Current Topics

Stimuli-Responsive Systems of Therapeutics

Review

Methods for Evaluating the Stimuli-Responsive Delivery of Nucleic Acid and Gene Medicines

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In this review, we have summarized evaluation methods for the analysis of external stimuli-mediated nucleic acid and gene delivery. Prior to reviewing these evaluation methods, we describe various delivery processes of nucleic acid and gene medicines (small interfering RNA (siRNA), micro RNA, mRNA, plasmid DNA, *etc.*), which include interaction with blood components, bio-distribution, disposition in the target tissue, cell entry, intracellular trafficking, nuclear localization, and dissociation from the carriers. Next, we discuss the advantages of external stimuli-mediated nucleic acid and gene delivery. External stimuli enable us to effectively deliver nucleic acids and genes to targeted regions. Evaluation methods are required to elucidate the behaviors of nucleic acid and gene medicines in the body. Quantitative analyses of the bio-distribution and *in situ* disposition in perfused organs, as well as visualization of bio-distribution, transgene expression in the body, and intracellular trafficking of nucleic acid and gene medicines, are all useful in evaluating not only the efficacy and safety of delivery, but also serve as guidelines for the further development of nucleic acid and gene medicines by elucidating delivery problems. Progress in evaluation methods, including tissue optical clearing and super resolution microscopy, will help to better elucidate the *in vivo* fate of nucleic acid and gene medicines.

Key words external stimuli; nucleic acid; gene delivery; evaluation method; transfection mechanism

1. Introduction

In this post genome era, causes of disease are now being elucidated at the genetic level. Thus, gene therapy is a promising approach for treating intractable diseases such as monogenic diseases and tumors. For gene therapy, plasmid DNA and mRNA are utilized to deliver foreign genes. Gene expression level can be regulated by small nucleic acids, including small interfering RNA (siRNA) and micro RNA (miRNA). Naked nucleic acids and genes themselves are generally ineffective in treating disease, due to their rapid degradation by nucleases and their inappropriate systemic distribution to reticuloendothelial systems (RES)[1,2]; thus, the development of effective delivery systems is essential. Among the current delivery systems for nucleic acids and genes, the stimuli-responsive delivery system is useful not only for effective *in vivo* transfection, but also in targeting specific tissues.[3–13] In this review, we focused on evaluation methods for the development of stimuli-responsive delivery systems. Prior to reviewing the evaluation methods, we summarized the delivery processes of nucleic acid and gene medicines. We then discussed the delivery advantages of external stimuli, and finally reviewed the evaluation methods.

2. Regulation Strategies for Nucleic Acid and Gene Delivery

To obtain optimum effectiveness of nucleic acid and gene medicines, we must control not only systemic bio-distribution but also the intracellular disposition in target cells. The final destination of mRNA, siRNA and miRNA in the target cells should be cytosol, whereas the nucleus is the ultimate goal of plasmid DNA; thus, we face a long, complex journey toward acquiring optimum effectiveness.[14] As to the delivery processes after intravenous administration, the first event for the vectors is interaction with blood components, including erythrocytes and plasma proteins.[15–21] In particular, interaction with plasma proteins is a determinant factor for tissue accumulation.[19–22] Interaction with plasma proteins is highly dependent on the composition of the vectors. The interaction of cationic liposome (1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/cholesterol)/plasmid DNA complex with fibronectin contributes to lung accumulation and gene expression after intravenous administration.[20] Ionizable lipid nanoparticles containing siRNA interact with apolipoprotein E, and subsequently accumulates in the liver.[21,22] After interaction with blood components, the vectors are distributed to tissues throughout the body through systemic circulation. Thus, targeting strategies are required to deliver vectors to a specific tissue. As a targeting strategy, utilization of the ligand against the specific receptor on the target cells is useful.[23–25] Also, the administration route of vectors is important in order to control bio-distribution of the vectors.[26,27] To inhibit distribution to the non-target tissues/cells, PEGylation of the carriers is a common strategy.[28–30] PEGylation reduces interaction with erythrocytes[30,31] and modulates interaction with plasma proteins.[32] However, PEGylation also reduces uptake by tar-

