Expert Opinion

Advances in non-viral gene delivery: using multifunctional envelope-type nano-device

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Background: Low transfection efficiency is an obstacle to the clinical use of non-viral gene vectors. Effective non-viral vectors require the ability to control intracellular trafficking of gene vectors for the delivery of exogenous DNA to the nucleus. *Objective*: To overcome multiple intracellular barriers, various types of devices must be integrated into one nano-particle so that each device performs its function at the appropriate location at the desired time. Such a strategy requires an understanding, based on quantitative information, of the rate-limiting processes that hinder intracellular trafficking. *Methods*: In this review, advancements in the development of multifunctional envelope-type nano-devices (MEND) are discussed. In particular, a novel method to quantitatively evaluate the rate-limiting steps in intracellular trafficking, based on a comparison of viral and non-viral gene-delivery systems, is described. *Conclusion*: MENDs are useful to integrate various kinds of devices to overcome intracellular barriers into one particle. Comparison of intracellular trafficking between adenoviruses and non-viral vectors indicates that a postnuclear delivery process is an important rate-limiting step for efficient transfection with non-viral vectors.

Keywords: gene delivery, intracellular trafficking, multifunctional envelope-type nano device (MEND), stearylated octaarginine (STR-R8)

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1. Introduction

Selective delivery of drugs to target organs, so that pharmacological activity is maximized and side effects are kept to a minimum, requires the ability to control the pharmacokinetics of a particular drug. Targeting drug delivery to specific organs is the ultimate research goal in the study of drug delivery systems. Over the past few decades, various kinds of drug carriers have been developed for the *in vivo* delivery of low-molecular weight drugs, including antitumor reagents [1-4]. One of the most innovative technologies in the field of drug delivery systems is liposomes that have been coated with hydrophilic polymers, such as gangliosides [5,6] or polyethylene glycol (PEG), the coating increasing their half-life in circulation, that is long-circulating liposomes [7,8]. In addition, restricting particle size to < 200 nm may result in effective particle accumulation in tumors, depending on the half-life in systemic circulation [9-12], due to the leaky endothelial junctions of the tumor vessels (enhanced retention and permeation [EPR] effect). In the case of antitumor drugs, they can spontaneously enter the cells after release from the liposomes, due to the hydrophobic nature of the molecule. In contrast, gene-delivery systems require strict control of intracellular trafficking, because DNA is highly hydrophilic and negatively charged. Thus, nuclear delivery of DNA requires optimization of intracellular trafficking of the therapeutic genes to target the pharmacologically active site. Therefore, gene-delivery systems require consideration

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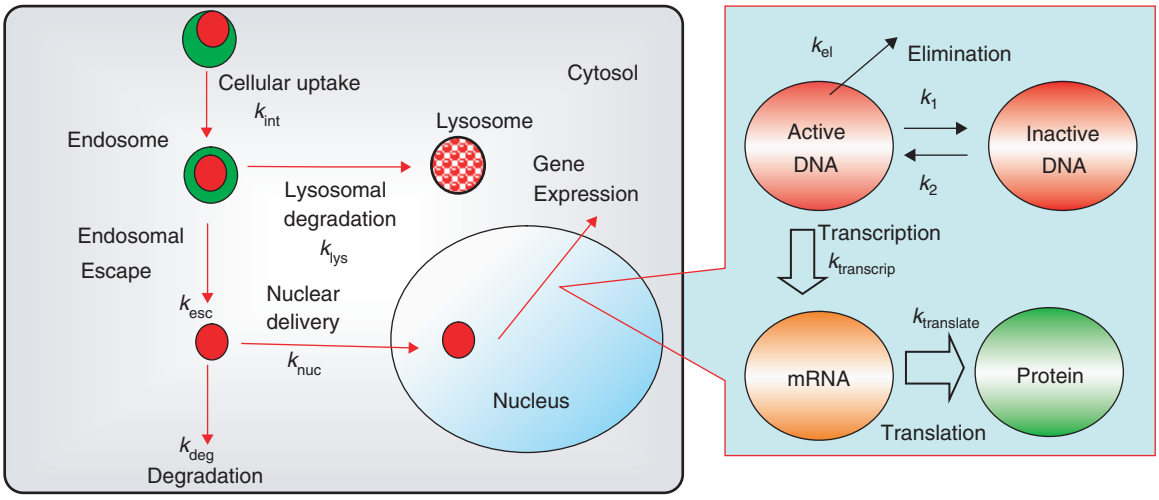

Figure 1. Schematic diagram illustrating the intracellular pharmacokinetics and nuclear dynamics of DNA. Intracellular barriers to the development of gene-delivery systems are shown. DNA carriers bind to the plasma membrane electrostatically and are internalized via endocytosis (k_{int}). Endosomal pDNA fused with lysosomes is degraded (k_{lys}). A certain fraction of the endosomal DNA is released into the cytosol (k_{esc}), where a fraction of the DNA is degraded by nucleases (k_{deg}) or partially translocates to the nuclear membrane (k_{nuc}). In the nucleus, active pDNA (e.g., the decondensed form of DNA) is subjected to transcription ($k_{transcript}$), followed by translation ($k_{translate}$).

of intracellular pharmacokinetics and nuclear dynamics, as well as conventional pharmacokinetics (**Figure 1**) [13].

In this review, the intracellular barriers that must be overcome for successful gene delivery will be discussed first. Next, the trials to overcome these barriers will be presented. It is vital to consider how intracellular trafficking is evaluated. In the majority of studies, only the final outcome – transfection activity – was evaluated, while the intracellular events required for transfection were not studied. To optimize transgene expression, the rate-limiting step(s) must also be identified. This requires quantitative evaluation of the subcellular distribution of genes. However, little quantitative information is currently available, primarily because adequate assay systems for quantifying the pDNA in each organelle are lacking. In the last section of this review, a method to quantify intracellular trafficking will be presented and the rate-limiting intracellular processes that determine the transfection efficiency of non-viral vectors will be discussed.

