value of less than 0.05 was

considered significant.

3. RESULTS

3.1. Physicochemical properties of liposomes

The particle size and ζ-potential of the liposomes were measured. The DOTMA-DOPE liposomes and DOTMA-CHOL liposomes showed 21.7 ± 9.2 nm and 34.1 ± 11.2 nm of a mean particle size, and 65.2 ± 1.1 mV and 65.7 ± 1.7 mV of a mean ζ-potential, respectively. The two liposomes showed extremely similar physicochemical properties.

3.2. Gel retardation assay

Complex formations of lipoplexes were examined by a gel retardation assay (Fig. 1). Naked plasmid DNA was detected as two bands on the agarose gel (Lane 1; A and B). At charge ratio 0.5 for both lipoplexes naked DNA and retarded DNA was detected (Lane 2; A and B). A slight amount of naked plasmid DNA was detected at the charge ratio 1.0 of DOTMA-CHOL lipoplexes (Lane 3; B). On the other hand, the bands of plasmid DNA were not detected at the charge ratio 1.0 of DOTMA-DOPE lipoplexes (Lane 3; A). The charge ratio 1.5 of DOTMA-DOPE lipoplexes and DOTMA-CHOL lipoplexes also showed no detection of plasmid DNA bands (Lane 4; A and B).

3.3. DOTMA-DOPE and DOTMA-CHOL lipoplexes mediated *in vivo* gene expression at various times after CCl_4 injection

In the previous report, liver injury in mice by CCl_4 was confirmed by measuring activity of serum asparete transaminase (AST) and alanine transaminase (ALT) (Sasaki, 2006). In this model, the activity of serum ALT and AST peaked (4033 IU/L of ALT, 10464 IU/L of AST) at

18 h after CCl$_4$ injection and dropped to one fortieth and one fifteenth at 48 h, respectively. And at 168 h after, the activities became the same as control mice. On the other hand, after intravenous injection of the complexes into mice, the luciferase activity of various organs was determined over time in the preliminary experiment. As the results, the luciferase activity peaked at 6 h, gradually decreased, and mostly disappeared at 24 h. We compared the luciferase activity 6 h after intravenous injection of the lipoplexes.

Fig. 2 shows luciferase expressions in the liver, kidney, spleen, heart, and lung of mice after administration of DOTMA-DOPE (A) and DOTMA-CHOL (B) lipoplexes at 18 h after CCl$_4$ injection. By the CCl$_4$ injection, the luciferase expressions of DOTMA-DOPE lipoplexes were significantly decreased in the liver and spleen ($P < 0.05$) and those of DOTMA-CHOL lipoplexes were not substantially affected.

The luciferase expressions of the each lipoplexes administrated at 48 h after CCl$_4$ injection were evaluated (Figs. 3A and 3B). The CCl$_4$ injection did not significantly decrease the luciferase expressions of DOTMA-DOPE lipoplexes. On the other hand, the luciferase expressions of DOTMA-CHOL lipoplexes were significantly increased in the liver by the CCl$_4$ injection ($P < 0.01$).

Figs. 4A and 4B show luciferase expressions of the each lipoplexes which administrated at 168 h after CCl$_4$ injection. In the both lipoplexes, the luciferase expressions of the CCl$_4$ treated mice were comparable to the control mice.

4. DISCUSSION

Among non-viral systems, cationic lipsosomes have been reported to be suitable for effective human gene therapy (Tsukamoto, 1995; Sorgi, 1997; Meyer, 1998; Woodle, 2001; Zhdanov, 2002). Cationic liposomes are constructed with cationic lipids and "helper-lipids". Cationic lipids, DOTMA, are often used for forming electrostatic complexes with DNA (Felgner, 1987). CHOL and DOPE are well known helper-lipids. CHOL was known to stabilize liposomal membrane. DOPE is a fusogenic lipid to destabilize lipid bilayers by promoting the transition from a lamellar to a hexagnonal phase, it is believed to be involved in endosomal disruption. The DOTMA-DOPE liposomes is popular commercial vector for gene delivery as 'lipofectine'. The liposomal vectors were studied in many reports, however, there are a few report that the transgene efficiencies of the liposomal vectors were affected by biological factors such as extent and stage of hepatic diseases.