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get cells. This problem is known as the 'polyethylene glycol (PEG) dilemma.'[33] To overcome this 'PEG dilemma,' detachable PEGylated lipids have been utilized.[34,35]

After reaching the target cells, the next step is cellular uptake. Endocytosis is a primary mechanism of vector uptake.[36–41] There are several endocytic pathways, including clathrin-, caveolae- and flotillin-mediated endocytosis, macropinocytosis, and phagocytosis.[42–44] These endocytic pathways seem to involve different intracellular trafficking.[44] *Via* clathrin-mediated endocytosis, a vector immigrates to endosome/lysosome compartments and subsequently degrades. In contrast, at least a portion of internalized substances uptaken *via* caveolae-mediated endocytosis and macropinocytosis bypass the immigration to lysosomal compartments.[45–49] To allow a vector to escape from endosomes/lysosomes, pH-responsive materials such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and polyethyleneimine are utilized.[50–53] For genes, delivery to the nucleus is essential. Certain sequences in plasmid DNA play an important role in delivering plasmid DNA into the nucleus *via* interaction with transcriptional factors having a nuclear localization signal.[54] The dissociation of nucleic acids and genes from the vectors is a limiting step for therapeutic effectiveness.[55] Certain dissociation strategies using disulfide bond that are responsive to reductive environment in the cytosol have been reported.[56,57] Overall, in order to develop effective vectors, it is important to understand the total process, from the administration of the vector to its modulation of gene expression.

3. Delivery Advantages Using External Stimuli

A stimuli-responsive system is a promising approach for the delivery of nucleic acid and gene medicines. External stimuli can activate the vector at the stimuli-applied site in the body. Thus, not only the organ but also a specific part of organ, such as a diseased site, can be targeted by the external stimuli.[12,58–60] The combination of external stimuli with other targeting strategies, such as usage of ligands, is another useful approach. Even when several types of cells express the same receptor for a ligand, the use of external stimuli allows us to specifically deliver nucleic acids and genes to the target cells in the stimuli-applied organ.[7,61] As external stimuli, electric pulse,[4,6,59,60] ultrasound,[5,7–9,58,61] tissue pressure,[10,11] tissue suction,[12,62] and tissue rubbing[13] have all been reported as modes for nucleic acid and gene delivery. These stimuli-based transfections are highly effective. The mechanism of this high transfection efficiency may be due to the generation of transient pores on the plasma membrane of cells.[63,64] Therefore, not *via* endocytosis, but through transient pores, nucleic acids and genes would enter the cytosol of the cells; thus, these delivery methods would bypass lysosomal degradation. Since naked nucleic acids and genes can be delivered *via* these external stimuli, it is also possible to bypass the dissociation process in their delivery. In the next chapter, we introduce methods for evaluating nucleic acids and gene delivery.

4. Evaluation Methods for Nucleic Acid and Gene Delivery

Our evaluation objectives include all processes in nucleic acid and gene delivery. Figure 1 and Table 1 summarize evaluation levels for each process subsequent to the administration of nucleic acids and gene medicines. There are four levels of evaluation: *in vivo* (live animals), *ex vivo* (extracorporeal methods), *in situ* (organ perfusion, *etc.*) and *in vitro* (cell culture, *etc.*). Each level has advantages and disadvantages in