2. Strategy to control intracellular trafficking of gene vectors

2.1 Significance of the condensation of plasmid DNA into nano-order size

The plasma membrane is the first barrier to gene carriers because electrostatic repulsion between the negatively charged cell surface and DNA molecules significantly inhibits association between DNA and the cell membrane. Condensation of DNA with cationic materials, such as cationic polymers [14-16] or cationic liposomes [17-20], neutralizes the negative charge. Moreover, surface modification with ligands, such as Tf [9,21,22], LDL [23], EGF [24], folate [25-27] and RGD peptide [28,29] enhance the interactions between the carrier and cell surface.

Generally, gene vectors are taken up into the cells via clathrin-mediated endocytosis. Because clathrin-coated vesicles are only ~ 200 nm in size, the condensed pDNA must be less than ~ 200 nm. After endocytosis, DNA has to escape the endosomes before it can fuse with the lysosomes. Lysomotropic reagents, such as chloroquine, which accumulate in the acidic lysosomes and destabilize the membrane by swelling, drastically enhance transfection efficiency, demonstrating the importance of the endosomal escape.

The next barrier to efficient transfection encountered by DNA is the cytoplasm. Naked DNA is rapidly degraded in the cytoplasm by nuclease digestion with a half-life of the order of dozens of minutes [30,31]. Condensation of DNA protects against degradation by nucleases.

Finally, DNA has to enter the nucleus prior to transcription. The nuclear membrane consists of a double-membrane and transport of various substances (that is ions, low-molecular compounds and proteins) occurs only via the nuclear pore complex (NPC). Generally, size determines what substances can be transported by the NPC – 9 nm appears to be the threshold for diffusion through the NPC [32]. In contrast, for proteins that possess a nuclear localization signal (NLS), the threshold is a diameter of 39 nm [32]. However, commonly used pDNA exceeds this threshold and is therefore too large to pass through the NPC [33-35]. Hagstrom *et al.* demonstrated that DNA fragments less than 200 bp are suited to nuclear import [33]. Therefore, it is assumed that exogenous DNA primarily enters the nucleus when the nuclear membrane is diminished during the M-phase [36-38]. In fact, the percentage of pDNA that reaches the nucleus

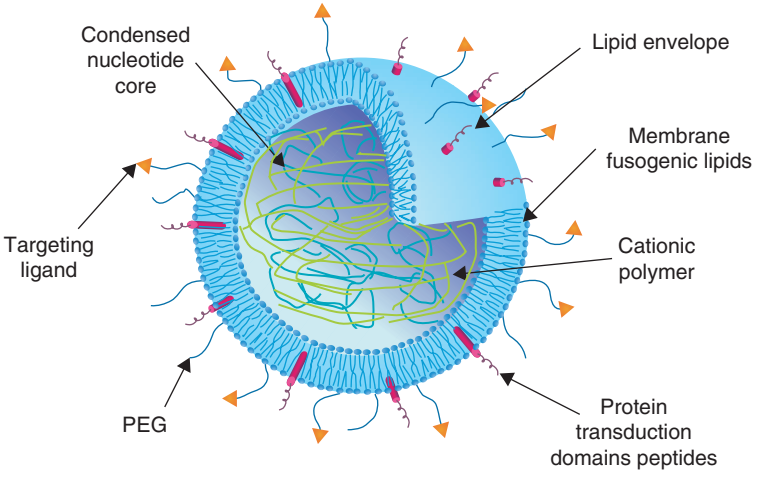

Figure 2. Schematic representation of multifunctional envelope-type nano-devices (MEND). MEND consist of condensed DNA molecules coated with a lipid envelope modified with functional devices, such as PEG, to increase half-life in systemic circulation, ligands for specific targeting, peptides containing a protein-transduction domain to increase intracellular availability and fusogenic lipids to enhance endosomal escape. Devices to increase nuclear transfer of DNA involve modification of the lipid envelope and/or the pDNA/polycation core.

is less than 1% of cytoplasm-microinjected DNA [39]. Approaches to overcome the nuclear membrane barrier are discussed below.

2.2 Multifunctional envelope-type nano-devices: a novel concept to control intracellular trafficking of DNA

As described above, gene vectors must overcome multiple barriers, including the plasma, endosomal and nuclear membranes. Thus, non-viral gene-delivery systems must be equipped with various functional devices: ligands for specific receptors; pH-sensitive fusogenic peptides for endosomal escape; and NLS for enhanced nuclear delivery. However, it is difficult to integrate all of these functional devices into a single system by simple mixing and to have each function operate at the appropriate time and place. Therefore, a new packaging concept called 'Programmed Packaging' has been proposed [40,41]. This concept consists of three components, as follows: i) a program to overcome all barriers; ii) design of functional devices and their three-dimensional assignment; and iii) nano-technology to assemble all devices into a nano-sized structure.

A novel non-viral gene delivery system – multifunctional envelope-type nano-devices (MEND) – has been recently proposed to realize Programmed Packaging [13,40-43]. The MEND, as shown in **Figure 2**, consists of a condensed DNA core and a lipid envelope equipped with the various functional devices. DNA condensation, the first step in preparing MEND, allows DNA protection from DNase, size control and improved packaging efficiency. In the second step, complexes are incorporated into the lipid envelopes. MEND were constructed using a novel assembly method [43] – the lipid-film hydration method. This packaging mechanism is based on electrostatic interactions between DNA, polycations and lipids.

pDNA was first condensed electrostatically with a polycation, such as poly-L-lysine (PLL), by vortexing at room temperature. Kinetic control of this process is important for determination of the size and charge of the condensed DNA. In the case of PLL, small (~ 100 nm) and positively charged (~ 30 mV) PLL/DNA complexes were prepared at a nitrogen/phosphate (N/P) ratio of 2.4:1.0. The lipid-film, containing a negatively charged lipid, such as cholesteryl hemisuccinate (CHEMS), was hydrated with an aqueous solution of DNA/PLL particles. The packaging of the PLL/DNA particles into the lipid bilayer was achieved by sonication in a bath-type sonicator. The diameter and zeta-potential of the MEND were approximately 300 nm and -40 mV, respectively. The encapsulation efficiency of the DNA was about 70% [43].

