International Journal of Pharmaceutics 363 (2008) 192–198

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Non-linear pharmacodynamics in the transfection efficiency of a non-viral gene delivery system

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ARTICLE INFO

Article history:
Received 26 December 2007
Received in revised form 5 June 2008
Accepted 21 July 2008
Available online 30 July 2008

Keywords:
Non-viral gene delivery systems
Nonlinearity
Pharmacodynamics
Intracellular pharmacokinetics
Intranuclear disposition

ABSTRACT

Transfection efficiencies using LipofectAMINE varied by more than three orders of magnitude depending on the concentrations of lipid and plasmid DNA (pDNA) used to prepare the lipoplexes. When lipoplexes were formed at lower concentrations a striking positive but non-linear relationship was found between dose and transfection efficiency, while at higher (i.e., normally used) concentrations a linear relationship was maintained. To determine the contribution of intracellular pharmacokinetics (PK) and pharmacodynamics (PD) to the observed nonlinearity, we quantified pDNA in whole cells and nuclei by real-time PCR and compared the results with the transfection efficiencies. There was no significant difference in the efficiency of intracellular PK; however, a remarkable difference was observed in the efficiency of PD. Analysis of individual cells by confocal laser scanning microscopy (CLSM) revealed that the amount of nuclear-delivered pDNA was higher for lipoplexes prepared at the normal concentration (NCL) compared to those of lipoplexes prepared at low concentration (LCL). Moreover, the size of the NCL was larger than that of the LCL. Both the size of the lipoplex particle and the dose appear to contribute to the non-linear efficiency of PD. These results emphasize the need to control not only intracellular PK, but also PD for the rational development of non-viral gene delivery systems.

1. Introduction

Quantitative evaluation of intracellular pharmacokinetics (PK) is important for rational development of effective non-viral gene delivery systems (Kamiya et al., 2003; De Laporte et al., 2006). Various methods have been used to investigate intracellular PK (Wattiaux et al., 2000; Varga et al., 2001; Clamme et al., 2003). For example, Akita et al. (2004) developed a quantitative, three-dimensional analytical system using confocal image-assisted three-dimensionally integrated quantification (CIDIQ) to evaluate the intracellular PK of non-viral delivery systems. Using this technique, Hama et al. (2006) found that LipofectAMINE PLUS (LFN) required three orders of magnitude more gene copies to achieve the same level of gene expression as adenoviruses. However, there was no significant difference between LFN and adenoviruses in intracellular PK, including such parameters as cellular uptake, endosomal escape and nuclear delivery. Unexpectedly, a 7000–8000-fold difference was observed in the efficiency of gene expression after delivery to the nucleus, which we define as pharmacodynamics (PD). These results are consistent with a report by Varga et al. (2005), which compared the intracellular trafficking of adenoviruses with that of polyethylenimine/DNA or Lipofectamine/DNA by mathematical modeling.

The importance of PD has been pointed out by Tachibana et al. (2002). They measured nuclear-delivered plasmid DNA (pDNA) by quantitative PCR and Southern blot analysis of AH130 cells transfected with cationic liposomes. They found a remarkable saturation in the level of transfection activity (reporter gene expression) versus the number of pDNA molecules in the nucleus, while no such saturation was observed between nuclear-delivered pDNA and the dose of transfection. Moreover, we have found a remarkably positive non-liner relationship between dose and gene expression of non-viral gene delivery systems, such as a LFN and our original gene delivery system octaarginine-modified multifunctional envelope-type nano-device (R8-MEND), although the cellular up-take mechanism and intracellular trafficking pathways were different between both non-viral vectors (Moriguchi et al., 2006). The efficiency of PD at high doses of luciferase-encoding

Abbreviations: pDNA, plasmid DNA; PK, pharmacokinetics; PD, pharmacodynamics; NCL, normal concentration lipoplex; LCL, low concentration lipoplex; CIDIQ, confocal image-assisted three-dimensionally integrated quantification; LFN, LipofectAMINE PLUS; R8-MEND, octaarginine-modified, multifunctional, envelope-type nano-device; ANOVA, analysis of variance; SDS, sodium dodecyl sulfate.

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doi:10.1016/j.ijpharm.2008.07.020

pDNA was more than 10-times higher than that at low doses, while no significant difference was found in the intracellular PK. Furthermore, the co-presence of GFP-encoding pDNA (dummy DNA) significantly enhanced the efficiency of PD without changing the amount of luciferase-encoding pDNA in the nucleus. From these results, we found that the nonlinearity of non-viral vectors is caused mainly by PD after the nuclear delivery of pDNA, and the pDNA delivered to the nucleus is transcribed synergistically depending on the amount in the nucleus (Moriguchi et al., 2006). Therefore, these results emphasize the need to control PD in the development of efficient non-viral gene delivery systems.

