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Note

An assessment of relative transcriptional availability from nonviral vectors

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Abstract

To design better delivery systems that enhance transfection efficiency of nonviral vectors, we need to improve our understanding of the mechanisms governing both the amounts of plasmid delivered to the nucleus and gene expression. What is needed is a measure of transcriptional availability (TA): the average level of gene expression per plasmid delivered to the nucleus over the course of an experiment. We describe a method to measure TA and demonstrate its application. The chloramphenicol acetyltransferase reporter gene was transfected into NIH/3T3 cells using either cationic liposomes (TFL-3; *O*,*O*′-ditetradecanoyl-*N*-(α-trimethylammonioacetyl) diethanolamine chloride (DC-6-14), dioleoylphosphatidylethanolamine (DOPE) and cholesterol, molar ratio 1/0.75/0.75) or cationic polymer (PEI; polyethylenimine). The time courses of both nuclear delivery of plasmids and reporter gene expression were measured for 4 h thereafter. For the conditions used, time courses of gene expression and plasmid nuclear delivery for the two vectors were different. To understand the origins of those differences, we applied a simple pharmacokinetic model, used the data to estimate the values of the model parameters, and interpret differences in estimated parameter values. The rate constant of delivery of plasmids into the nucleus for the TFL-3 vector was twice that of the PEI vector, whereas rate constant of elimination of plasmids in the nucleus for the PEI vector was four times that for the TFL-3 vector. The gene expression rate constant for the TFL-3 vector was estimated to be seven times larger than that of the PEI vector for the conditions used. The pharmacokinetically determined average exposure of a nucleus to plasmid was about 17 times larger for the TFL-3 vector, relative to the PEI vector. That greater exposure resulted in increased relative gene expression. Overall, the TA from the TFL-3 vector was about 13 times greater than from the PEI vector. The experimental design combined with the adoption of pharmacokinetic concepts and principles provide a method to measure TA along with detailed insights into the mechanisms governing gene delivery and expression.

Keywords: Nonviral gene delivery; Nuclear delivery; Kinetic analysis; Cationic liposome; Cationic polymer; Polyethylenimine

A desire for safe, efficient and effective gene delivery technologies motivates nonviral gene delivery research. Nonviral gene delivery is expected to have several advantages over viral gene delivery, including low immunogenicity, low acute toxicity, ease of han-

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dling, and suitability for large-scale production. Their main shortcoming, relative to viral vectors, is low transfection efficiency. To enable enhancements in gene expression efficiency, it is essential to improve our understanding of the intracellular controlling mechanisms (Tachibana et al., 2001).

Expression of a plasmid DNA is preconditioned on cellular delivery followed by translocation into the nucleus. The nuclear membrane is believed to be one of the more important barriers to nonviral gene delivery (Zabner et al., 1995; Pollard et al., 1998; Escriou et al., 1998). It is therefore not surprising that it is commonly hypothesized that if one delivers more plasmids to the nucleus, then gene expression will correspondingly increase. To test that hypothesis, it is necessary to quantitatively assess both plasmid delivery into the nucleus and gene expression. We recently reported using both PCR and Southern analysis to quantify gene expression in AH130 cells (a rat ascites hepatoma line) following plasmid transfection using cationic liposomes (Tachibana et al., 2002a). We also described the quantitative relationships between delivered plasmid and a measure of gene expression efficiency. Some of the results demonstrated a lack of proportionality between gene expression and the measured amount of plasmid delivered into the nucleus. The relationship between these two events appeared nonlinear and time dependent. Consequently, the relationship between these two events cannot be established by a single measurement. A deeper understating requires a kinetic