3. Control of intracellular trafficking with MEND

3.1 Control of the cellular uptake pathway

Recent studies have revealed that protein transduction domains (PTDs) are promising devices for improving the delivery of various types of biologically active molecules [44,45], such as proteins [46-49], nucleic acids [50-53] and liposomes [54,55]. One of the most widely studied carrier peptides, which consists of six arginine and two lysine residues, is derived from human immunodeficiency virus-type 1 (TAT) [56,57]. Based on the high arginine content within the TAT sequence, Futaki *et al.* synthesized polypeptides that consist solely of arginine residues. Octamers of arginine (R8), which were internalized via macropinocytosis – a

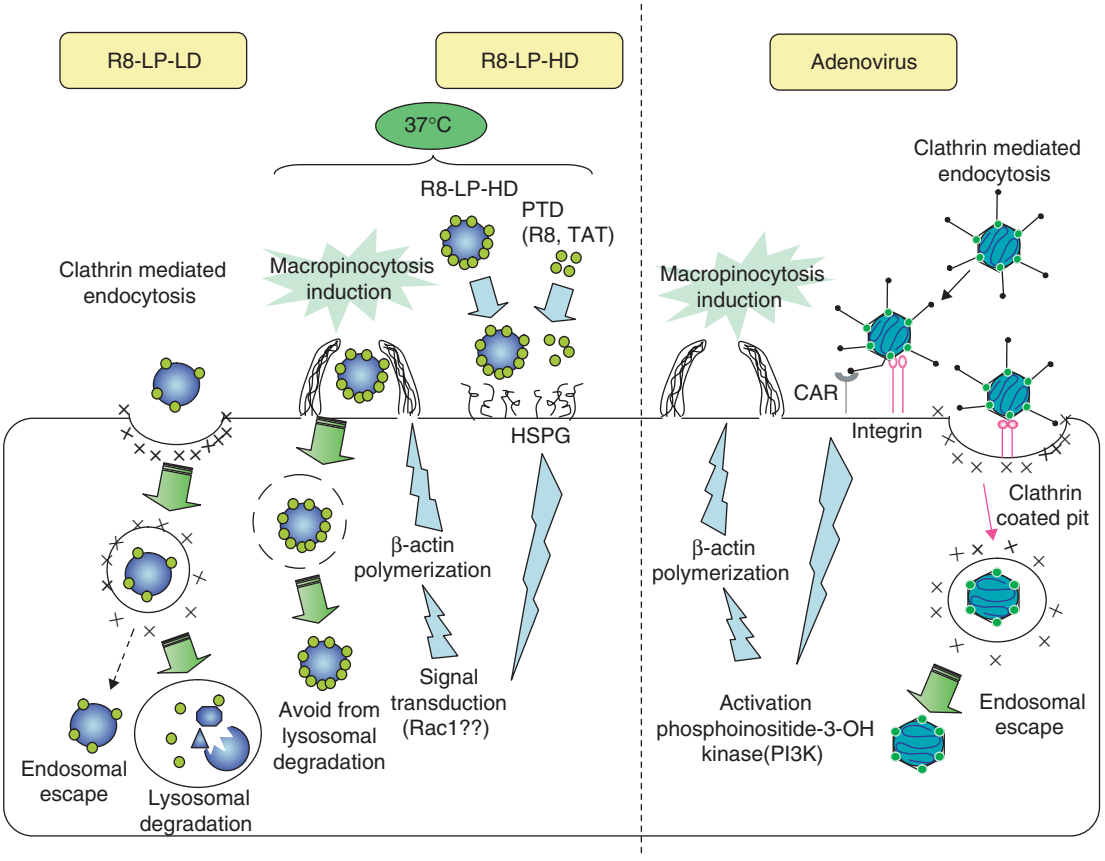

Figure 3. Summary of the cellular uptake mechanism of R8-modified liposomes and adenoviruses. Cellular uptake mechanisms of liposomes modified with R8 at high density (R8-LP-HD) and low density (R8-LP-LD), and adenovirus are shown. R8-LP-LD are taken up via clathrin-mediated endocytosis, whereas R8-LP-HD are taken up via macropinocytosis, which is an advantageous pathway as it avoids lysosomal degradation. It is possible that induction of a cellular uptake pathway by PTD or R8-LP-HD is closely related to signal transduction after the interaction with heparin sulfate proteoglycan (HSPG). Induction of the macropinocytosis pathway is also observed in adenoviral infection. Another unique characteristic of R8-LP-HD is its potential to penetrate cells at 4°C, conditions under which the majority of cellular uptake pathways are inhibited.

non-classical uptake pathway [58-60], showed the most efficient internalization. Thus, the R8 peptide is a promising device for internalization of MEND via a non-endocytic pathway.

For the surface modification of R8, stearylated R8 was incubated with liposomes. The stearyl group can efficiently incorporate in the lipid envelope, and result in a surface display of R8 on the liposome surface. The cellular uptake pathway can be distinguished by the evaluation of cellular uptake in the presence of specific inhibitors (chlorpromazine, filipin and amiloride are useful inhibitors of clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis, respectively). Interestingly, the density of R8 on the liposomal surface determines the cellular uptake pathway (**Figure 3**) [42]. R8-liposomes with low R8 surface density (R8-LP-LD) are taken up via clathrin-mediated endocytosis, whereas R8-liposomes with high R8 surface density (R8-LP-HD) are taken up via macropinocytosis. The cellular uptake pathway determines, in part, the intracellular fate. Confocal microscopic images revealed that R8-LP-LD highly co-localize with a lysosomal marker, whereas only partial colocalization was observed for R8-LP-HD. This observation indicates that internalization through macropinocytosis may be an effective means of avoiding lysosomal degradation [42].