However, it still remains to be elucidated how pDNA works synergistically in the nucleus. The understanding of this mechanism would be expected to lead to a breakthrough in the area of non-viral gene delivery. In the present study, we examined the relationship between nonlinearity and physical properties of non-viral vectors using lipoplex to clarify the mechanism of the phenomenon we found previously (Moriguchi et al., 2006). Interestingly, the lipoplexes prepared under different conditions showed different relationships between dose and gene expression. At this point, intracellular PK and PD were evaluated in detail and the effect of physical properties of lipoplexes on PD was discussed.

2. Materials and methods

2.1. Materials

LipofectAMINE and PLUS reagents were purchased from Invitrogen Corp. (Carlsbad, CA, USA). pCMV-luc (7037 bp) encoding luciferase was prepared using an Endfree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Other chemicals used were commercially available reagent grade products. NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

2.2. Gene transfection

Transfection of pDNA was performed using LipofectAMINE PLUS reagent as follows. Briefly, NIH3T3 cells were seeded at a density of 4×10^4 1 day before transfection. Four microliters of PLUS reagent and 0.4 μg of pDNA were incubated in 21 μl of serum-free medium (DMEM(−)) for 15 min at room temperature and mixed with DMEM(−) including 1 μl of lipofectAMINE, followed by further incubation at room temperature for 15 min. This concentration for lipoplex formation was according to the manufacturer's instructions and was defined as concentration ratio 1. To prepare lipoplex at low concentration, pDNA, PLUS and LipofectAMINE were diluted with DMEM(−) before mixing. The lipoplex suspension containing 0.02 μg of pDNA was diluted to 0.25 ml by adding DMEM(−) and then incubated with cells for 3 h at 37 °C (0.5 pg pDNA/cell). Next, 1 ml of DMEM containing 10% fetal calf serum was added to the cells, followed by further 45 h incubation. The cells were then washed and solubilized with reporter lysis buffer (Promega, Madison, WI). The luciferase reaction was initiated by the addition of 50 μl of luciferase assay reagent (Promega) to 20 μl of the cell lysate, measured using a luminometer (Luminescencer-PSN, ATTO, Japan). The amount of protein in the cell lysate was determined using a BCA protein assay kit (PIERCE, Rockford, IL). Data are shown as the mean ± S.D. ($n = 3$).

2.3. Electrophoresis and size measurement of the lipoplex

For agarose gel electrophoresis of lipoplexes, samples containing 64 ng of pDNA were treated with various concentrations of sodium dodecyl sulfate (SDS) to release the pDNA. Electrophoresis was performed on a 0.8% agarose gel at 100 V for 30 min, and the gel was then stained with ethidium bromide. The diameters of the lipoplexes were measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka electronics, Japan).

2.4. Imaging of intracellular pDNA by confocal laser scanning microscopy

Transfection with rhodamine-labeled pDNA was performed to observe intracellular trafficking of lipoplex as reported previously (Akita et al., 2004). Briefly, 6×10^4 cells were seeded in 2 ml of cell culture medium on a 35-mm/GLASS BASE DISH (IWAKI, Chiba, Japan) for 2 days. Lipoplexes containing 1 μg of rhodamine-labeled pDNA suspended in 1 ml of DMEM(−) were added to the cells and incubated for 1.5 h at 37 °C. After incubation, the nucleus was stained with Hoechst 33342. Confocal images were captured by a Zeiss Axiovert 2000 inverter fluorescence microscope equipped with a 63 × NA 1.4 Planachromat objective (Carl Zeiss Co. Ltd., Jena, Germany). For CIDIQ analysis, 20 Z-series images obtained from the bottom of the dish to the top of the cells were recorded. Each 8-bit TIFF image was transferred to an Image-Pro Plus version 4.0 (Media Cybernetics, Inc.) to quantify the pixel area of each cluster in each nucleus.