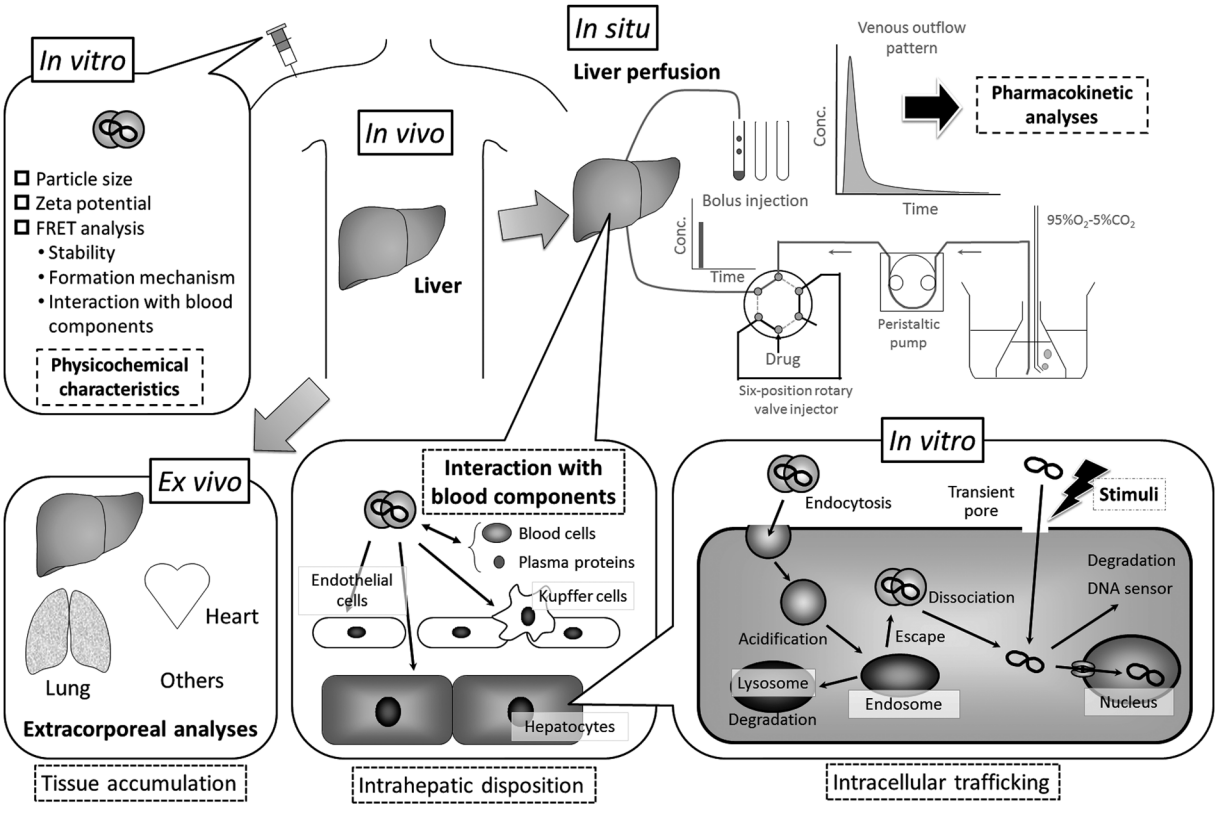

Fig. 1. Schematic Illustration of Delivery Processes and Evaluation Levels for Stimuli-Responsive Nucleic Acid and Gene Delivery

Table 1. Capacity of Each Experimental Level for Evaluation of Delivery Processes

Processes	*In vivo*	*Ex vivo*	*In situ*	*In vitro*
Tissue clearance	Possible	Good	Difficult	Impossible
Interaction with blood components	Marginal	Possible	Possible	Possible
Penetration through endothelium	Marginal	Possible	Possible	Impossible
Diffusion in the target tissue	Marginal	Possible	Possible	Impossible
Binding with the target receptors	Difficult	Possible	Possible	Good
Cellular uptake	Difficult	Marginal	Marginal	Good
Intracellular trafficking	Difficult	Marginal	Marginal	Good
Penetration of nucleus membrane	Difficult	Marginal	Marginal	Good

Table 2. Examples of Evaluation Methods for Nucleic Acid and Gene Delivery at Each Experimental Level