The mechanism for the different routes of cellular uptake of R8-LP-LD and R8-LP-HD remain to be clarified. Induction of the macropinocytosis pathway has also been observed in adenoviral infection (**Figure 3**). The adenovirus first binds to the cell surface via the ligand/receptor interaction between the coxsackie ligand and adenovirus receptor (CAR)/fiber [61-63], and integrin/RGD in the penton base [64-67]. Internalization of Ad5 was increased by integrin-dependent activation of phosphoinositide 3-OH kinase (PI3K) [68]. It has been hypothesized that signals derived from binding of adenovirus to the cell-surface also activate kinases downstream from macropinocytosis [69,70]. Nakase *et al.* demonstrated that F-actin organization is accompanied by macropinocytosis of R8 and TAT [58].

Furthermore, it is possible that this event is closely related to activation of Rac1 after interaction with heparin sulfate proteoglycan (HSPG) [59]. HSPG-dependent uptake of R8-modified liposomes is also supported by results from our laboratory, demonstrating that cell-surface binding of R8-modified liposomes is prevented by heparan sulfate treatment [71,72]. Therefore, R8 or R8-modified MENDs induce certain types of signal transduction that activate cellular uptake pathways, as has also been observed in the case of adenovirus.

3.2 Control of endosomal escape

Our group has optimized the endosomal escape process for efficient gene delivery. As discussed in Section 2.2, above, the lipid envelope of MEND plays an important role in endosomal escape. When MEND modified with high density R8 (R8-MEND-HD) were prepared with phosphatidylcholine (EPC)/cholesterol (Chol), which is commonly used in the study of liposomes, transfection activities were higher than those of PLL/DNA complexes [40]. However, transfection activity remained low when liposomes were prepared using the EPC/Chol modification. Substitution of EPC with the fusogenic lipid, dioleoylphosphatidylethanolamine (DOPE), increased the transfection activities by approximately 20-fold. Furthermore, transfection activity increased by approximately two orders of magnitude when Chol was replaced with the negatively charged fusogenic lipid, cholesteryl hemisuccinate (CHEMS) [40].

This result can be explained by lipid-mixing between the endosomal membrane and cationic liposome, which leads to membrane disruption and release of DNA into the cytosol. This mechanism is supported by enhanced transgene expression using cationic liposomes modified with dioleoylphosphatidyl ethanolamine (DOPE) [73,74]. This lipid forms a stable lipid bilayer at physiological pH (~ 7.0), whereas a hexagonal-II structure is formed at acidic pH (5 – 6), which facilitates membrane fusion [75,76].

The transfection activity of R8-MEND-HD with optimized lipid envelope composition was compared with that of an adenovirus [40]. The transfection activities of the adenovirus increased with the applied dose in HeLa cells. 1×10^5 particles/cell was the maximum dose, as toxicity was evident above this dose. Transfection activities of R8-MEND-HD were as high as those of the adenovirus at 1×10^5 particles/cell. As judged from the protein content in cell lysates after transfection, the R8-MEND-HD showed no significant cytotoxicity, while higher doses of the adenovirus produced significant cytotoxicity (~ 50% loss of protein content). Amiloride significantly inhibited transfection activity of the R8-MEND (reduced by ~ 95%), indicating that macropinocytosis is the major determinant of efficient gene transfection of R8-MEND-HD [72]. Since pharmacological activity of short interference RNA (siRNA) can be achieved only when it is delivered to the cytosol, R8-MEND was also applied to the siRNA delivery system. For the encapsulation of siRNA to the MEND, siRNA was incubated with stearylated R8 to form a core complex (~ 50 – 70 nm), and was then encapsulated to the lipid envelopes by lipid hydration technique. As a result, efficient (approximately 80%) gene-knockdown can be achieved, suggesting that siRNA core were released to the cytosol with an efficient membrane fusion between endosome and lipid envelope [77].

Another option is the use of pH-sensitive fusogenic peptide. This peptide was first enlightened from the mechanism of the endosomal escape of influenza virus, an envelope-type RNA virus. In this virus, endosomal escape is conferred by the function of the hemagglutinin (HA) protein on the envelope membrane, which changes its conformation to the α-helix structure in the acidic compartment [78,79]. Similarly, artificial amphiphatic peptide (e.g., GALA [80,81] and JTS1 [82]) are also synthesized and applied to the various gene vectors. Recently, we have synthesized a cholesterol conjugate of GALA. Hydrophobic cholesterol structure can be readily incorporated to the lipid envelope, and then GALA was displayed on the surface of MEND. Modification of transferring and GALA on the surface of MEND can confer the targeting to the clathrin-mediated endocytosis pathway and subsequent endosomal escape [83,84].

3.3 Control of nuclear delivery

After cytoplasmic transport, pDNA must then translocate through the nuclear membrane. To achieve nuclear transport, NLS derived from simian virus 40 T-antigen (NLS_{SV40}) was directly attached to the pDNA [35,85,86]. However, direct chemical modification of the pDNA with NLS_{SV40} resulted in only a very slight increase in transgene expression [85,86]. Alternatively, condensation of pDNA with nucleus-targeting polycations appears to be a promising strategy for enhancing nuclear delivery. Based on this second strategy, DNA was condensed with polycations possessing NLS function, such as protamine [68,87]. A novel condenser also was synthesized. The μ (mu) peptide derived from an adenovirus was modified with NLS_{SV40}, which is a highly potent condenser of DNA [88]. As a result, a significant increase in transfection activity was observed. However, using this strategy it is difficult to control the NLS density on the surface of the particles because cationic residues in NLS_{SV40} interact with pDNA via strong electrostatic interactions.