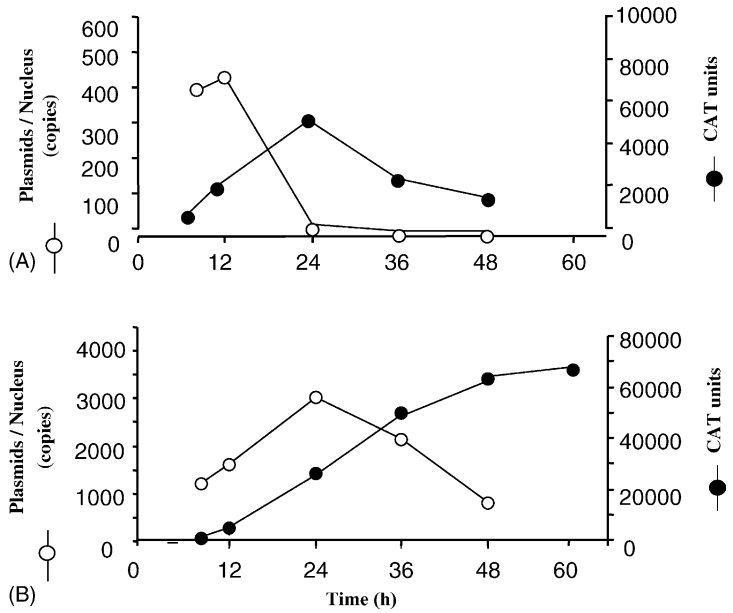


Fig. 1. Time course for plasmid delivery into the nucleus and for gene expression in NIH/3T3 cells (a murine embryo fibroblast cell line) following transfection mediated by cationic polymer (A) and cationic liposomes (B).Recombinant plasmids, pGEM/SV2CAT, were used as previously described (Tachibana et al., 2002a). The plasmid–PEI complexes (800 kDa, Fluka, France) were prepared as described previously (Boussif et al., 1995). To determine the optimal ratio of PEI nitrogen to plasmid DNA phosphate (N/P ratio), cells were transfected using five N/P ratios: 1.5, 3, 6, 9, and 18, and efficiency measured. In our experiments, an N/P ratio of 6 was the best, and so it was that ratio that we used throughout the other experiments.Cationic liposomes (TFL-3; DC-6-14/DOPE/cholesterol in the molar ratio 1/0.75/0.75, 2.5 μmol lipid per ml) were prepared as described previously (Kikuchi et al., 1999). The ratio of plasmid to cationic liposome is 12.5 (nmol μg^{-1}). Ten micrograms of plasmid were transfected to 10^6 cells in 5 ml of fresh serum-free culture OPTI-MEM medium (Invitrogen Co., CA, USA). After incubation for 4 h, the medium was replaced with DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, and the cells were incubated further. After incubation, cells were washed with phosphate-buffered saline and subdivided for measurements of CAT activity and intranuclear plasmid. Measurements of CAT activity (in CAT units) followed a standard procedure (Sambrook et al., 1989); the CAT unit is pmol of acetylated chloramphenicol per milligram protein per hour. The protocols for preparation of the nuclear fraction and for quantification of intranuclear plasmids by Southern analysis were the same as those described previously (Tachibana et al., 2002a). The results shown are typical of those obtained from three independent experiments.

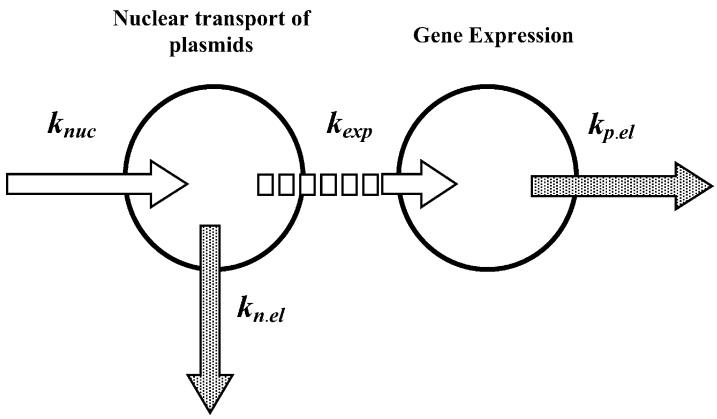
analysis of these events, and that is what this report provides.

With the above hypothesis in mind, we designed and executed new experiments. We followed the kinetics of gene delivery and expression in NIH/3T3 cells following transfection of a reporter gene using either cationic liposomes (TFL-3) or the cationic polymer polyethylenimine (PEI). Plasmid delivery to the nucleus was measured as before using Southern analysis (Tachibana et al., 2002a). The time course of nuclear delivery and gene expression was measured for each vector. The kinetics of these events were then analyzed to estimate the efficiencies of plasmid delivery to the nucleus and gene expression.