2.5. Quantification of cellular uptake and nuclear delivery of pDNA

Cellular uptake and nuclear delivery of pDNA were quantified using the method described in previous reports (Moriguchi et al., 2006). Briefly, 2×10^5 cells were seeded on a six-well culture plate for 24 h. Then, lipoplexes containing 0.1 μg or 2 μg of pDNA suspended in 1.25 ml of DMEM(−) were added to the cells (0.5 pg pDNA/cell or 10 pg pDNA/cell) with incubation for 1.5 h at 37 °C. The cells were trypsinized and suspended in 100 μl of CellScrub Buffer (Gene Therapy Systems, Inc.) followed by gentle shaking at 4 °C for 30 min. The cell suspension was then centrifuged and the precipitate was subjected to DNA purification as the whole-cell sample. For further purification of the nuclear fraction, the trypsinized cells were suspended in 187.5 μl of CellScrub Buffer, and 62.5 μl of cell lysis solution (2% IGEPAL CA630, 40 mM NaCl, 12 mM $MgCl_2$, and 40 mM Tris–HCl, pH 7.4) was then added. The suspension was centrifuged at 9200 × g for 2 min at 4 °C, and the supernatant was removed. This procedure was repeated three times. DNA was purified from either the cell lysates or the isolated nuclei using a GeneElute Mammalian Genome DNA Miniprep kit (Sigma–Aldrich, St. Louis, MO). Real-time PCR was performed using a PCR apparatus (7500 Real Time PCR System, Applied Biosystems Co.) according to the manufacturer's instructions with 0.5 μM of two types of primers (luc(+): GGTCCTATGATTATGTCCGGTTATG and luc(−): ATGTAGCCATCCATCCTTGTCAAT, or Beta-actin forward: AGAGGGAAATCGTGCCTGAC and Beta-actin reverse: CAATAGTGATGACCTGGCCGT) and SYBR Green Real Time PCR Master Mix (TOYOBO Co.).

2.6. Statistical analysis

Data are presented as mean ± S.D. With noted exceptions, comparisons between multiple treatments were made with one-way or two-way analysis of variance (ANOVA) followed by the Scheffe's F-test. A Mann–Whitney-test was applied in Fig. 6. A value of $P < 0.05$ was considered significant.

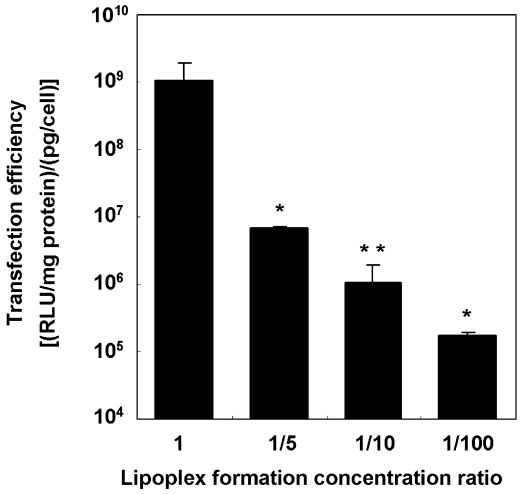

Fig. 1. Transfection efficiencies of lipoplexes formed at different concentrations. The concentration used to prepare lipoplex according to the manufacturer's instruction was defined as concentration ratio 1. The lipoplexes were prepared at four different concentrations (1, 1/5, 1/10, 1/100) and added to NIH3T3 cells at a dose of 0.5 pg pDNA/cell. Luciferase activities were measured at 48 h after transfection. Transfection efficiencies were estimated by dividing the luciferase activities by the dose of pDNA. Data are presented as mean ± S.D. ($n = 3$). Asterisks indicate statistically significant differences (*$P < 0.05$ and **$P < 0.01$ vs. concentration ratio 1).

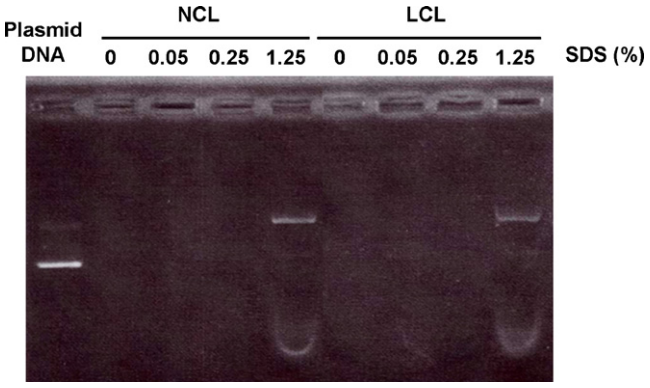

Fig. 2. Comparison of release of pDNA from lipoplexes prepared at normal and low concentrations. Lipoplexes formed at normal concentration (NCL) or low concentration (LCL) were mixed with different concentrations of sodium dodecyl sulfate (SDS) and the release of pDNA was visualized by agarose gel electrophoresis, followed by staining with ethidium bromide.