Recently, we proposed a third strategy for nuclear delivery, which mimics the nuclear gene delivery system of adenoviruses. In adenoviruses, NLS are spontaneously displayed on the adenovirus particle via a well-ordered assembly of capsid proteins around the DNA core. To control the topology of NLS on the particle surface, a lipid derivative of a nuclear-targeting device was synthesized with the lipid moiety incorporated into the envelope and the NLS spontaneously oriented outward towards the MEND surface. This strategy was effective in the transfection of non-dividing cells (i.e., primary cultured dendritic cells) [89].

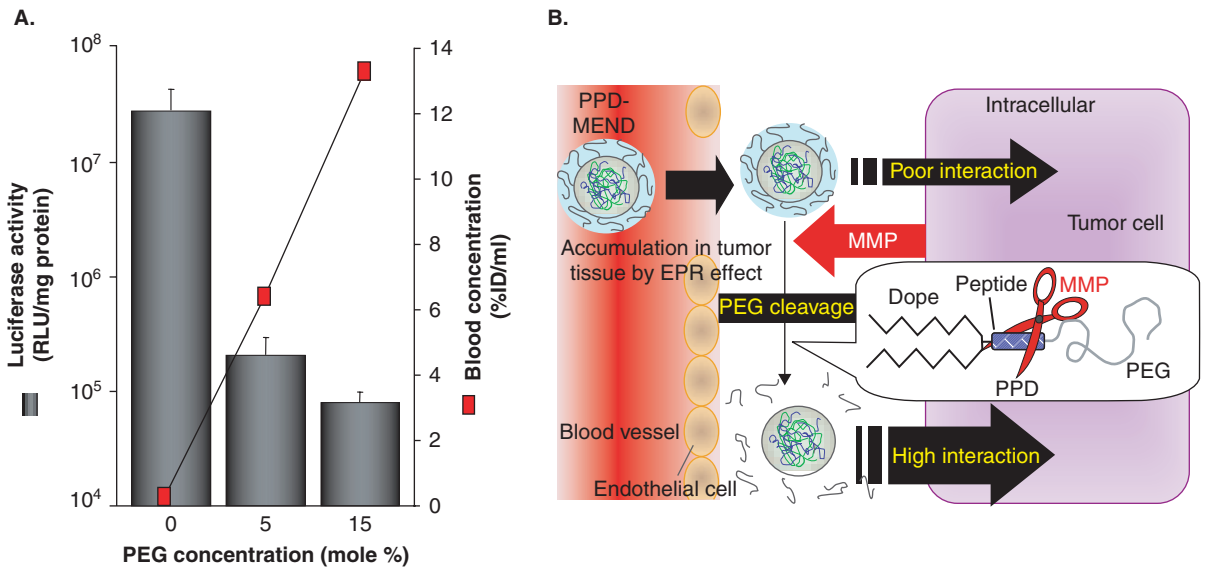

Figure 4. A schematic diagram illustrating the strategy used to resolve the dilemma associated with the use of PEG. A. The dilemma associated with the use of PEG is shown. The line graph shows the blood concentration of PEG-modified or PEG-unmodified MEND 6 h post-administration. Stability in circulation blood is improved depending on the PEG density. In contrast, the transfection activity is drastically decreased by tPEG modification. Therefore, a dilemma exists regarding the use of PEG; it is useful for conferring stability in systemic circulation, but it has undesirable consequences in cellular uptake and endosomal escape. **B.** A schematic diagram illustrating the strategy used to overcome the PEG dilemma. (i) By modifying the gene carrier with PPD, half-life in systemic circulation is prolonged, and accumulation in tumor cells is increased due to the enhanced retention and permeation (EPR) effect. (ii) After extravasation from capillaries in the tumor tissue, the PEG–peptide–lipid ternary complex (PPD) is cleaved by an extracellular matrix metalloprotease (MMP) secreted from tumor cells. (iii) PEG dissociates from the gene carrier and the naked carrier can then associate efficiently with the tumor cell surface.

Moreover, our group also has focused on sugars as nuclear-targeting devices. In recent decades, it was reported that bovine serum albumin (BSA) modified with certain kinds of sugars accumulated in the nucleus [90,91]. By modifying the surface of R8-MEND with sugars (i.e., *N*-acetylglucosamine), transfection activity was increased by 10-fold [92]. Investigation of the mechanism of enhanced transfection and optimization of sugar-modified MENDs is ongoing in our laboratory.

4. *In vivo* application of MEND

4.1 Topical application

R8-MEND were applied topically to the skin of a 4-week-old mouse for gene delivery to hair follicles. Expression of the transgene was observed in the hair shaft and follicle cells, which were treated with R8-MEND encapsulating LacZ- or GFP-encoding pDNA. The gene transfer efficiency of R8-MEND was significantly higher than that of lipoplexes made of the commercially available reagent, Lipofectamine, and pDNA. Next, R8-MEND containing DNA encoding bone morphogenetic protein receptor type 1A (BMPR1A), which is related to the hair growth cycle [40], also was applied topically. The R8-MEND formulated BMPR1A gene extended the duration of hair growth.