The time course of the two processes, presented in Fig. 1, was strikingly different for each of the two vectors. For the PEI vector, gene expression, measured by CAT (chloramphenicol acetyltransferase) units, peaked at 24-h post-transfection. Whereas, nuclear delivery of plasmids within these same cells peaked at 12-h post-transfection. The pattern of events was quite different for the cationic liposome vector (hereafter referred to as the TFL-3 vector). The expected peak in gene expression did not occur during the 60-h experiment. However, plasmid nuclear delivery did peak at 24 h. Plasmid delivery by the TFL-3 relative to the PEI vector was higher at all sampling times. Furthermore, the duration of nuclear delivery, as measured by peak time, for the TFL-3 vector was twice that of the PEI vector (24 h versus 12 h). Maximal plasmid delivery for the two vectors, as measured by plasmid copies per nucleus, differed by a factor of 7: 3059 and 434 for the TFL-3 and PEI vectors, respectively. Clearly, delivery efficiency cannot be determined using measurements made at a single time point.

Having an adequate estimate of the amount of plasmid in the nucleus is essential for this research. Does extranuclear plasmid contribute significantly to the values measured? To address this contamination question, we added microgram of plasmid to the cell extract from which the nuclei were isolated, and then conducted a Southern analysis. We detected an average of 10.7 copies of plasmid per nucleus. Because the nonviral vectors deliver thousands of copies per nucleus, the observed level of contamination does not significantly impact our results.

The model in Scheme 1 can reasonably account for the measured events in Fig. 1. To keep the model simple, we elected to represent each process as being a reaction that is controlled by either a zero or a first-order rate constant. The processes of plasmid delivery and gene expression are thought to be controlled by factors other than effective plasmid concentration, and are consistent with delivery of plasmid into the nucleus (k_{nuc}) and gene expression (k_{exp}) being zero-order processes. Two major mechanisms of nuclear transport of plasmid are considered, one via the nuclear pore complex (NPC; Pollard et al., 1998;



Scheme 1. Pharmacokinetic model of nuclear transport of plasmids and gene expression. White arrows represent an apparent zero-order process; gray arrows represent an apparent first-order process. The dotted arrow represents gene transcription, not a transport process. Each process is controlled by one of four system average rate constants: k_{nuc} is for plasmid delivery into the nucleus; k_{exp} is for gene expression; $k_{n\cdot el}$ is for elimination of plasmid in the nucleus; and $k_{p\cdot el}$ is for decrease of expression.

Colin et al., 2000, 2001; Dean et al., 1999a,b), and the other occurring during mitosis (Mortimer et al., 1999; Tseng et al., 1999; Brunner et al., 2000; Escriou et al., 2001). Dean et al. (1999a,b) and Colin et al. (2001) report that plasmid DNA itself or plasmid DNA in the presence of cationic polymer, are translocated to the nucleus via the NPC. In the case of transport via NPC, it is known that cytosolic transport factors such as Importin α/β are necessary (Imamoto, 2000). Gene expression becomes saturated when an excess amount of plasmids is delivered to the nucleus (Tachibana et al., 2002a). A plausible explanation of this phenomenon is that limited amounts of transcription factors become exhausted. Their observations suggest that the two processes should be represented as being zero order. Our preliminary computer simulations (data not shown) supported that position, and so that is how we represent them.

The processes responsible for elimination of plasmids from the nucleus ($k_{n\cdot el}$) are clearly dependent on plasmid level, so they are treated as being first order. The process leading to a decrease of expression ($k_{p\cdot el}$) is slow and may be equally well represented as being zero or first order. We elected to represent it as being first order. Results of our preliminary computer simulations supported the reasonableness of that decision. Initial estimates for the two zero-order rate constants were obtained from the initial slope of the data. Estimates of the two first-order parameters were obtained from the terminal slope of a semilogarithmic plot of the data. The results are listed in Table 1. The value of k_{nuc} for the TFL-3 vector was about twice as large as the corresponding value for the PEI vector. In contrast, the value of $k_{n\cdot el}$ for the TFL-3 vector was only about 20% of the PEI vector value. These values strongly suggest that plasmids delivered by the TFL-3 vector are transferred to the nuclei faster and they remain there longer than their PEI vector counterparts. The rate constant for gene expression, k_{exp}, from the TFL-3 vector was about seven times larger than that for PEI vector. The rate constant governing decrease of expression, $k_{p\cdot el}$, was measurable for the PEI vector, but not for the TFL-3 vector. In the latter case it could not be estimated because gene expression from the TFL-3 vector was increasing until 60 h.

