DDT Vol. 8, No. 21 November 2003

Pharmacokinetic and pharmacodynamic considerations in gene therapy

Hiroyuki Kamiya, Hidetaka Akita and Hideyoshi Harashima

During gene therapy the concentration of plasmid DNA or oligonucleotides in the plasma can be quite different from their concentrations in the nucleus or cytosol where they exert their actions. For a better understanding of the apparent discrepancies between pharmacokinetics (PK) and pharmacodynamics (PD), a new concept for intracellular PK with an emphasis on the final efficacy of gene transcription is needed. Here, the conventional PK and intracellular PK and PD of non-viral gene delivery systems are discussed, together with a new concept, referred to as controlled intracellular disposition, which integrates these factors to gain a better understanding of gene expression in the nucleus. The importance of optimizing the system from a transcriptional point of view in the nucleus is also discussed. These new concepts must be integrated to develop an optimized non-viral gene delivery system.

Hiroyuki Kamiya, Hidetaka Akita Hideyoshi Harashima*
Laboratory for Molecular Design of Pharmaceutics
Graduate School of Pharmaceutical Sciences
Hokkaido University
Kita 12, Nishi 6, Kita-ku
Sapporo City
Hokkaido 060-0812, Japan
*e-mail: harasima@pharm.hokudai.ac.jp

▼ It is widely accepted that pharmacokinetic (PK) and pharmacodynamic (PD) considerations are important in terms of understanding the efficacy, as well as the toxicity, of a drug. In most cases, PK and PD can be separated by measuring the drug concentration in plasma [1]. It is generally assumed that plasma concentration is proportional to the concentration at the site-of-action, and that the effect of a drug is controlled by its concentration in plasma. However, in the case of gene therapy, the concentration of plasmid DNA or oligonucleotides in plasma is quite different from their concentration in the nucleus or cytosol where they exert their action [2]. Therefore, it has generally been recognized that knowledge of intracellular PK is required to integrate conventional PK and PD [3]. The focus of this review is the conventional PK, and the intracellular PK and PD of a non-viral gene delivery system for use in developing an optimized system (Figure 1).

PK and PD considerations on gene delivery

When 'naked' DNA is administered intravenously, it is subjected to rapid degradation in the blood by DNase and uptake by the liver. Therefore, it is essential to protect DNA from the action of DNase. Cationic lipids or cationic polymers are typically used for the compaction of plasmid DNA as well as for enhanced cellular delivery.

Compacting plasmid DNA

The first cationic lipid used in gene delivery, DOTMA (2,3-dioleyloxypropyl-1-trimethyl ammonium bromide) was introduced by Felgner *et al.* in 1987 [4]. Although cationic lipids can form complexes with plasmid DNA, their ability to condense is limited, compared with cationic polymers. Huang *et al.* prepared a core particle of condensed plasmid DNA with poly-L-lysine (PLL), which was coated with a lipid bilayer to form positively charged, small sized (<100 nm) particles (LPD1) [5]. Particle size is important in terms of their selective targeting for internalizing into cells via receptor-mediated endocytosis. After intravenous administration, transgene expression was observed in all major organs, including the lung, heart, liver, spleen and kidney. The highest expression was found in the lung. The transfected cells were found to be primarily vascular endothelial cells, especially lung endothelial cells.

Polyethyleneimine (PEI) is one of the most frequently used cationic polymers [6]. When plasmid DNA is complexed with PEI and administered to mice intravenously, gene expression can be observed in the lung and liver. The highest gene expression is usually observed in the lung, whereas most plasmid DNA are

PII: S1359-6446(03)02889-7

taken up by the liver. The extensive degradation of plasmid DNA in the liver is a major reason for this discrepancy between organ distribution and transgene expression, which clearly indicates the importance of the intracellular availability of plasmid DNA for efficient transgene expression.

Tumor targeting

For successful *in vivo* tumor targeting, a long circulating carrier system is required to protect plasmid DNA from DNase in the plasma and to pass through the endothelial barrier to reach the extracellular space in tumor tissues. Long circulating properties can be achieved by incorporating polyethyleneglycol (PEG) to prevent recognition by opsonins, as well as macrophages in the liver and spleen [7]. Stabilized plasmid–lipid particles (SPLP) were developed to encapsulate plasmid DNA in a cationic lipid bilayer, which was surface-coated with PEG [8]. The SPLP had long circulating properties and permitted the tumor delivery of plasmid DNA [9]. Although the cellular uptake of SPLP was minimal in *in vitro* experiments, tumor-specific gene expression was observed after intravenous administration [10]. Although plasmid DNA was distributed in the liver, spleen, lung and tumor, among others, gene expression was observed only in the tumor. By contrast, recent studies report that incorporation of PEG did not necessarily extend blood circulation time [11,12].