4.2 Systemic application

A gene vector that can be used for transfection after intravenous administration is one of the ultimate goals of gene therapy research. For intravenous administration of anticancer gene therapy to be successful, the carriers must be stable in systemic circulation and association between the DNA and tumor cells must occur after accumulation of the carrier in the tumor tissue. PEG-modification is useful for prolonging carrier stability in systemic circulation, however, a dilemma exists regarding the use of PEG. It is useful for conferring stability in systemic circulation, but it has undesirable consequences on cellular uptake and endosomal escape (**Figure 4A**) [93]. To overcome this dilemma, a novel PEG–peptide–lipid ternary complex (PPD) was synthesized, in which the peptide substrate for matrix metalloprotease (MMP) was inserted between PEG and the lipid [93]. Since MMP expression is high in tumor tissue, PPD-modified MEND is specifically activated in tumor cells in conjunction with the release of PEG from the surface of MEND (**Figure 4B**). In this study, serum-resistant cationic lipids composed of DOPE, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol were used to coat the DNA/polycation core. An *in vitro* study revealed that transfection activity of PPD-modified MEND is dependent on the level of MMP expression in the host cells. *In vivo*

studies further revealed that PPD effectively stabilized MEND in systemic circulation, thereby facilitating tumor accumulation. Moreover, intravenous administration of PPD- or PEG/PPD dually-modified MEND enhanced pDNA expression in tumor tissue compared with conventional PEG-modified MEND at 48 h after transfection. Thus, MEND modified with PPD is a promising device, which has the potential to make *in vivo* anticancer gene therapy a viable treatment option [93].

5. Identification of the rate-limiting step in gene delivery

5.1 Quantitative analytical method

As described above, intracellular trafficking of pDNA can be controlled using particles developed based on the concept of Programmed Packaging. For the development of new-generation gene-delivery vectors, a strategy based on quantitative analysis of intracellular trafficking is needed. This information would enable us to clearly recognize which barriers need to be overcome to improve transfection activity. Recently, we proposed a novel strategy to quantify the distribution of pDNA in the cytosol, endosomes/lysosomes and nucleus simultaneously, using sequential Z-series images captured by confocal laser scanning microscopy – a process that we refer to as Confocal Image-assisted 3-Dimensionally Integrated Quantification (CIDIQ) [94]. A schematic diagram and numerical formulae illustrating CIDIQ are summarized in **Figure 5A**. After transfection with rhodamine-labeled pDNA, acidic compartments (endosomes/lysosomes) and the nucleus were stained with LysoSensor DND-189 (green) and Hoechst 33342 (blue), respectively to visualize the subcellular localization of pDNA. Soon after transfection, pDNA is detected as clusters. Pixel areas of pDNA clusters were used as a quantitative index of the amount of pDNA. First, the total pixel area for the clusters of pDNA in each subcellular compartment was determined in each X–Y plane. Then, these values were further integrated and the values representing the amount of pDNA in each organelle were denoted S(cyt), S(endosome/lysosome) and S(nucleus). The total cellular uptake of pDNA into one cell was determined by integrating the organelle values and was denoted S(tot). Finally, the percentage of the total cellular pDNA in each organelle was calculated. This method is applicable to intracellular pharmacokinetic analysis of the various gene vectors and ought to be used in the development of new gene delivery systems.

5.2 Quantitative comparison of intracellular trafficking between viral and non-viral vectors

Various different kinds of physical methods have been developed, such as hydrodynamic delivery of naked DNA to the liver [95,96]. In addition, recent progress in non-viral vectors includes a triggered activation system in response to the intracellular microenvironment (i.e., intracellular reducing condition), which can also achieve a high level of transfection activity even in the postmitotic cell [97]. For the future *in vivo* application of promising non-viral vector (especially via systemic administration), it is desirable to achieve a high transfection activity at a lower dose. In other word, it is important to maximize transgene expression efficiency per one copy of DNA. Viruses have evolved sophisticated mechanisms to overcome these barriers, so that delivery of the viral genome to the host nucleus for viral replication occurs. As a result, transfection efficiency of viral vectors is superior to non-viral vectors. Therefore, it is essential to clarify why and to what extent current non-viral vectors are inferior to viral vectors from the point of view of intracellular trafficking [13]. This information would enable us to clearly recognize which barriers need to be overcome to improve transfection activity, and to what extent transfection activity might be improved if the barriers were overcome. Recently, our group compared intracellular trafficking between adenovirus and LipofectAMINE PLUS (LFN), as models of a viral and non-viral vector, respectively [98]. First, transfection activities were compared. When used according to the manufacturer's protocol, the time course of pDNA expression with LFN was comparable to that of the adenovirus. However, based on the dose–response curves, LFN required three to four orders of magnitude more gene copies than the adenovirus to achieve comparable transgene expression (**Figure 5B**). Therefore, it is important to clarify which intracellular process is responsible for such a large difference in transfection activity.

First, the cellular uptake of pDNA transfected using LFN and adenovirus were quantified. One hour after transfection, cells were collected and cellular uptake was evaluated using real time polymerase chain reaction (PCR). The uptake of DNA using LFN was approximately 15,000-fold greater than that using adenovirus. Normalizing cellular uptake to the applied dose, more than 40% of the pDNA was taken up by the cell with LFN, whereas for pDNA in the adenovirus this value was only 10%. The intracellular distribution of pDNA and adenovirus was then quantified with TaqMan PCR and CIDIQ. One hour after transfection, adenovirus delivered its DNA to the nucleus more efficiently than LFN. However, the nuclear transport efficiency of adenovirus was only twofold greater than that of LFN. Thus, the large difference in transfection efficiency cannot be explained by intracellular trafficking. Finally, comparison of the nuclear delivery of DNA revealed that three to four orders of magnitude more gene copies were necessary when using LFN to obtain a transfection activity comparable to that of adenovirus (**Figure 5B**). Transcription efficiency was calculated as expression divided by gene copies in nucleus. As a result, adenovirus was 8100-fold more efficient at nuclear transcription than LFN [98]. This result indicates that a postnuclear delivery process is critical to the difference in transfection efficiency between LFN and adenovirus.