These data fail to shed light on the form(s) of the plasmid that is involved in intracellular trafficking, the plasmid alone, the plasmid–polycation complex, or some combination thereof. Furthermore, the actual rate-determining process that is represented by the rate constant in Scheme 1 remains unknown. However, we can identify four possibilities: (1) cellular uptake, (2) escape from endosome, (3) delivery from cytosol to the nucleus, and (4) nuclear uptake.

The model in Fig. 1 is analogous to those used in the pharmacokinetics field (Rowland and Tozer, 1980a; Gibaldi and Perrier, 1982). Pharmacokinetic models and equations are used to estimate the fraction of a dose that is bio-available, and fraction of a dose that passes through a site where drug can be directly measured, such as blood. These same concepts apply here. For the model in Scheme 1, when the elimination process is first order, the total amount X passing through the measurement site, in this case the nucleus, is given by $X = k \times \mathrm{AUC}$, where AUC is the total area under the curve of measured values versus time, and k is an apparent first-order rate constant for elimination from the measurement site. In the case of the plasmid, X is a

Table 1
Results of pharmacokinetic analysis

		TFL-3	PEI	TFL-3/PEI ratio
Nuclear transport of plasmid	k_{nuc} (copies h^{-1})	110	50	2.2
	$k_{n\cdot el}$ (h^{-1})	0.078	0.35	0.22
	AUC_{nuc} (copies h)	1.0×10^5	5.9×10^3	17
Gene expression	F	4.3×10^{-3}	1.1×10^{-3}	3.9
	k_{exp} (units h^{-1})	1.9×10^3	2.8×10^2	6.8
	$k_{p\cdot el}$ (h^{-1})	–	0.032	–
Gene expression efficiency	AUC_{exp} (units h)	4.3×10^6	1.9×10^4	2.3×10^2
	AUC_{exp}/AUC_{nuc} (units copies^{-1})	43	3.2	13

Values for rate constants and pharmacokinetic terms were obtained as described in the text.

measure of the plasmid "dose" that made it to the nucleus (a small portion of the dose applied to the cells), and AUC is a measure of exposure of the plasmid to the nucleus. Greater exposure may enable more gene expression. The plasmid dose was known for each experiment. If we assume that the fraction of the dose delivered to each cell in the system was approximately the same, and that none of that plasmid dose was precluded from being available for other, unknown reasons, then the efficiency F of plasmid nuclear delivery can be calculated as $F = X/\text{Dose}$, and percent efficiency is $100F$. Dose is the amount of plasmid applied per cell.

The above methodology was applied to both sets of plasmid delivery and gene expression data in Fig. 1. AUC_{nuc} values were calculated using the trapezoidal rule (Rowland and Tozer, 1980b) and are listed in Table 1. Because dose is the same for the two vector types, nuclear delivery efficiency F is directly calculable, and was 0.4% for the TFL-3 vector, which was four times the efficiency of the PEI vector.

Because the duration of the experiment was unexpectedly insufficient to measure the decline in gene expression from the TFL-3 vector, it is not possible to directly measure the area under the expression versus time curve for that vector. However, if we make the conservative assumption that the value of $k_{p\cdot el}$ for the TFL-3 vector is about the same as that for the PEI vector, then AUC is calculable. When we did that, the estimated efficiency of the TFL-3 vector was about 230 times greater than that of the PEI vector. Such a dramatic difference is not unexpected. Kikuchi et al. (1999) report that the TFL-3 vector has a higher transfection efficiency than the commercially available Lipofectin, LipofectACE, and LipofectAMINE cationic liposome reagents.