In this study, we used DOTMA-DOPE and DOTMA-CHOL liposomes as non-viral vectors. Physicochemical characteristics such as particle sizes and ζ-potentials of those liposomes were determined. Both liposomes showed extremely similar particle sizes and ζ-potentials. These liposomes can formulate the electrostatic complexes (lipoplexes) with plasmid DNA, which was identified by gel electrophoresis (Fig. 1). In 1.5 charge ratio, the plasmid DNA was completely complexed with each liposomes, migrations of plasmid DNA and plasmid DNA bands not observed. However, obvious differences were found in the DNA condensation capability of two cationic liposomes. DOTMA-DOPE liposomes were completely complexed with plasmid DNA at a lower theoretical charge ratio than DOTMA-CHOL liposomes. The strong binding and condensed conformation of DOTMA-DOPE liposomes with plasmid DNA might be explained by high fluidity of lipid membrane because DOPE is a fusogenic lipid

although CHOL makes liposome membrane stable.

Most gene delivery systems, including viral vectors as well as non-viral vectors, e.g., lipoplexes, polyplexes and lipopolyplexes, are rapidly cleared from the circulation and are preferentially taken up by the 'first-pass' organs such as liver, lung and spleen. The gene expression efficiency in various tissues was examined after intravenous administration of these lipoplexes into mice (Figs. 2, 3, and 4). The high gene expression efficiency was observed in the liver and spleen for DOTMA-DOPE lipoplexes although it was shown not only in the liver and spleen but also in the lung for DOTMA-CHOL lipoplexes. It was reported that DOTMA-DOPE lipoplexes showed lower transgene efficiency than DOTMA-CHOL lipoplexes in the lung and it was caused by agglutination with erythrocytes of DOTMA-DOPE lipoplexes (Sakurai, 2001a, b). The agglutination made DOTMA-DOPE lipoplexes embolized in the lung capillary with erythrocytes, however, DOTMA-CHOL lipoplexes did not agglutinate with erythrocytes and they could transfer to the lung capillary endothelial cells. On the other hand, the decreased gene expression of spleen at 168 h was observed in Fig. 4. It may be explained by the influence of aging. Actually, it was reported that the aged mice showed lower gene expression than young mice (Hamilton, 1997; Johnson, 1998).

We prepared a mouse model of experimental hepatitis with CCl_4 according to previous method (Sasaki, 2006). CCl_4 hepatotoxicity depends on the reductive dehalogenation of CCl_4 catalyzed by cytochrome P-450 in the liver cell endoplasmic reticulum. A cascade of secondary mechanisms is evoked by the initial events of CCl_4 metabolism, and the secondary mechanisms are responsible for ultimate plasma membrane disruption and cell death (Recknagel, 1989; Lesiuk, 2003; Muriel, 2003). A significant increase of the activity of serum ALT and AST was observed after CCl_4 subcutaneous injection. Those activities were significantly

elevated and peaked at 18 h after CCl$_4$ injection.   In macroscopic observation, the liver had a

lighter color and dull surface with multiple white spots.   The activities were dropped to one

fortieth in ALT and one fifteenth in AST at 48 h, respectively.   At 168 h, the transaminase

activity was in the normal range as in the control.   We determined the gene expression

efficiency in several tissues at 6 h following administration of the lipoplex at 18 h, 48 h, and 168

h after CCl$_4$ injection.

DOTMA-DOPE lipoplexes were administered intravenously into CCl$_4$-treated mice.

CCl$_4$ injection significantly reduced the transgene expression induced by DOTMA-DOPE

lipoplexes at 18 h in the liver and spleen where high transgene expressions were observed in the

control mice.   The reduction of transgene expression by DOTMA-DOPE lipoplexes in spleen

was still observed at 48 h.   The present results at 18 h corresponded with previous reports.