3. Results

3.1. Effect of concentrations of pDNA and lipid in lipoplex preparations on transfection efficiency

We initially examined the effect of concentrations of pDNA and lipid during lipoplex formations on transfection efficiency at a certain lipid/pDNA ratio (LipofectAMINE reagent 2.5 μl/μg pDNA). Lipoplexes of luciferase-encoding pDNA and LFN were prepared at four different concentrations, and transfection efficiencies were calculated as luciferase activity divided by the dose of luciferase pDNA per cell. In Fig. 1, the concentration ratio of 1 refers to the concentrations of pDNA and lipid recommended by the manufacturer (i.e., 8 μg/ml pDNA, 40 μg/ml lipid). The other lipoplexes (1/5, 1/10, and 1/100) were prepared by mixing serially diluted pDNA, PLUS reagent and LipofectAMINE. After lipoplex formation, the lipoplexes were diluted to the same concentration of pDNA with medium, followed by incubation with cells. The dose and concentration of pDNA in the cell culture medium were the same among the lipoplexes, while the concentrations during lipoplex formation were different. As shown in Fig. 1, the transfection efficiencies of the lipoplexes were significantly different despite using the same overall dose of pDNA (0.5 pg/cell) and lipid/pDNA ratio. Surprisingly, the transfection efficiencies decreased in a non-linear fashion compared to the starting LFN lipoplex formulation, by factors of 1/160, 1/1300, and 1/6300 for the 1/5, 1/10, and 1/100 concentrations, respectively. This result indicated that lipoplex concentration is an important determinant of lipoplex activity. Thus, the lipoplexes prepared at concentration ratios of 1 and 1/5 were selected for further study and termed normal concentration lipoplex (NCL) and low concentration lipoplex (LCL), respectively.

3.2. Effect of concentrations of pDNA and lipid on physical properties of the lipoplex

We speculated that the interaction between pDNA and cationic lipid is affected by their concentrations in the lipoplex. The release of pDNA from NCL and LCL was compared using agarose gel electrophoresis after addition of different concentrations of SDS. No band of pDNA was observed in the absence of SDS. In contrast, a single band appeared following decondensation of the lipoplex in the presence of 1.25% of SDS (Fig. 2). However, no difference in the release of pDNA was found between NCL and LCL. Measurement of the diameter of the lipoplexes revealed LCL (850 ± 40 nm) to be smaller than NCL (1430 ± 110 nm).

3.3. Comparison of intracellular trafficking of NCL and LCL

Intracellular trafficking of NCL and LCL was observed by confocal laser scanning microscopy (CLSM). Using 1 μg of each lipoplex (17 pg pDNA/cell) to enable clear detection, the difference in transfection efficiency was 10-fold higher for NCL (8.5×10^8 (RLU/mg protein)/(pg/cell)) compared to LCL (7.7×10^7 (RLU/mg protein)/(pg/cell)). After transfection of lipoplexes containing rhodamine-labeled pDNA, the nuclei were stained with Hoechst 33342. As shown in Fig. 3, uptake and nuclear delivery of pDNA were observed for both lipoplexes, and there were no obvious differences in these parameters that would account for the observed 10-fold difference in gene expression levels. However, the size of the vesicular-shaped NCL appeared to be larger than that of the LCL. This difference in size is consistent with the measurements of lipoplex diameter determined above. Therefore, we hypothesized that size of the lipoplexes can influence gene expression. In addition, the difference in transfection efficiency between NCL and LCL was 160-fold at 0.5 pg pDNA/cell (Fig. 1), whereas it decreased to 10-fold at a higher dose (17 pg pDNA/cell) (Fig. 3). This suggests that the dose of pDNA also has an effect on gene expression. Therefore, the relationship between dose and transfection efficiency was investigated in more detail.

3.4. Relationship between dose and transfection efficiency of lipoplexes

As shown in Fig. 4, the transfection efficiencies of NCL were higher (> 10^9 (RLU/mg protein)/(pg/cell)) at all doses tested compared to those of LCL. LCL showed a significantly lower transfection efficiency (10^6 (RLU/mg protein)/(pg/cell)) at 0.5 pg/cell and increased significantly as the dose increased. At 10 pg/cell, LCL showed almost the same transfection efficiency as NCL. There was a significant effect from the dose of lipoplex on the mean transfection efficiency of LCL ($P < 0.001$) but not for NCL ($P > 0.05$). Moreover, two-factor ANOVA revealed a significant interaction from the dose of lipoplex and concentration of pDNA and lipid in lipoplex prepa-