To more precisely discuss means for improving gene delivery efficiency, it is convenient to introduce and define a new term, transcriptional availability. The ability of the cell's transcriptional machinery to first access and then complete the transcription of a delivered gene can change depending on a number of variables, including how that gene is delivered to the nucleus, the site of transcription, and the physical condition of the gene and its carrier (the plasmid—associated factors and reagents) upon arrival in the nucleus. For a combination of reasons, not all plasmid DNA delivered to the nucleus will be equal. Some genes will be inaccessible and so will not be transcribable. In other cases, incomplete, nonfunctional transcripts will be produced. Over the course of a study, the transcriptional availability of the delivered genes can be defined as a measure of the number of functional transcripts produced relative to the number of genes delivered to the nucleus. For simplicity, we assume that once the transcript has been completed and released, the subsequent translation rate per transcript, for the same conditions, cell type, and cell phase, is relatively constant and independent of the location of the originating gene, i.e. the transcript has no memory of its place of origin. An optimal measure of transcriptional availability is thus a measure of the average level of gene expression per plasmid delivered to the nucleus over the course of an experiment. The ratio AUC_{exp}/AUC_{nuc} provides such a measure. The data in Table 1 show that the transcriptional availability from the TFL-3 vector is more than an order of magnitude greater than from the PEI vector. This is a dramatic difference. Clearly, additional research is needed to gain a better understanding of the causal influences that can impact transcriptional availability. For this purpose, we utilized recently developed the RTS 500 Rapid Translation System (Roche Diagnostics K.K., Tokyo, Japan) (Tachibana et al., 2002b). This system, which enabled detailed studies of the transcription of plasmids under a variety of conditions, was used as a model system for transcription, and the effect of cationic liposomes on the process was evaluated. Transcription of DNA/cationic liposome complexes was significantly inhibited even in the typical ratio of the complexes used in transfection studies although no inhibitory effect was observed when plasmids and cationic liposomes were separately added to the reaction device.

The events involved in intracellular trafficking of plasmids that are being delivered by cationic liposomes is believed to be different from those for plasmid–cationic polymer complexes such as the PEI vector studied here. In the latter case, Boussif et al. (1995) posit that PEI acts like a "proton sponge." Once inside the endosome, the polymer is thought to adsorb large numbers of protons, and that causes osmotic swelling and a pH shift. The increased osmotic pressure disrupts the endosomal membrane allowing escape of the now altered PEI vector. Cationic lipo-

somes, on the other hand, are believed to undergo a type of fusion with the endosomal membrane that results in both local weakening of the endosome and decreased electrostatic interaction between the plasmid and the cationic liposomal lipids. As a consequence, some plasmids are envisioned being released into the cytosol (Xu and Szoka, 1996), where a fraction of these plasmids become transcriptionally available. When plasmid–cationic liposome complexes are injected directly into the nucleus, gene expression does not occur (Zabner et al., 1995; Pollard et al., 1998)—the genes are not transcriptionally available.

From the data presented here and in the above citations, it appears that the post-transfection processing of the reporter gene delivered by the cationic liposome and by PEI vectors differs in four important ways. First, the intracellular trafficking and processing pathways are different. Transcriptional availability is different. As a consequence of these differences, the time courses of gene expression are different. Finally, the time for maximum gene expression is different. These differences underscore the fact that, in order to comment on the efficiency of a vector or relative differences in the efficiencies of different vectors, it is essential to have time course data, or the equivalent. Experimental interpretations based on a single time point (or even two) are seriously at risk of being misleading.

The extension of pharmacokinetic concepts to the gene delivery context is conceptually appropriate and useful. It is also clear that the kinetic model in Scheme 1 is, from a biological perspective, overly simplistic. However, it adequately accounts for the data, it has enabled extraction of new and informative insights, and it is proving useful for generating new hypotheses. We limited attention to evaluating relative therapeutic availability for two nonviral vectors under specific experimental conditions. The methodology and concepts, however, are expected to be generalizable to assessment of viral vectors as well as other nonviral vectors.

Our current interpretation of events is a reasonable first step. The actual network of factors that influence transcriptional availability is complicated. Key causal variables may change with cell type and cycle, with experimental conditions, and with biological setting. Some influential factors may have capacity limitations that shift their relative importance. By systematically extending the experimental approach used here, we will be better able to understand these processes, and will be better positioned to engineer superior nonviral vectors.