Our recent study indicated that postnuclear delivery process is also a key factor for the heterogeneity of transgene

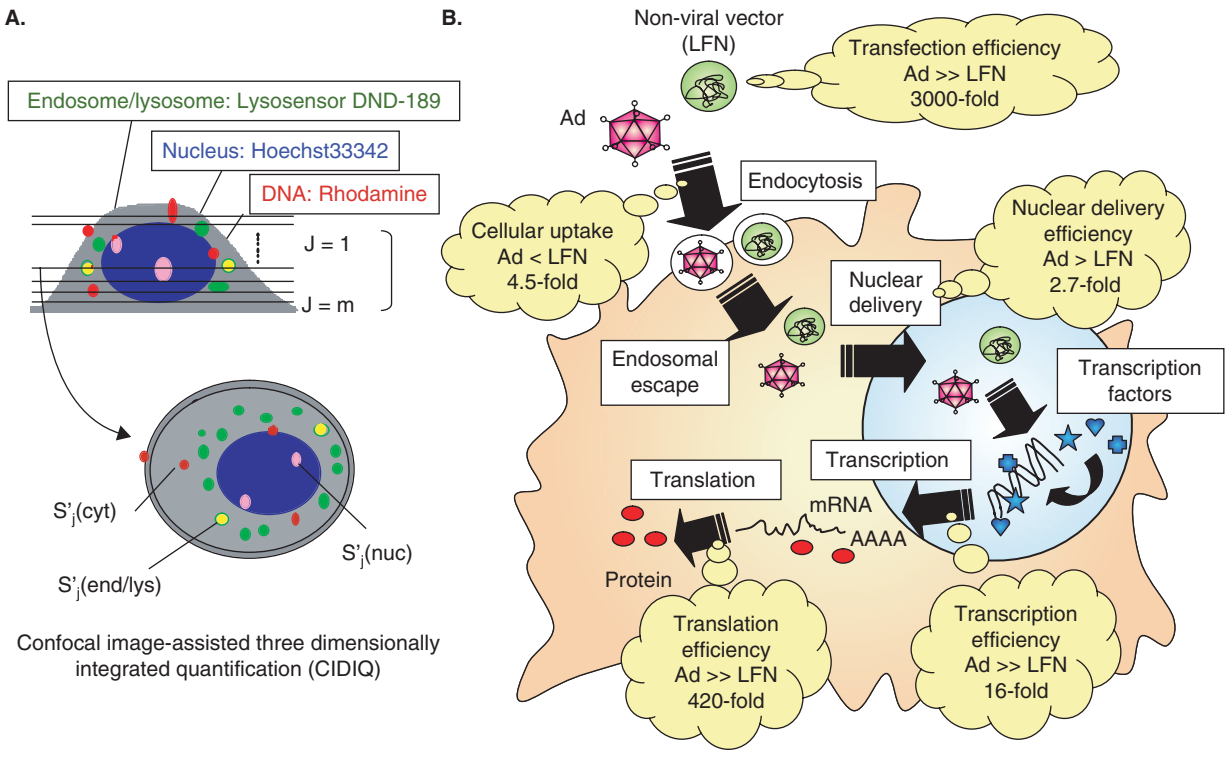

Figure 5. Quantitative comparison of intracellular trafficking between Lipofectamine PLUS (LFN) and an adenovirus. **A.** Schematic diagram illustrating a method of quantifying intracellular trafficking. Rhodamine-labeled pDNA was transfected into cells. Three hours after transfection, endosome/lysosome and nucleus compartments were stained with LysoSensor DND-189 and Hoechst 33342, respectively. The pixel areas of the pDNA in each X–Y plane were summed for each compartment and are denoted as $S'_j(i)$, where i represents each compartment (e.g., membrane-bound, endosome/lysosome, cytosol, perinucleus and nucleus). $S'_j(i)$ represents the total pixel area (which corresponds to the amount of plasmid DNA) in each X–Y plane for each compartment (i). $S'_j(i)$ was summed up in Z-series to obtain S(i), which represents the total pixel area in a cell for each compartment (i). All the S(i) values were combined to calculate the S(tot) reflecting the total pixel area in a cell. F(i), representing the fraction of the plasmid DNA in each compartment in the cell, was calculated by dividing S(i) by S(tot). **B.** By measuring cellular uptake and nuclear delivery, it was determined that the large difference in transfection efficiency between adenovirus and LFN is primarily due to a postnuclear delivery process. Furthermore, by measuring mRNA expression, this difference can be divided into transcription and translation. A difference of three orders of magnitude in postnuclear delivery process was due to a one order of magnitude difference in transcription efficiency and two orders of magnitude difference in translation efficiency.

expression in non-viral vectors. In an attempt to investigate the mechanism underlying heterogeneity, the relationship between efficiency of nuclear delivery of pDNA and trans-gene expression was examined in individual cells transfected with LFN as a model artificial gene vector [99]. Out of 204 randomly selected cells, 46 cells (22.5%) possessed nucleus-delivered pDNA. As expected, marker gene expression was below the detection limit in almost all of the nuclear pDNA-negative cells. However, simultaneous detection of nuclear pDNA and trans-gene expression showed that LacZ activity was only detected in 15 out of 46 nuclear pDNA-positive cells (32.6%), suggesting that nuclear delivery was not sufficient for the trans-gene expression.

Collectively, it is clearly indicated that improvement of postnuclear events is essential to achieve a transgene expression comparable to the viral vector, and to resolve heterogeneity.

5.3 Mechanism for the difference in postnuclear delivery events between adenovirus and LFN

To apply the results described above to the development of artificial vectors, we have to understand why the transcription activity of adenoviral vectors is so high. To address the influence of genome structure and sequence on transfection activity, adenoviral DNA and pDNA encoding GFP were microinjected into the nucleus, and GFP expression efficiency was evaluated. GFP expression efficiency was comparable between Ad genome and pDNA. Therefore, differences in DNA sequence and structure cannot explain the difference in transcription efficiency between adenovirus and LFN [100].