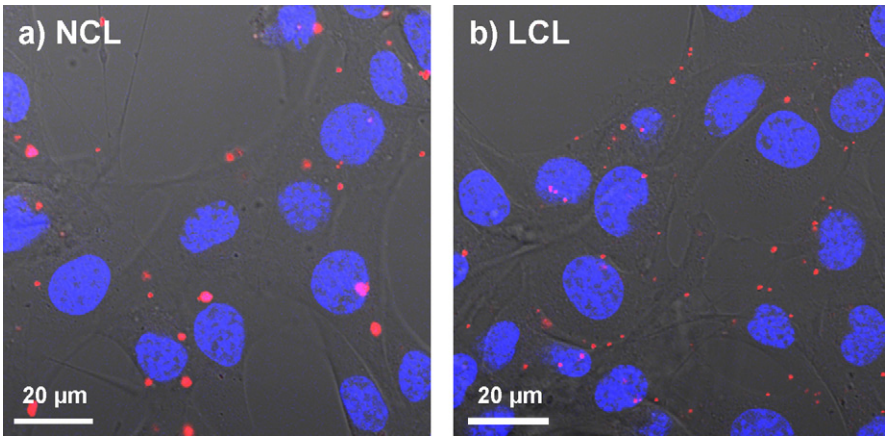

Fig. 3. Intracellular trafficking of lipoplexes formed at different concentrations. NCL (a) and LCL (b) containing 1 μg of rhodamine-labeled pDNA (red) were added to NIH3T3 cells. After 1.5 h incubation, nuclei were stained with Hoechst 33342 (blue), and the cells were observed by confocal laser scanning microscopy. Bars, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ration on mean transfection activity. Thus, in the case of LCL a remarkable positive non-linear relationship was observed between transfection efficiency and dose of lipoplex.

3.5. Intracellular PK and PD of NCL and LCL at different doses

The efficiency of intracellular PK can be estimated by measuring the cellular uptake and nuclear delivery of pDNA, and the efficiency of PD can be estimated when the transfection efficiency is divided by the amount of pDNA delivered to the nucleus. To distinguish between intracellular PK and PD as a cause of nonlinearity, we quantified the amount of pDNA in both solubilized whole cells and isolated nuclei by real-time PCR after transfection of either NCL or LCL at 0.5 or 10 pg/cell (Fig. 5a). The differences in copy number of pDNA in whole cells and nuclei between NCL and LCL were approximately three and five times, respectively, while the transfection efficiency for NCL was two orders of magnitude higher than for LCL at 0.5 pg/cell (Fig. 4). These results indicate that the uptake and nuclear transfer processes are not the determining factors of transfection efficiency. Moreover, as shown in Fig. 5b, no significant difference was found in the efficiency of intracellular PK between NCL and LCL, suggesting that intracellular trafficking is not responsible for the nonlinearity. However, the efficiency of PD for LCL at 0.5 pg/cell was significantly lower than it was for NCL (Fig. 5c). These results clearly indicate that the nonlinearity in transfection efficiency, which is dependent on both the concentration of pDNA used in preparing the lipoplex and the dose of lipoplex, results from processes occurring after nuclear transfer of the pDNA.

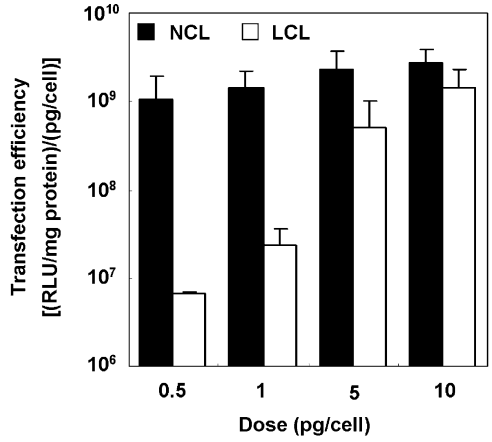

Fig. 4. Transfection efficiency of lipoplexes at different doses. Luciferase activities were measured after transfection of different doses of NCL (closed bar) or LCL (open bar), and transfection efficiencies were estimated by dividing the luciferase activities by the dose of pDNA. Data are presented as mean ± S.D. ($n = 3$). Two-factor ANOVA reveals a significant interaction of dose of lipoplex and concentration of pDNA and lipid in lipoplex preparation on the mean transfection activity ($P < 0.001$).

3.6. Quantification of pDNA in a single cell after transfection of lipoplexes

No significant difference between NCL and LCL was observed by real-time PCR in the amount of pDNA transferred to the nucleus. However, the estimate of nuclear delivery of pDNA determined by real-time PCR is a mean value calculated for 2×10^5 cells and does not reflect uptake by an individual cell. To evaluate intranuclear pDNA on a cell-by-cell basis, we previously developed a novel analytical technique, the CIDIQ method (Akita et al., 2004). Using this method, we captured the fluorescent images of cells transfected with either NCL or LCL under the same conditions as used in Fig. 3. The fluorescence intensity was not high enough to be analyzed after transfection at lower doses (0.5 pg/cell). Therefore, this evaluation was performed at a dose (17 pg/cell) different from that used in the transfection experiments. The pixel areas of clusters of rhodamine-labeled pDNA in each nucleus were measured as an index of the amount of pDNA in 30 cells. Nuclear transfer of pDNA was detected in 14 and 19 cells for NCL and LCL, respectively. Fig. 6 shows the amount of pDNA in each nucleus where nuclear transfer was detected. Total pixel area in nuclei calculated from 30 cells was 1.2×10^3 pixels and 8.3×10^2 pixels for NCL and LCL, respectively. Although no significant difference was observed between NCL and LCL, NCL produced a very high nuclear transfer in some cells, and the variation of NCL was significantly larger than that of LCL.