Levels	Evaluation methods/equipment	Evaluation objectives	Refs.
In vivo	Live imaging by bioluminescence	Transgene expression	109)
	Live imaging by positron-emission tomography	Bio-distribution of siRNA and lipoplex	110)
Ex vivo	Fluorescence imaging of tissues by IVIS	Bio-distribution of siRNA and LNP	111)
	Observation of imprinted mesothelial cells with confocal microscopy	Cellular uptake mechanism of plasmid DNA	41)
	3D-imaging using tissue clearing with confocal microscopy	Spatial distribution of transgene expression in tissues	87)
		Intracellular disposition of fluorescently-labeled plasmid DNA in tissues	
In situ	Organ perfusion	Disposition of ^{32}P-labeled plasmid DNA in the liver	73,74)
In vitro	Live cell fluorescence resonance energy transfer (FRET) imaging	Duplex siRNA intracellular trafficking	112)
	Real-time confocal microscopy of live cells	Generation of transient pores on cell membranes	94)
	Total internal reflection fluorescence/epifluorescence structured illumination microscopy	Cellular uptake and intracellular trafficking of hydrophobically modified siRNA	113)
	Super resolution imaging with structured illumination microscopy	Intranuclear chromatin binding of polyplex	114)

Table 3. Summary of Detection Methods Useful for Evaluation of Nucleic Acid and Gene Delivery

Objectives	MRI	PET, SPECT	Bioluminescence	Fluorescence
Transgene expression	Impossible	Tissue level	Tissue level (require substrate)	Tissue level, cell level
Bio-distribution	Require contrast agent	Good (tissue level)	Marginal	Possible (tissue and cell level)
Tissue structure	Tissue level	Tissue level	Tissue level	Tissue and cell level
Biological function (ex. cell signaling)	Impossible	Difficult	Impossible	Good
Cellular states (survival, death)	Difficult	Possible	Difficult	Good
Multiple events	Difficult	Difficult	Difficult	Excellent

evaluating the delivery processes. We can obtain macroscopic information by *in vivo* methods, while *in vitro* methods are good at microscopic evaluations. Table 2 shows examples of evaluation methods at each level. Through multiple evaluations by these methods, integration of information allows us to more thoroughly judge the effectiveness and safety of the delivery systems. The next section summarizes evaluation points in stimuli-responsive nucleic acid and gene delivery.

4.1. Evaluation Points As to the efficacy of nucleic acid and gene delivery, evaluation of gene expression is necessary. In gene delivery, gene expression is monitored using reporter genes such as luciferase[65] and secreted alkaline phosphatase (SEAP)[66] for quantitative evaluation. Also, use of fluorescent proteins is a useful option for determining the percentage of transgene-positive cells, as well as cell species.[27,67] In the case of nucleic acid medicines, the inhibition efficiency of gene expression is directly monitored by mRNA or protein levels. For mRNA level, real-time PCR is often utilized. For protein level, Western blotting and enzyme-linked immunosorbent assay (ELISA) are common representative methods. In addition, disposition of the nucleic acids, genes and delivery carriers are thoroughly analyzed, from the whole body level to the cellular level. Delivery efficiency to target tissue/cells and intracellular disposition are both important factors in determining the efficacy of nucleic acid and gene medicines. Yet, mechanisms are also important. Among the many evaluation points mentioned above, bio-distribution and intracellular disposition have significant impact on therapeutic efficacy. Thus, in the next section we focus on evaluation methods for the bio-distribution and intracellular disposition of nucleic acid and gene medicines.

4.2. Evaluation of Bio-Distribution It is important to evaluate the bio-distribution of nucleic acids, genes and the carrier. To evaluate distribution, the proper selection of detection methods is necessary. Table 3 summarizes detection methods for nucleic acids, genes, and carriers.

Nucleic acids and genes can be labeled by a radio isotope ^{32}P for quantification of tissue accumulation.[23,68] ^{18}F-, ^{99m}Tc- and ^{111}In-labeled nucleic acids and genes have also been developed.[69–72] Theoretically, nucleic acids and genes labeled with

^{18}F, ^{99m}Tc and ^{111}In can be visualized by positron emission tomography (PET) and by single photon emission computed tomography (SPECT) imaging. Systemic distribution, as well as local disposition of nucleic acid and gene medicines, are useful information in evaluating therapeutic efficacy. We previously analyzed the hepatic disposition of galactosylated liposome/plasmid DNA complex and polyethyleneimine/plasmid DNA complex using a rat liver perfusion system.[73,74] Organ perfusion experiments are useful, since it is possible to control perfusion conditions such as flow rate and composition of the perfusate. We also analyzed the role of blood components in the hepatic disposition of galactosylated liposome/plasmid DNA complex using the liver perfusion system.[16]