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References

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7300.

Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M., Wagner, E., 2000. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther. 7, 401–411.

Colin, M., Maurice, M., Trugnan, G., Kornprobst, M., Harbottle, R.P., Knight, A., Cooper, R.G., Miller, A.D., Capeau, J., Coutelle, C., Brahimi-Horn, M.C., 2000. Cell delivery, intracellular trafficking and expression of an integrin-mediated gene transfer vector in tracheal epithelial cells. Gene Ther. 7, 139–152.

Colin, M., Moritz, S., Fontanges, P., Kornprobst, M., Delouis, C., Keller, M., Miller, A.D., Capeau, J., Coutelle, C., Brahimi-Horn, M.C., 2001. The nuclear pre complex is involved in nuclear transfer of plasmid DNA condensed with an oligolysine–RGD peptide containing nuclear localization properties. Gene Ther. 8, 1643–1653.

Dean, D.A., Byrd Jr., J.N., Dean, B.S., 1999a. Nuclear targeting of plasmid DNA in human corneal cells. Curr. Eye Res. 19, 66–75.

Dean, D.A., Dean, B.S., Muller, S., Smith, L.C., 1999b. Sequence requirements for plasmid nuclear import. Exp. Cell Res. 253, 713–722.

Escriou, V., Ciolina, C., Helbling-Leclerc, A., Wils, P., Scherman, D., 1998. Cationic lipid-mediated gene transfer: analysis of cellular uptake and nuclear import of plasmid DNA. Cell Biol. Toxicol. 14, 95–104.

Escriou, V., Carriere, M., Bussone, F., Wils, P., Scherman, D., 2001. Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. J. Gene Med. 3, 179–187.

Gibaldi, M., Perrier, D., 1982. Pharmacokinetics, second ed. Dekker, New York, pp. 145–198.

Imamoto, N., 2000. Diversity in nucleocytoplasmic transport pathways. Cell Struct. Funct. 25, 207–216.

Kikuchi, A., Aoki, Y., Sugaya, S., Serikawa, T., Takakuwa, K., Tanaka, K., Suzuki, N., Kikuchi, H., 1999. Development of novel cationic liposomes for efficient gene transfer into peritoneal disseminated tumor. Hum. Gene Ther. 10, 947–955.

Mortimer, I., Tam, P., MacLachlan, I., Graham, R.W., Saravolac, E.G., Joshi, P.B., 1999. Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 6, 403–411.

Pollard, H., Remy, J.S., Loussouarn, G., Demolombe, S., Behr, J.P., Escande, D., 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Biol. Chem. 273, 7507–7511.

Rowland, M., Tozer, T.N., 1980a. Clinical Pharmacokinetics: Concepts and Applications. Lea & Febiger, Philadelphia, pp. 109–123.

Rowland, M., Tozer, T.N., 1980b. Clinical Pharmacokinetics: Concepts and Applications. Lea & Febiger, Philadelphia, pp. 288–291.

Sambrook, J., Fritstch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tachibana, R., Harashima, H., Shinohara, Y., Kiwada, H., 2001. Quantitative studies on the nuclear transport of plasmid DNA and gene expression employing nonviral vectors. Adv. Drug Deliver. Rev. 52, 219–226.

Tachibana, R., Harashima, H., Ide, N., Ukitsu, S., Ohta, Y., Suzuki, N., Kikuchi, H., Shinohara, Y., Kiwada, H., 2002a. Quantitative analysis of correlation between number of nuclear plasmids and gene expression activity after transfection with cationic liposomes. Pharm. Res. 19, 377–381.

Tachibana, R., Harashima, H., Ishida, T., Shinohara, Y., Hino, M., Terada, H., Baba, Y., Kiwada, H., 2002b. Effect of cationic liposomes in an in vitro transcription and translation system. Biol. Pharm. Bull. 25, 529–531.

Tseng, W.C., Haselton, F.R., Giorgio, T.D., 1999. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. Biochim. Biophys. Acta 1445, 53–64.

Xu, Y., Szoka, F.C., 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. Biochemistry 35, 5616–5623.

Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A., Welsh, M.J., 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. 270, 18997–19007.