4. Discussion

Many factors are known to affect the transfection efficiency of lipoplexes, such as lipid composition, lipid/pDNA ratio, liposome size, mixing procedure, net charge, and transfection protocol (Felgner et al., 1994; van der Woude et al., 1995; Eastman et al., 1997; Regelin et al., 2000; Simberg et al., 2001; Koumbi et al., 2006). Recently, we found a positive non-linear relationship between the applied dose and the gene expression of non-viral vectors, i.e., the gene expression exponentially increased with an increase in the dose of pDNA (Moriguchi et al., 2006).

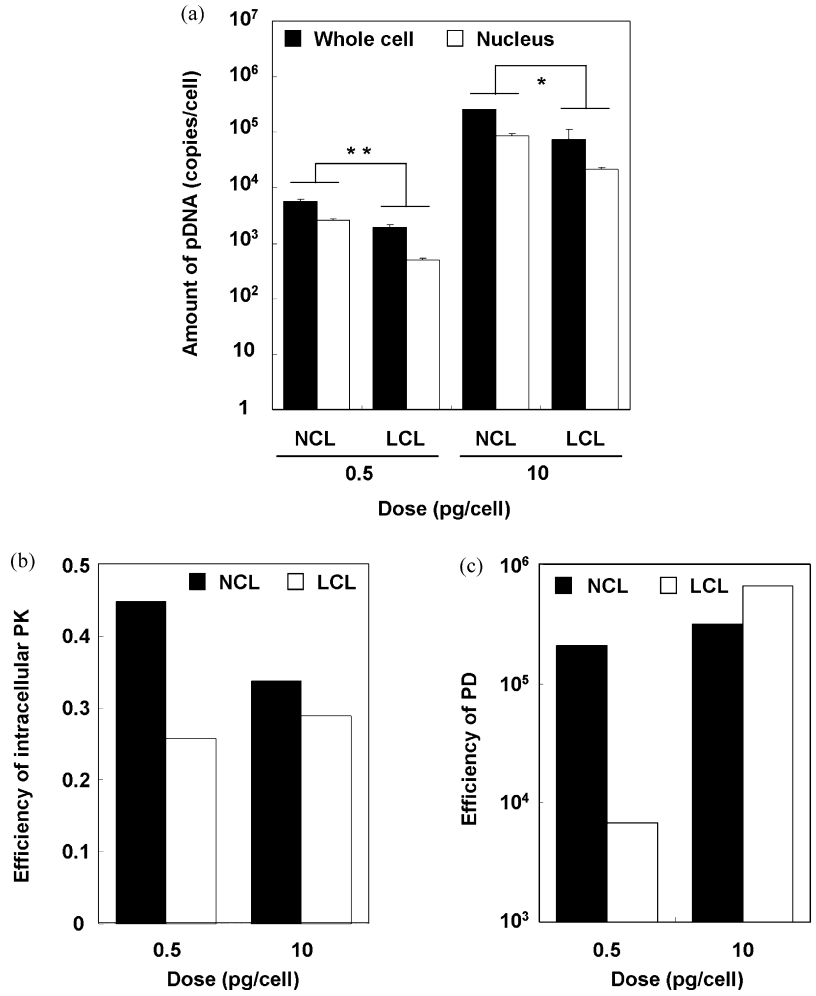

Fig. 5. Comparison of efficiencies of intracellular PK and PD between NCL and LCL. (a) Number of luciferase pDNA copies in whole cells (closed bars) and nuclei (open bars) after transfection of NCL or LCL at different doses. Data are presented as mean ± S.D. ($n = 3$) Asterisks indicate statistically significant differences determined by Student's t-test ($^{*}P < 0.01$ and $^{**}P < 0.001$). (b) The efficiencies of intracellular PK are shown as the ratios of mean intranuclear pDNA per mean whole-cell pDNA. (c) The efficiencies of PD were estimated by dividing the mean transfection activities by the mean number of pDNA copies in the nucleus. Closed and open bars represent the efficiencies of NCL and LCL, respectively.