Finally, differences in intranuclear distribution of DNA were examined by visualizing a decondensed form of DNA using *in situ* hybridization. It was confirmed that the ODN probe can access a specific sequence in naked

DNA but not in complexed DNA. As a result, the pDNA signal was less prominent compared with adenoviral genome, even though the total amount of nuclear DNA was higher in LFN compared with adenoviral transfection. These results strongly suggest that poor decondensation is responsible for the less efficient nuclear transcription with LFN.

Furthermore, we need to remember that transgene expression of nucleus-delivered DNA is limited by transcription and translation. Therefore, the contributions of these two processes to the overall differences in efficiency of postnuclear processes were quantitatively evaluated by measuring the amount of cellular mRNA. Transcription efficiency was calculated as mRNA expression divided by nuclear DNA [100]. Similarly, translation efficiency was calculated as transgene expression divided by mRNA expression. Translation efficiency with adenovirus transfection was approximately 16-fold higher than that of LFN. Furthermore, the translation efficiency of adenovirus was 420-fold higher than LFN. Therefore, the three orders of magnitude difference in postnuclear delivery process was due to a one order of magnitude difference in transcription efficiency and two orders of magnitude difference in translation efficiency (**Figure 5B**).

The mechanisms underlying the prominent differences in translation were examined. Because RNA is negatively charged, LFN may interact with mRNA via electrostatic interactions. If this assumption is correct, two hypotheses can explain the difference in translation efficiency. The first is that LFN may entrap mRNA in the nucleus and interfere with its nuclear export. The second is that the recognition of cytoplasmic mRNA by ribosomal RNA or another translation-related protein is inhibited. To examine these hypotheses, the nuclear distribution of mRNA was compared for adenovirus- and LFN-mediated transfection. Three hours after transfection, cellular and intranuclear mRNA was quantified by real time RT–PCR. Nuclear distribution of mRNA was comparable between LFN and adenovirus. Therefore, electrostatic interactions between LFN and mRNA do not inhibit nuclear export of mRNA. To compare the effect of LFN and adenovirus vectors on cytoplasmic translation, mRNA encoding luciferase was subjected to *in vitro* translation with or without adenovirus and LFN. When adenovirus was applied, protein synthesis was inhibited by 20% compared with no treatment. In contrast, when LFN was added, protein synthesis was drastically inhibited – by more than 90% [100]. These data indicate that the inhibition of translation due to electrostatic interactions between LFN and mRNA is a significant contributor to the difference in translation efficiency between LFN and adenovirus.

In summary, a novel strategy to control transgene intranuclear disposition (i.e., intranuclear decondensation and localization) and to minimize cytoplasmic interactions between mRNA and vectors, is needed to further improve non-viral vectors.

6. Conclusion

MEND were developed based on the novel concept of 'Programmed Packaging'. MEND are composed of a condensed DNA core, which is covered with lipid bilayer that mimic envelope-type viruses. These nano-structures are useful because they allow the integration of various kinds of devices to overcome intracellular barriers (i.e., endosomal membrane and nuclear membranes) into one particle. Studies comparing adenoviruses and non-viral vectors indicate that a postnuclear delivery process is the rate-limiting step for efficient transfection with non-viral vectors. The development of MEND equipped with devices to control intranuclear disposition (i.e., nuclear matrix-targeting signals) is the next strategy to be developed to bring transfection efficiency of non-viral vectors closer to that of viral vectors.

7. Expert opinion

In this review, we introduced the concept of Programmed Packaging for the development of non-viral gene delivery vector. According to this concept, various kinds of devices are integrated into one particle, so that each function operates at the appropriate time and place. As a model vector, our group developed MEND, in which the devices for endosomal escape and nuclear delivery can be modified by altering the lipid membrane or pDNA/polycation core. For the development of a new generation of gene-delivery vectors, information on intracellular trafficking enables us to clearly recognize which barriers need to be overcome to improve transfection activity. Most current research efforts have focused on individual intracellular barriers to enhance transgene expression. However, it is possible that increasing the efficiency of one process may reduce the efficiency of other processes. For example, it is plausible that tight condensation of pDNA with polycations may produce small particles, which increases cellular uptake; however, excess condensation inhibits transcription. To optimize intracellular trafficking, it is necessary to balance all intracellular processes. Establishment of an integrated intracellular kinetic model, which integrates kinetic parameters (i.e., first-order kinetic rate constant: time^{-1}) for cellular uptake, endosomal release, nuclear binding, nuclear translocation, decondensation, transcription and translation first-order mass-action kinetics, would be useful.

One method to quantify intracellular distribution is CIDIQ. With this method, confocal laser scanning microscopy is used to quantify pDNA by measuring the pixel area of the fluorescent signals. In the future, an application that enables automated quantification procedures is needed because this method requires the analysis of a large number of confocal images to obtain an accurate time course for the determination of kinetic parameters.

One of the most significant findings in our recent studies is the importance of postnuclear delivery processes to

the development of non-viral vectors. Results from our laboratory demonstrate that intranuclear disposition differs between viral and non-viral vectors. Just as intracellular space is occupied by various organelles, intranuclear space also is compartmentalized, each compartment having a unique function. Decondensation efficiency is higher in adenoviral compared with non-viral vectors. In addition, the adenovirus genome localizes specifically in the euchromatin region where transcription activity is enriched. However, why adenoviruses have such a high decondensation efficiency and/or euchromatin-targeting activity remains unclear at present. Investigation of the molecular mechanisms of these phenomena is important to the future design of non-viral vectors. Furthermore, recent progress in epigenetics has revealed that intra-nuclear transcription is also dynamically regulated by acetylation/methylation of histones, methylation of DNA *per se* via the CpG motif, and by chromosome organization. In the future, an understanding of the structure, function and dynamics of the intranuclear space is required to design devices that control intranuclear trafficking. Current work to improve the MEND system is focused on the rational design of polycations, with particular attention on decondensation efficiency and euchromatin targeting.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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