Sasaki et al. stated that PEI polyplexes showed the remarkable reduction of high gene

expression efficiency in the spleen at 18 h after CCl$_4$ injection into mice (Sasaki, 2006).   Yu et

al. reported that adenovirus-mediated gene transfer was reduced by liver injury induced by bile

duct ligation or repeated administrations of CCl$_4$ (Yu, 2002).   Tyler et al. found that

monocrotaline-induced endovascular inflammation markedly reduced the efficacy of

lipid-mediated pulmonary vascular gene transfer (Tyler, 1999).   On the other hand, Sasaki et

al. reported that PEI polyplexes showed an inductive transgene expression in the liver at 48 h

after CCl$_4$ injection into mice (Sasaki, 2006), although the significant induction of transgene

expression was not observed in DOTMA-DOPE lipoplexes at 48 h.   DOTMA-DOPE

lipoplexes may not be delivered to proliferous hepatic cells through sinusoidal membrane by

agglutination.   No significant difference in the transgene expression by DOTMA-DOPE

lipoplexes was observed at 168 h, indicating that the influence of CCl$_4$-induced hepatitis by

DOTMA-DOPE lipoplexes-mediated gene delivery was reversible.

Further, DOTMA-CHOL lipoplexes were administered intravenously into CCl_4-treated mice. The transgene expression was not decrease in the liver at 18 h after CCl_4 injection into mice; furthermore, the high transgene expression was induced in the liver at 48 h after CCl_4 injection (Fig. 3). The activity of serum ALT and AST at 48 h after CCl_4 injection decreased to one fortieth and one fifteenth of those at 18 h. The macroscopic damage subsequently recovered although slight enlargement of the liver was observed. Sasaki et al. also reported that PEI polyplexes significantly enhanced transgene expression in the liver compared with control mice at 48 h after CCl_4 injection. It was demonstrated that the transfection efficiency of non-viral vectors critically depends on the cell cycle and is enhanced by mitotic activity in the regeneration (Mortimer, 1999; Collins, 2007). Actually, Tada et al. reported that partial hepatectomy of mice increased transgene expression of PEI/pDNA complexes in the liver with increased liver weight (Tada, 2006). Liver regeneration was considered to be triggered by multiple pathways and cytokine interaction. The reason for increase in the transgene expression was not clear in the present study; however, the cationic liposome-mediated gene vector containing CHOL was the best choice for the gene therapy of murine hepatitis induced by CCl_4 among our experiments. The distribution of PEI/pDNA complexes in the liver may be enhanced by the hepatitis. There is also a possibility that the hepatitis changes the process of gene transfection such as taken up by endocytotic pathway, releasing pDNA into the cytoplasm, and the gene entry into the nucleus. On the other hand, hepatitis is also able to change the plasma protein in the blood and may influence the lung delivery of DOTMA-CHOL lipoplexes by affecting the agglutination. Further study is necessary to elucidate the influencing mechanism of hepatitis on cationic liposomes-mediated gene expression.

In conclusion, our findings demonstrate that murine hepatitis induced by CCl_4 can influence not only polyplex- but also lipoplex-mediated plasmid DNA delivery according to the disease stage. The influence of hepatitis was smaller in lipoplex-mediated plasmid DNA delivery than polyplex-mediated plasmid DNA delivery. Among the lipoplex-mediated plasmid DNA deliveries, the extent of influences was dramatically affected by lipid contents. These results indicate the necessity of considering the formulation for gene therapy according to the disease stage.

ACKNOWLEDGMENTS

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Figure legends

Fig. 1   Gel detergent assay.   Plasmid DNA was complexed with DOTMA-DOPE liposomes (A) or DOTMA-CHOL liposomes (B) at 0 (Lane1), 0.5 (Lane2), 1 (Lane3), and 1.5 charge ratio (Lane4).