Wagner *et al.* used transferrin (Tf) to reduce the surface charge of the complex and to increase selective internalization via receptor-mediated endocytosis [13]. As a result, the transgene expression of the Tf–PEI–DNA complex was one order of magnitude higher because of its reduced hepatic uptake, as well as enhanced tumor delivery.

Brain targeting

Brain targeting is one of the most difficult goals in the field of gene delivery because the blood–brain barrier (BBB) resists the passage of molecules across it. Pardridge *et al.* succeeded in overcoming this barrier by encapsulating plasmid DNA into long circulating liposomes with targeting ligands, such as transferrin receptors or insulin receptors and encapsulating plasmid DNA into liposomes [10,14]. A receptor-mediated transcytosis system exists in brain endothelial cells for the transport of transferrin and insulin. PEGylated immunoliposomes were developed; these contain a monoclonal antibody to the insulin receptor, and they encapsulated the plasmid DNA encoding β-galactosidase. This system was capable of delivering a reporter gene to brain neuronal cells and expressing the β-galactosidase activity [15], although this delivery system delivered only 0.06% of the injected dose per gram of brain in the case of transferrin receptors [14]. The precise mechanism for how these carriers pass through endothelial cells without releasing the encapsulated plasmid DNA in endothelial cells, as well as how the encapsulated plasmid DNA is released from endosomes to deliver them to the nucleus in parenchymal cells, is unknown.

As described, discrepancies between organ distribution and the gene expression of non-viral gene delivery system continue to exist. To better understand this and to develop a more sophisticated delivery system, a new approach to the intracellular PK and PD of such systems is required.

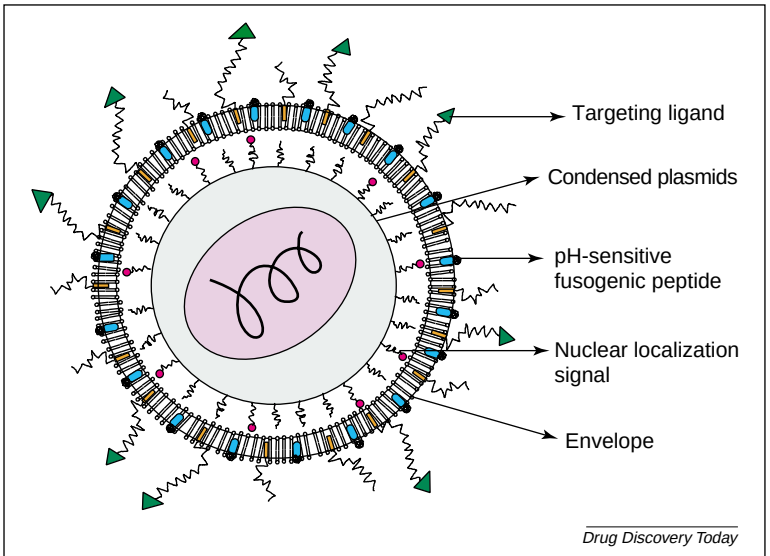

Figure 1. An optimized/ideal multifunctional non-viral gene delivery system. A multifunctional, enveloped type gene delivery system was designed that has specific ligands for selectively internalizing into cells during a long blood circulation time. After receptor-mediated endocytosis, this system can escape from endosomes by a pH-sensitive fusion mechanism similar to that of the influenza virus, and reach the nucleus using a sorting device such as a nuclear localization signal. This system also has a specific mechanism to enhance intranuclear transcriptional efficiency (dumbbell-shaped), thus maximizing the efficacy of gene expression.

Regulation of intracellular trafficking of genes and carriers: a new concept of intracellular PK

Intracellular barriers governing the gene expression efficiency

Although receptor-mediated endocytosis is a promising strategy for achieving selective cellular targeting, plasmid DNA must be released from the endosomes to the cytosol so as to avoid lysosomal degradation. Therefore, the endosomal membrane is the first barrier in the intracellular trafficking of plasmid DNA for efficient transfection. Most viruses, such as influenza or adenoviruses, have developed sophisticated mechanisms to escape from endosomes in response to the acidic endosomal pH (~5.0). Advantage has

Table 1. Peptides to regulate the intracellular disposition of genes, proteins and macromolecules