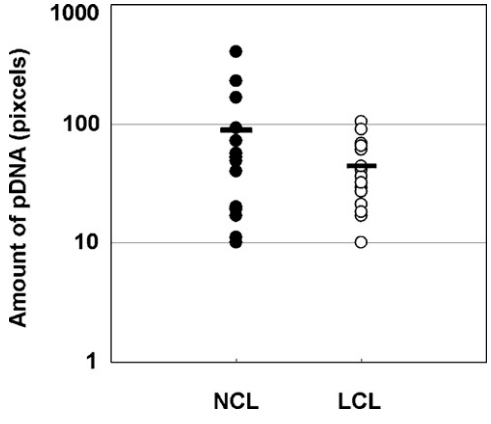

Fig. 6. Quantification of pDNA in each nucleus by CIDIQ analysis after transfection of the lipoplexes. Nuclear delivery of pDNA by NCL and LCL was quantified based on CLSM images (Fig. 3), as described in Section 2. Nuclear transfer of pDNA was detected in 14 of 30 cells with NCL, 19 of 30 cells with LCL, respectively. Black bars represent the mean values. The difference between NCL and LCL was not significant, as determined by the Mann–Whitney test.

In this study, we focused on the concentrations of pDNA and liposome components used for lipoplex formation and examined their effects on the physical properties of lipoplexes as well as on intracellular PK and PD. We found that the transfection efficiency decreased significantly as a function of the decreasing concentration of LipofectAMINE PLUS used to prepare the lipoplexes (Fig. 1). Initially we suspected there might be differences in decondensation abilities between lipoplexes formed at low (LCL) versus normal (NCL) concentrations. However, no differences were found in decondensation abilities (Fig. 2). In fact, the only physicochemical difference we detected was in the size of lipoplexes; NCL are about 70% larger than LCL. For *in vivo* applications, such as intravenous administration, the accepted practice is to use small-sized vectors in the belief that they achieve efficient delivery by avoiding uptake by the reticulo-endothelial system, while passing directly through capillary vessels to the target tissue (Templeton et al., 1997). However, it should be emphasized that smaller lipoplexes showed lower transfection activities, especially at lower doses.

The relationship between the concentration of lipid and pDNA used to prepare lipoplexes versus transfection activities was previously discussed by Staggs et al. (1996). They showed that lower concentrations of lipid and pDNA caused a reduction in both lipoplex size and transfection activities. Our results are consistent

with theirs; however, those authors did not examine the underlying mechanisms. Other factors also influence lipoplex size, including size of the liposomes used, lipid/pDNA ratio, and the presence of serum or salt (Felgner et al., 1994; Tomlinson and Rolland, 1996; MacDonald et al., 1999; Almofti et al., 2003; Koumbi et al., 2006). A large lipoplex can be advantageous for transfection. For example, Ross and Hui (1999) reported that the size of lipoplexes composed of DOTAP and DOPE (1:1) could be regulated by incubating the lipoplexes in media containing polyanions for different periods. Transfection activity was found to increase with an increase the size of the lipoplex, regardless of liposome type. Moreover, linear relationships were seen in plots of size versus cellular association and uptake, indicating that those cellular parameters are important determinants of transfection activity (Ross and Hui, 1999). However, in our quantification of cellular uptake by real-time PCR, NCL showed only a 3-fold higher uptake than LCL at a dose of 0.5 pg/cell (Fig. 5a), which cannot explain the 160-fold difference in gene expression levels. Similar results were reported by other groups (Escriou et al., 1998; Turek et al., 2000). Escriou et al. (1998) have shown a positive correlation between the size and transfection activity of lipoplexes formed at different lipid/pDNA ratios in the presence or absence of serum. They suggested that the difference in transfection activity resulted from differences in intracellular trafficking, since the amounts of pDNA and lipid taken up by the cells were not related to the transfection activities. In addition, by fluorescent microscopic observation, they found that large lipoplexes were taken up within large and heterogeneous intracellular vesicles, whereas small lipoplexes were internalized as smaller, homogeneous vesicles. They concluded that the large vesicles could be easily disrupted, releasing pDNA into the cytoplasm efficiently. On the contrary, we found no significant difference in the efficiency of intracellular PK, including endosomal escape, between NCL and LCL (Fig. 5b). This suggests that intracellular trafficking is not the principal basis for the observed size-dependent differences in lipoplex transfection efficiency. Furthermore, we can exclude the possibility that LCL might be inactivated by either the cell medium or the cell during incubation because LCL showed almost identical ability to deliver pDNA to the nucleus.