Fig. 2   Luciferase expression in the liver, kidney, heart, and lung of mice after the administration of DOTMA-DOPE lipoplexes (A) and DOTMA-CHOL lipoplexes (B) at 18 after CCl_4 injection.   All data points are the mean values S.E. (n=3-7).   *; $p < 0.05$ compared with control mice at the corresponding time.   Control mice (closed bars), CCl_4-treated mice (open bars).

Fig. 3   Luciferase expression in the liver, kidney, heart, and lung of mice after the administration of DOTMA-DOPE lipoplexes (A) and DOTMA-CHOL lipoplexes (B) at 48 after CCl_4 injection.   All data points are the mean values S.E. (n=4-7).   **; $p < 0.01$ compared with control mice at the corresponding time.   Control mice (closed bars), CCl_4-treated mice (open bars).

Fig. 4   Luciferase expression in the liver, kidney, heart, and lung of mice after the administration of DOTMA-DOPE lipoplexes (A) and DOTMA-CHOL lipoplexes (B) at 168 after CCl_4 injection.   All data points are the mean values S.E. (n=3-5).   Control mice (closed bars), CCl_4–treated mice (open bars).

Fig. 1

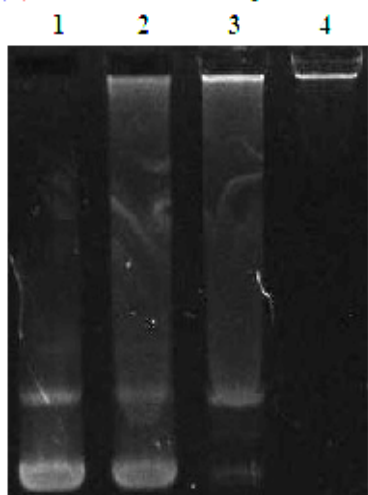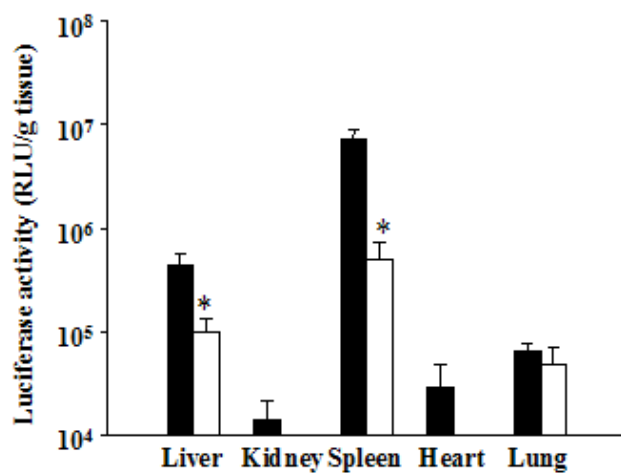(A) DOTMA-DOPE liposomes
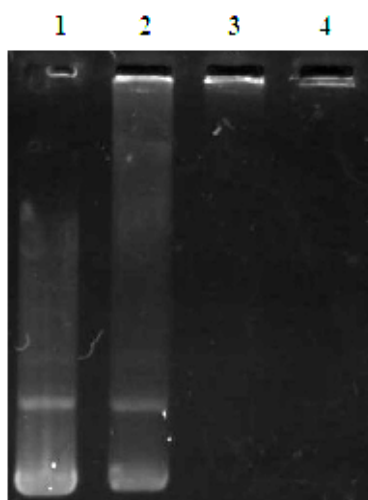
(B) DOTMA-CHOL liposomes