Function of the peptide	Subtype	Examples	Refs
Fusogenic peptide	pH-insensitive (virus)	HIV-1 gp41 (N-terminus)	[52]
		SIV gp32(N-terminus)	[53]
		Sendai virus F1(N-terminus)	[54]
	pH-sensitive (virus)	Influenza HA2(N-terminus)	[19]
		Semliki Forest virus E1(internal)	[55]
		Sindbis virus E1(internal)	[56]
	pH-sensitive (synthetic)	IFN 1-10	[20]
		GALA	[57]
		KALA	[20]
		ppTG1,ppTG20	[58]
		JTS-1	[59]
Membrane permeable basic peptide	Cell permeable peptide	HIV-1 Tat-(48–60)	[60]
		Antennapedia (Antp) (43–58)	[60]
		Oligo arginine	[61]
		HIV-1 Rev-(34–50)	
	RNA binding peptide	FHV coat-(35–49)	
		BMV Gag-(7–25)	[62]
		HTLV-II Rex-(4–16)	
		P22 N-(14–30)	
		Human U2AF-(142–153)	
	DNA-binding peptide	Human cFos-(139–164)	
		Human cJun-(252–279)	[63]
		Yeast GCN4-(231–252)	
Nucear localization signal (NLS)	NLS in mammalian genes	Cardiac muscle factor 1 (CMF1)	[64]
		Mitosin	[65]
		Myc	[66]
		NF-κB	[66]
		DNA helicase Q1	[67]
		M9	[68]
	NLS in polyoma virus	Large T (SV40)	[66]
		Vp1 (SV40)	[69]
	NLS in lenti virus	IN (HIV-1)	[70]
		MA (HIV-1)	[71]

Abbreviations: BMV, Brome mosaic virus; FHV, feline herpesvirus; HA, hemagglutinin protein; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus; IN, integrase; MA, matrix protein; SIV, simian immunodeficiency virus; U2AF, U2 auxiliary factor;.

been taken of this in designing non-viral gene vectors to enhance gene expression [16–20]. Artificial devices such as pH–sensitive lipids [21,22], dioleoylphosphatidyl ethanolamine (DOPE), polyethyleneimine (PEI) [23,24] and amphiphilic peptides [19,20,25–27], have also been developed for this purpose (Table 1).

Once the plasmid DNA has escaped from the endosomes, its stability in the cytosol needs to be considered. Because plasmid DNA is subject to hydrolysis by DNase in the cytosol [28,29], it must be protected until it enters into the nucleus. In most cases, plasmid DNA is condensed with cationic lipids or polymers, which are resistant to DNase, and it is important to control the dissociation of plasmid DNA from the cationic counterparts to enhance nuclear delivery and transgene expression. If the dissociation is too rapid, then most of the plasmid DNA will be degraded. If the dissociation is too slow, the accessibility of transcription factors in the nucleus might be impaired and the expression might be low. Therefore, the stability of the complex between plasmid DNA and cationic counterparts should be optimized

in the cytosol, as well as in the nucleus for enhanced gene expression.

The second barrier is the nuclear membrane, which is composed of double lipid bilayers and transport between the cytosol and nucleus are regulated by nuclear pore complexes (NPC), which restrict the passive diffusion of molecules >40–45 kDa. Plasmid DNA are usually larger than this threshold, therefore, the nuclear translocation of plasmid DNA are strictly limited. It is generally accepted that free plasmid DNA can enter the nucleus when the nuclear membrane disappears during mitosis [30]. By contrast, the NPC can transport various karyophilic proteins >45 kDa only when they have a nuclear localization signal (NLS), which are partially listed in Table 1. NLS has been attached to the plasmid DNA itself, along with DNA condensing proteins, and polycations in attempts to improve nuclear delivery and transgene expression [31–37]. However, strategies for enhanced nuclear delivery using NLS remain controversial. Further challenges remain for overcoming this barrier in the development of a non-viral gene delivery system.