More importantly, the transfection efficiency of NCL increased linearly in proportion to the dose of pDNA, whereas LCL showed an exponential increase over the same dose range (Fig. 4). Furthermore, our data suggest that the difference in transfection efficiency is due to the difference in PD, based on the quantification of pDNA delivered to the nucleus (Fig. 5c). We previously showed that a positive non-linear relationship exists between pDNA delivered to the nucleus and gene expression (Moriguchi et al., 2006). Therefore, the nonlinearity in PD can explain the increase in efficiency of PD as a function of the dose of LCL. On the other hand, NCL showed no significant difference in the efficiencies of PD (Fig. 5c), indicating that other factors must contribute to the nonlinearity.

The images obtained by confocal laser scanning microscopy showed that the amounts of pDNA in the nucleus varied widely among the cells, and this heterogeneity seemed to be greater for the larger lipoplexes (Fig. 3). LFN-mediated transfection of pDNA encoding green fluorescent protein resulted in expression in 40–50% of total cells at 24 h, whereas adenovirus-induced 100% transduction (unpublished data). Therefore, heterogeneity should be considered in evaluating intracellular trafficking. Thus, we quantified the amount of pDNA in the nucleus using the CIDIQ method (Akita et al., 2004). At the dose where NCL showed a 10-fold higher expression than LCL, total pixel area in the nuclei calculated from 30 cells was 1.4-fold higher for NCL than for LCL. The mean value and coefficient of variation of the amount of pDNA in the nucleus was approximately two-times higher for NCL than for LCL (88 pixels/cell and 1.24 with NCL, 44 pixels/cell and 0.59 with LCL, respectively,

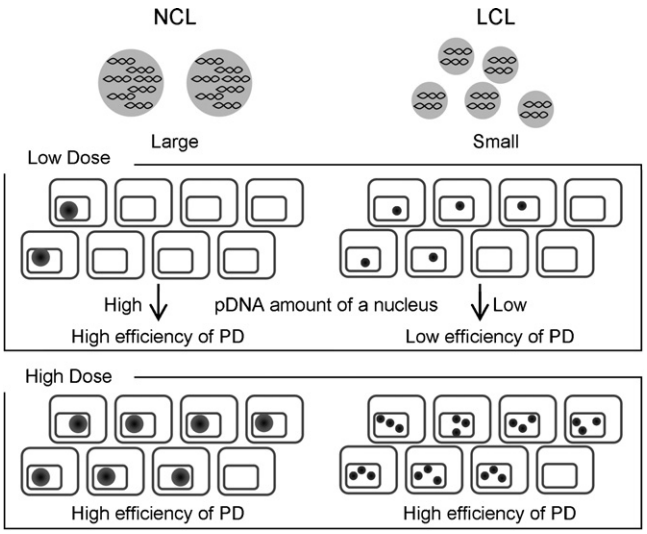

Fig. 7. A model of the effect of lipoplex size on nuclear delivery and gene expression at different doses. At a low dose of pDNA, small lipoplexes (LCL) can deliver a small number of pDNA copies to many cells, while large lipoplexes (NCL) can deliver a large number of pDNA copies to fewer cells, although the total amounts of nuclear-delivered pDNA are similar in both cases. Since pDNA can work synergistically into the nucleus, the large lipoplex, which can deliver more pDNA to one nucleus, shows a higher efficiency of PD. On the other hand, at a high dose of pDNA, a sufficient amount of pDNA is delivered to nuclei not only by large lipoplexes but also by small ones.

Fig. 6). The >2-fold higher nuclear delivery could explain the order of magnitude difference in transfection activities when the positive nonlinearity in PD is considered.

These results lead to a possible mechanism of the effect of lipoplex size on gene expression, as explained in Fig. 7. At a low dose of pDNA (0.5 pg/cell), many small lipoplexes are taken up by many cells because of the large number of particles present, whereas large lipoplexes being fewer in number are taken up by fewer cells, although the overall amounts of nuclear-delivered pDNA are nearly the same for both sizes of lipoplex. We propose that large lipoplexes effectively deliver more copies of pDNA to the nucleus than small lipoplexes, since large lipoplexes contain more copies of pDNA per particle. As we reported previously (Moriguchi et al., 2006), the relationship between nuclear-delivered pDNA and gene expression shows positive nonlinearity, which suggests that pDNA once delivered to the nucleus can work synergistically. Therefore, large lipoplexes show higher efficiency in PD leading to higher gene expression than small lipoplexes. However, when the dose is increased, the amount of pDNA delivered to a nucleus is sufficient to result in transfection activities not only for large lipoplexes but also for small ones.

In conclusion, this study examined nonlinearity in the transfection efficiency of a non-viral gene vector and evaluated the contribution of PD to the non-linear relationship. Lipoplex size appears to be fundamental in determining the non-linear relationship. These findings provide important information for the development and optimization of non-viral vectors.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Japan Society for the Promotion of Science, and by Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We also thank Dr. Milton S. Feather for helpful advice in the use of English in this manuscript.

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