Fig. 2
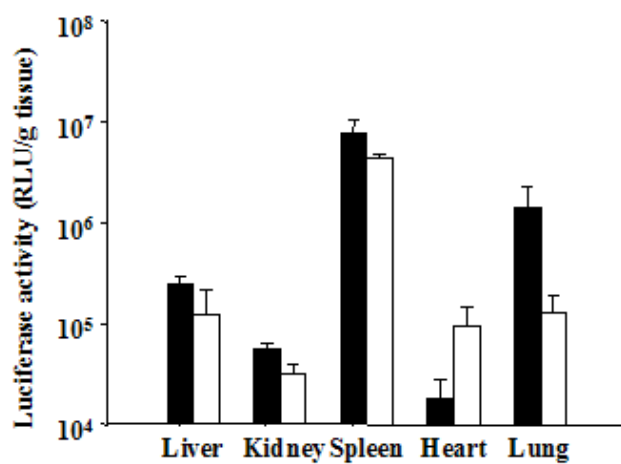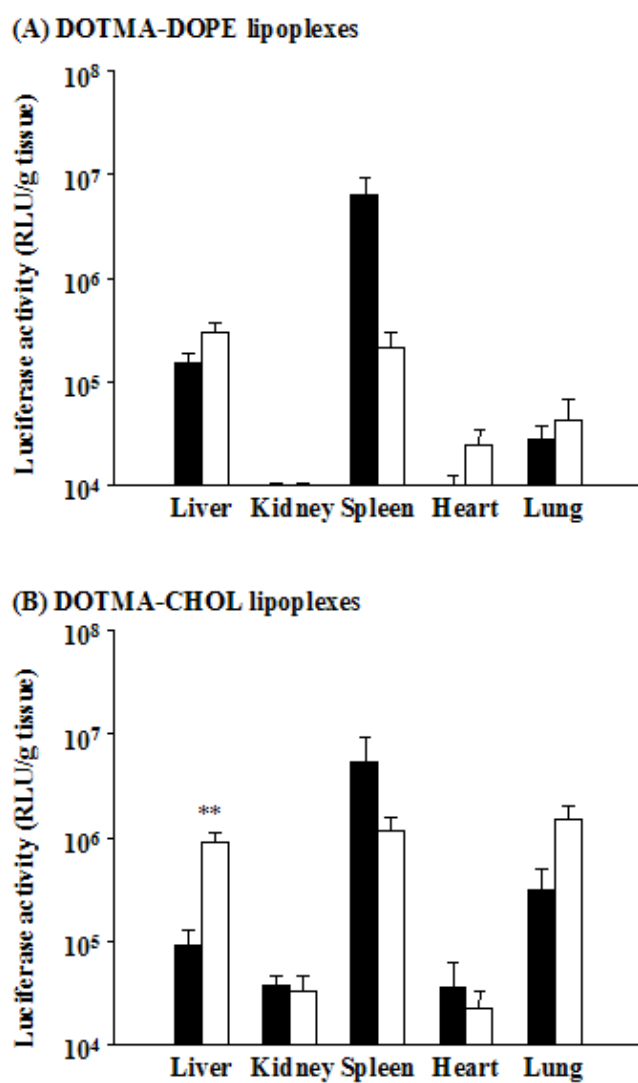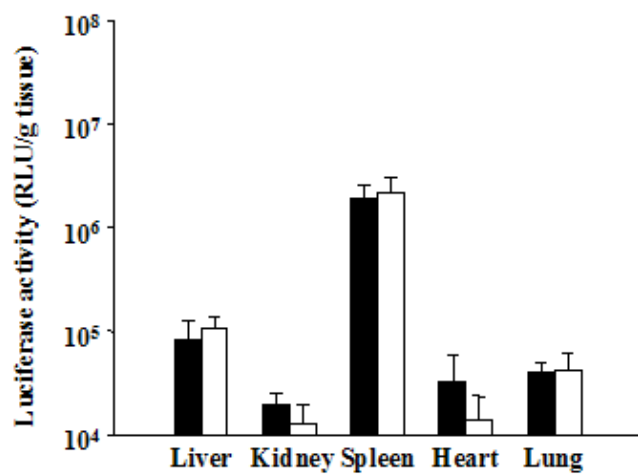

(A) DOTMA-DOPE lipoplexes

(B) DOTMA-CHOL lipoplexes

Fig. 3

(A) DOTMA-DOPE lipoplexes

(B) DOTMA-CHOL lipoplexes

Fig. 4

(A) DOTMA-DOPE lipoplexes

(B) DOTMA-CHOL lipoplexes