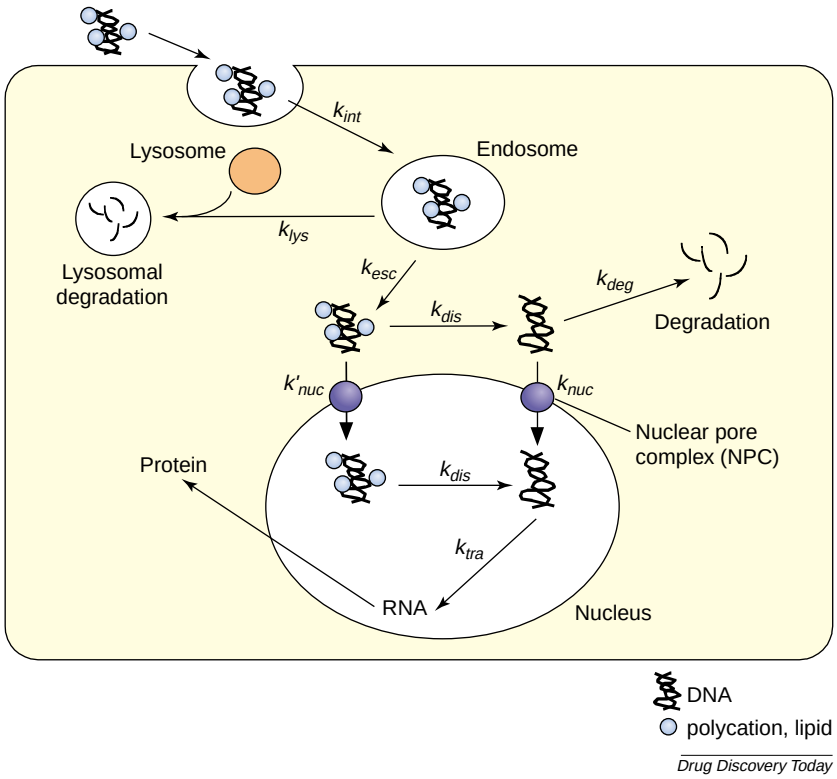

Figure 2. A schematic diagram illustrating the intracellular pharmacokinetic model of the genes. The intracellular disposition of the gene is represented. A complex of DNA and cationic lipids/polycations bind to the plasma membrane electrostatically and is internalized via endocytosis (k_{int}). Endosomal plasmid DNA fused with a lysosome is degraded (k_{lys}). A certain fraction of the endosomal DNA is released into the cytosol (k_{esc}) where a part of the DNA dissociates from the cationic lipids/polycations (k_{dis}). Free plasmid DNA is subject to degradation by nucleases (k_{deg}) or is partially translocated to the nucleus (k_{nuc}). Alternatively, DNA enters the nucleus in a complexed form with cationic lipids/polycations (k'_{nuc}) followed by dissociation in the nucleus (k_{dis}). Finally, free DNA in the nucleus are transcribed (k_{tra}).

Intracellular PK for an optimized gene delivery system

To maximize the transgene expression of a non-viral gene delivery system, an intracellular kinetic model (Figure 2) was developed to analyze the rate-limiting process and to optimize the intracellular trafficking of the delivered plasmid DNA [2]. Kinetic modeling enables us to predict the effect of variations of multiple parameters on gene expression. Varga *et al.* collected kinetic parameters from the literature and reported on an integrated kinetic model [38]. This model predicted the effect of the polymer length of the vectors on transgene expression. The model successfully predicted the experimentally observed biphasic gene expression efficiency by the length of the polymers used.

Despite the advantages of such a kinetic modeling, little information has appeared to date, owing to a lack of quantitative measure of the distribution of plasmid DNA in each subcellular compartment. Therefore, it is important to evaluate the kinetics of the intracellular distribution of plasmid DNA. We have traced the kinetics of transfected plasmid DNA and observed its rapid nuclear delivery by confocal laser scanning microscopy [39]. We subsequently established a 3D quantitative assay system to analyze the distribution of plasmid DNA in endosomes and/or lysosomes, cytosol and nucleus simultaneously by confocal laser scanning microscopy [40]. Using this system, the intracellular disposition of the plasmid DNA transfected with octaarginine (R8), stearyl octaarginine (STR-R8) and LipofectAMINE plus (LFN) was compared. A kinetic analysis of the intracellular trafficking satisfactorily explained the order of gene expression (R8<<STR-R8<<LFN). These results are consistent with our mechanistic study on R8 and St-R8 [41]. These kinetic analyses enabled us to clarify the rate-limiting step and to propose a new mechanism of gene trafficking from a kinetic point of view. In addition, these quantitative/kinetic analyses represent a useful strategy for optimizing the intracellular trafficking of a non-viral gene delivery system.

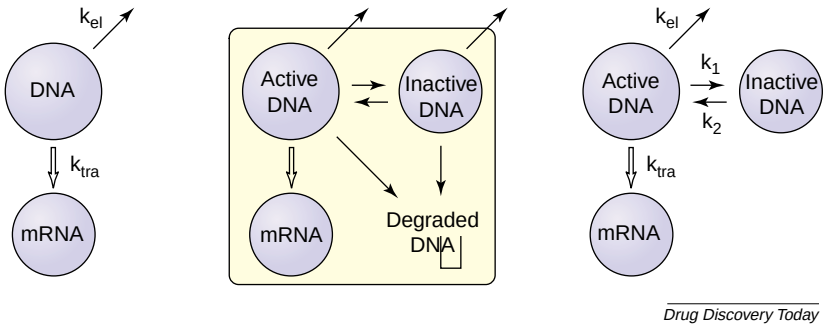

Figure 3. A pharmacokinetic/pharmadynamic (PK/PD) model of transgene gene expression in the nucleus. **(a)** The simplest PK-PD model in which a direct correlation of the amounts of DNA and mRNA is hypothesized. **(b)** A model of intranuclear disposition of exogenous DNA. The two states, active and inactive, are hypothesized. **(c)** Our simplified PK/PD model based on the intranuclear disposition model shown in **(b)**.

In addition to the quantification using confocal microscopy, a subcellular fractionation method was also used. The nuclear delivery of the plasmid DNA or adenovirus genome DNA was evaluated using PCR and/or Southern blotting after subcellular fractionation [42–45]. Although the PCR technique can be used to quantify the plasmid DNA in endosomes and/or lysosomes after subcellular fractionation [46], problems can arise as a result of uncertainties of the recovery of the endosome/lysosome fraction, or the leakage of DNA from the endosome/lysosome fraction to the cytosolic fraction. These problems should be carefully addressed when quantifying plasmid DNA in these fractions.

Following the PCR technique, Tachibana *et al.* measured the nuclear delivered plasmid DNA and transgene expression [42,43]. They reported a linear increase in plasmid DNA in the nucleus with increasing dose, and a remarkable saturation in transgene expression [42,43]. These results clearly indicate that it is necessary to enhance not only the nuclear delivery of plasmid DNA, but also the transcription efficiency in the nucleus.

PD of gene expression: towards controlled intranuclear disposition

The simplest PK/PD model of gene expression

After entering the nucleus, the disposition of DNA carrying a target gene depends upon the origin of the DNA – from what type of DNA it is derived. In general, a transgene that is inserted into the genome of the host is maintained for a long period. However, the possibility that the integration of the exogenous DNA might induce carcinogenesis can not be excluded. By contrast, DNA that exists as extrachromosomal DNA is likely to be safe from the viewpoint of cancer promotion, and should be the first choice in clinical applications of exogenous DNA. However, the copy number will decrease in response to cell division (in dividing cells) and by degradation, and a plasmid-based transgene is integrated into the host genome at low levels. Thus, the expression of a gene is transient.

It is generally considered that the total amount of transcript (mRNA) depends upon the amount of exogenous DNA in the nucleus. Each DNA molecule is assumed to act as a template with the same efficiency during transcription. Based on this assumption, a direct linear correlation between the amount of DNA and that of the mRNA would be expected, as shown in Figure 3a. This is the simplest PK/PD model for transgene expression in the nucleus. The elimination constant – k_{el} – represents the rate of division of exogenous DNA (in dividing cells) and degradation by nuclease(s). The PD parameter – k_{tra} – represents the transcription efficiency. According to this model, the efficiency of gene delivery into the nucleus (amount of the exogenous DNA) appears to be the only determinant for transgene expression.

A PK/PD model based on the intranuclear disposition

However, when the number of plasmid DNA molecules and the activity of the encoded enzyme (chloramphenicol acetyltransferase) were analyzed after transfection with cationic liposomes, a dramatic saturation of the enzymatic activity was found [42,43]. This result suggests that it is necessary to optimize not only the nuclear delivery of the genes but also the subsequent transcriptional process after nuclear entry. We recently found that transgenes (luciferase and green fluorescence protein genes) on linearized DNA capped at each end with a highly stable loop (dumbbell-shaped constructs, DNA dumbbells) were expressed about tenfold more than those on circular plasmid DNA of the same size when the same amount of DNA was transfected or intranuclearly microinjected into simian COS-7 cells ([47], Yamazaki *et al.*, unpublished data). It was also found that transgene expression is dependent on the length of the DNA dumbbell. Moreover, intranuclear microinjection and transfection of plasmid DNA isolated from a *dcm*$^-$ *Escherichia coli* strain, which lacks the major cytosine methylase (the Dcm protein), into simian COS-7 cells revealed an enhanced expression of the gene on the unmethylated molecule, compared with the conventional, methylated DNA (Yamazaki *et al.*, unpublished data). Although the actual molecular mechanisms of the effects of DNA structure and

methylation on expression are currently being examined in our laboratory, these results suggest that not every exogenous DNA molecule is transcribed with the same efficiency. Gene expression in the nucleus is regulated by various factors, including histone modification [48–50]. Thus, exogenous DNA molecules might be present in various states, from the maximally expressing state to the completely suppressed state. To simplify, we hypothesize the presence of the two kinds of exogenous DNA molecules in a PK/PD model: one, 'active DNA', that is transcribed and the other 'inactive DNA', in which transgene expression is suppressed.

As described previously, the expression of a transgene on a plasmid is transient. This is partly due to the degradation of exogenous DNA by cellular nuclease(s) and to cell division (in dividing cells). Recently, Ludtke *et al.* reported that plasmid DNA was mainly cytoplasmic in divided cells, even after an intranuclear microinjection, suggesting that the exogenous DNA is excluded during division [51]. Thus, the exclusion of exogenous DNA from the nucleus and its loss by division in dividing cells must be considered in addition to its degradation.

Based on these factors, the intranuclear disposition of exogenous DNA could be described as shown in Fiure 3b. To simplify a PK/PD model, only a decrease in active DNA (or inactive DNA) can be considered. Figure 3c shows the simplified PK/PD model for exogenous DNA. The elimination constant – k_{el} – represents the rate of degradation, division and exclusion from the nucleus of the exogenous DNA. The constants k_1 and k_2 represent the conversion efficiencies from the active to the inactive state, and from the inactive to the active state, respectively. The effects of DNA structure and methylation on expression, as described previously, can be regarded as changes in the ratio of k_1 to k_2. Alternatively, the effects might be a result of a decrease in the elimination constant, k_{el}. At present, the measurement of amounts of DNA in the active and inactive DNA compartments is difficult. However, the molecular mechanism(s) corresponding to the conversion efficiencies from the active to the inactive, and from the inactive to the active state, will help to establish a method to estimate the amounts of DNA in the active and inactive compartments.

As described here, an improvement in transcription efficiency in the nucleus is also important in developing non-viral vector systems for clinical applications. To this end, the disposition of transcriptionally active DNA (molecules in the active DNA compartment) should be regulated properly. In our model shown in Figure 3, the constants, k_1, k_2 and k_{el} are key parameters that determine the efficacy (amounts of the corresponding mRNA and therapeutic protein). This 'controlled intranuclear disposition' of exogenous DNA can possibly be achieved by alternation of the DNA structure and base modification, as shown in our recent study ([49], Yamazaki *et al.*, unpublished data).

Conclusion

There are apparent discrepancies between PK and PD in gene delivery. The principal reason was that intracellular events were considered as a black box. For an improved understanding, a new concept – controlled intracellular disposition – was introduced, which integrates conventional PK, intracellular PK and intranuclear disposition. The new concept postulated here is useful for the rational design of gene delivery vectors and could open up a new field in drug delivery system.

References

1 Harashima, H. *et al.* (2002) Pharmacokinetics of targeting with liposomes. *Crit. Rev. Ther. Drug Carrier Syst.* 19, 235–275
2 Harashima, H. *et al.* (2001) Intracellular control of gene trafficking using liposomes as drug carriers. *Eur. J. Pharm. Sci.* 13, 85–89
3 Kamiya, H. *et al.* (2001) Intracellular trafficking and transgene expression of viral and non-viral gene vectors. *Adv. Drug Deliv. Rev.* 52, 153–164
4 Felgner, P.L. *et al.* (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U. S. A.* 84, 7413–7427
5 Gao, X. and Huang, L. (1996) Potentiation of cationic liposome-mediated gene delivery by polycations. *Biochemistry* 35, 1027–1036
6 Ogris, M. *et al.* (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* 6, 595–605
7 Harashima, H. *et al.* (1998) Identification of proteins mediating clearance of liposomes using a liver perfusion system. *Adv. Drug Deliv. Rev.* 32, 61–79
8 Wheeler, J.J. *et al.* (1999) Stablized plasmid-lipid particles: construction and characterization. *Gene Ther.* 6, 271–281
9 Tam, P. *et al.* (2000) Stabilized plasmid-lipid particles for systemic gene therapy. *Gene Ther.* 7, 1867–1874
10 Monck, M.A. *et al.* (2000) Stabilized plasmid-lipid particles: pharmacokinetics and plasmid delivery to distal tumors following intravenous injection. *J. Drug Target.* 7, 439–452
11 Mullen, P.M. *et al.* (2000) Strength of conjugate binding to plasmid DNA affects degradation rate and expression level *in vivo*. *Biochim. Biophys. Acta* 1523, 103–110
12 Oupicky, D. *et al.* (2002) Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol. Ther.* 5, 463–472
13 Kircheis, R. *et al.* (2001) Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther.* 8, 28–40
14 Shi, N. and Pardridge, W.M. (2000) Noninvasive gene targeting to the brain. *Proc. Natl. Acad. Sci. U. S. A.* 97, 7567–7572
15 Shi, N. *et al.* (2001) Brain-specific expression of an exogenous gene after i.v. administration. *Proc. Natl. Acad. Sci. U. S. A.* 98, 12754–12759
16 Curiel, D.T. *et al.* (1991) Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc. Natl. Acad. Sci. U. S. A.* 88, 8850–8854
17 Wu, G.Y. *et al.* (1994) Incorporation of adenovirus into a ligand-based DNA carrier system results in retention of original receptor specificity and enhances targeted gene expression. *J. Biol. Chem.* 269, 11542–11546
18 Wagner, E. *et al.* (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. U. S. A.* 89, 7934–7938
19 Plank, C. *et al.* (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J. Biol. Chem.* 269, 12918–12924

20 Wagner, E. (1999) Application of membrane-active peptides for nonviral gene delivery. *Adv. Drug Deliv. Rev.* 38, 279–289
21 Friend, D.S. *et al.* (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochim. Biophys. Acta* 1278, 41–50
22 Zhou, X. and Huang, L. (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim. Biophys. Acta* 1189, 195–203
23 Kichler, A. *et al.* (2001) Polyethylenimine-mediated gene delivery: a mechanistic study. *J. Gene Med.* 3, 135–144
24 Klemm, A.R. *et al.* (1998) Effects of polyethyleneimine on endocytosis and lysosome stability. *Biochem. Pharmacol.* 56, 41–46
25 Simoes, S. *et al.* (1999) Transfection of human macrophages by lipoplexes via the combined use of transferrin and pH-sensitive peptides. *J. Leukoc. Biol.* 65, 270–279
26 Wyman, T.B. *et al.* (1997) Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry* 36, 3008–3017
27 Chaki, S. *et al.* (2002) Intracellular controlled trafficking after endocytosis with pH-sensitive liposomes and GALA-delivertives. In *Advances in Biomaterials and Drug Delivery Systems* (Hsiue, G. *et al.*, eds), pp. 527–536, Princeton International Publishing
28 Lechardeur, D. *et al.* (1999) Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther.* 6, 482–497
29 Lechardeur, D. and Lukacs, G.L. (2002) Intracellular barriers to non-viral gene transfer. *Curr. Gene Ther.* 2, 183–194
30 Tseng, W.C. *et al.* (1999) Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. *Biochim. Biophys. Acta* 1445, 53–64
31 Sebestyen, M.G. *et al.* (1998) DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. *Nat. Biotechnol.* 16, 80–85
32 Zanta, M.A. *et al.* (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. U. S. A.* 96, 91–96
33 Subramanian, A. *et al.* (1999) Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat. Biotechnol.* 17, 873–877
34 Hagstrom, J.E. *et al.* (1997) Nuclear import of DNA in digitonin-permeabilized cells. *J. Cell Sci.* 110, 2323–2331
35 Ludtke, J.J. *et al.* (1999) A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *J. Cell Sci.* 112, 2033–2041
36 Chan, C.K. and Jans, D.A. (2002) Using nuclear targeting signals to enhance non-viral gene transfer. *Immunol. Cell Biol.* 80, 119–130
37 Nagasaki, T. *et al.* (2003) Can nuclear localization signals enhance nuclear localization of plasmid DNA? *Bioconjug. Chem.* 14, 282–286
38 Varga, C.M. *et al.* (2001) Quantitative analysis of synthetic gene delivery vector design properties. *Mol. Ther.* 4, 438–446
39 Kamiya, H. *et al.* (2002) Visualization of intracellular trafficking of exogenous DNA delivered by cationic liposomes. *Biochem. Biophys. Res. Commun.* 298, 591–597
40 Akita, H. *et al.* (2003) Semi-quantitative analysis of the intracellular trafficking of plasmid DNA with confocal lazer scanning microscopy. *Mol. Ther.* 7, S214
41 Khalil, I. *et al.* Mechanism of improved gene transfer by the N-terminal stearylation of octaarginine: Enhanced cellular association by hydrophobic core formation. *Gene Ther.* (in press)
42 Tachibana, R. *et al.* (2001) Quantitative studies on the nuclear transport of plasmid DNA and gene expression employing nonviral vectors. *Adv. Drug Deliv. Rev.* 52, 219–226
43 Tachibana, R. *et al.* (2002) Quantitative analysis of correlation between number of nuclear plasmids and gene expression activity after transfection with cationic liposomes. *Pharm. Res.* 19, 377–381
44 Oh, Y.K. *et al.* (2002) Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA. *Gene Ther.* 9, 1627–1632
45 Miyazawa, N. *et al.* (1999) Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. *J. Virol.* 73, 6056–6065
46 Colin, M. *et al.* (2000) Cell delivery, intracellular trafficking and expression of an integrin- mediated gene transfer vector in tracheal epithelial cells. *Gene Ther.* 7, 139–152
47 Kamiya, H. *et al.* (2002) Size and topology of exogenous DNA as determinant factors of transgene transcription in mammalian cells. *Gene Ther.* 9, 1500–1507
48 Curradi, M. *et al.* (2002) Molecular mechanisms of gene silencing mediated by DNA methylation. *Mol. Cell. Biol.* 22, 3157–3173
49 Irvine, R.A. *et al.* (2002) DNA methylation has a local effect on transcription and histone acetylation. *Mol. Cell. Biol.* 22, 6689–6696
50 Wang, H. *et al.* (2001) Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* 293, 853–857
51 Ludtke, J.J. *et al.* (2002) The effect of cell division on the cellular dynamics of microinjected DNA and dextran. *Mol. Ther.* 5, 579–588
52 Sackett, K. and Shai, Y. (2002) The HIV-1 gp41 N-terminal heptad repeat plays an essential role in membrane fusion. *Biochemistry* 41, 4678–4685
53 Martin, I. *et al.* (1991) Fusogenic activity of SIV (simian immunodeficiency virus) peptides located in the GP32 NH2 terminal domain. *Biochem. Biophys. Res. Commun.* 175, 872–879
54 Peisajovich, S.G. *et al.* (2002) Sendai virus N-terminal fusion peptide consists of two similar repeats, both of which contribute to membrane fusion. *Eur. J. Biochem.* 269, 4342–4350
55 Kielian, M. and Helenius, A. (1985) pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. *J. Cell. Biol.* 101, 2284–2291
56 Boggs, W.M. *et al.* (1989) Low pH-dependent Sindbis virus-induced fusion of BHK cells: differences between strains correlate with amino acid changes in the E1 glycoprotein. *Virology* 169, 485–488
57 Simoes, S. *et al.* (1999) Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Ther.* 6, 1798–1807
58 Rittner, K. et al. (2002) New basic membrane-destabilizing peptides for plasmid-based gene delivery *in vitro* and *in vivo*. *Mol. Ther.* 5, 104–114
59 Vaysse, L. and Arveiler, B. (2000) Transfection using synthetic peptides: comparison of three DNA-compacting peptides and effect of centrifugation. *Biochim. Biophys. Acta* 1474, 244–250
60 Wadia, J.S. and Dowdy, S.F. (2002) Protein transduction technology. *Curr. Opin. Biotechnol.* 13, 52–56
61 Futaki, S. et al. (2001) Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjug. Chem.* 12, 1005–1011
62 Tan, R. and Frankel, A.D. (1995) Structural variety of arginine-rich RNA-binding peptides. *Proc. Natl. Acad. Sci. U. S. A.* 92, 5282–5286
63 Futaki, S. *et al.* (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* 276, 5836–5840
64 Redkar, A. *et al.* (2002) Characterization of cardiac muscle factor 1 sequence motifs: retinoblastoma protein binding and nuclear localization. *Gene* 282, 53–64
65 Li, S. *et al.* (1998) Identification of a novel cytoplasmic protein that specifically binds to nuclear localization signal motifs. *J. Biol. Chem.* 273, 6183–6189
66 Nadler, S.G. *et al.* (1997) Differential expression and sequence-specific interaction of karyopherin alpha with nuclear localization sequences. *J. Biol. Chem.* 272, 4310–4315
67 Miyamoto, Y. *et al.* (1997) Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. *J. Biol. Chem.* 272, 26375–26381
68 Siomi, H. and Dreyfuss, G. (1995) A nuclear localization domain in the hnRNP A1 protein. *J. Cell. Biol.* 129, 551–560
69 Wychowski, C. *et al.* (1986) A domain of SV40 capsid polypeptide VP1 that specifies migration into the cell nucleus. *EMBO J.* 5, 2569–2576
70 Gallay, P. *et al.* (1997) HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci. U. S. A.* 94, 9825–9830
71 Gallay, P. *et al.* (1996) Role of the karyopherin pathway in human immunodeficiency virus type 1 nuclear import. *J. Virol.* 70, 1027–1032