On the other hand, fluorescence can be used for both quantitative and qualitative analyses ranging from the whole body level to the cellular level. For live animal imaging using fluorescence, near infrared fluorescence is useful since there are several bio-optical windows in which light absorbance by tissues is low compared with visible light.[75,76] Near infrared fluorescence dyes such as Cy5.5 are suitable for *in vivo* tracking of nucleic acids and genes.[77,78] Tissues are generally opaque; thus, thin tissue sections are required for microscopy. However, spatial information is lost in the observation of thin tissue sections. Here, tissue optical clearing is a useful strategy for the imaging of spatial distribution of fluorescence in tissues.[79–85] Using the tissue optical clearing method with confocal laser scanning microscopy or two photon excitation microscopy,[86] the observation of a thicker tissue sample, or multiple thin tissue sections at a time, is possible. We previously demonstrated the imaging of spatial distribution of transgene expression in the liver after hydrodynamics-based injection in mice.[87] Cell-to-cell variability and intra-tissue variability can be visualized by tissue optical clearing. One reason for the opacity of tissues is light scattering.[88] Mechanisms for clearing up opaque tissues are dependent on each visualization method. Dehydration, homogenization of the refractive index, and lipid removal are commonly used mechanisms.[88,89] Tissue clearing allows for observation under conditions ranging from ultraviolet to infrared rays. Therefore, not only red fluorescent protein, but also green fluorescent protein can be visualized in cleared tissues.[87] Tissue clearing methods can be categorized into lipid structure-preserved and non-preserved methods. Detergents are often used in tissue clearing methods to remove lipids or to enhance the clearing reagent penetration. Among reported tissue clearing reagents, clear, unobstructed brain imaging cocktails and computational analysis (CUBIC) has demonstrated the best performance,[82,83,87] but CUBIC removes lipids; lipid structures are destroyed. Preserving lipid structures has several merits, as follows. First, blood vessels can be stained with lipophilic carbocyanine dyes such as 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI).[90] Moreover, by preserving lipid structure, we can evaluate the spatial distribution of lipidic carriers. Detergent-free tissue clearing methods such as Clear^{T} and SeeDB can preserve lipid structures.[80,81] However, the tissue clearing efficiencies of Clear^{T} and SeeDB are insufficient for allowing the observation of spatial distribution.[84,87] Therefore, the challenge remains to develop tissue clearing methods that preserve lipid structures but have sufficient clearing efficiency.

4.3. Evaluation of Intracellular Disposition The intracellular disposition of nucleic acid and gene medicines largely contributes to their efficacy; thus, evaluation of their intracellular disposition is useful for further development of effective nucleic acid and gene medicines. Cell entry, endosomal escape and dissociation of nucleic acids and genes from the carriers are major important processes determining therapeutic efficacy. Again, fluorescence is often utilized for the visualization of the intracellular disposition.[91] Using tissue optical clearing, we obtained multicolor deep imaging of the spatial distribution of transgene expression and of fluorescently-labeled plasmid DNA after hydrodynamics-based injection.[87] We revealed that transgene-positive cells were also positive in co-delivered fluorescent dextran, indicating that the mechanism was confirmed to be hydroporation, as previously reported.[92] Moreover, several molecules of plasmid DNA were co-delivered to the nuclei of transgene-positive cells. There should be specific mechanisms for the co-delivery of several molecules of plasmid DNA, such as interaction with certain proteins having a specific nuclear localization signal. The cell entry process affects subsequent intracellular disposition including localization in cytosol, as mentioned above. Here, several physical stimuli, including electroporation,[63] sonoporation[93] and hydroporation,[92] can directly deliver nucleic acids and genes into cytosol *via* transient pores on the cell membranes. Hu *et al*. observed and proved transient membrane perforation and recovery using real-time confocal microscopy.[94] Evaluation of each delivery process can elucidate the behavior of nucleic acid and gene medicines, and provide useful information for their further development.

5. Perspectives

In this review, we mainly focused on methods of evaluation for efficacy. Safety concerns must also be solved, thus evaluation methods for safety must be studied as well. Some external stimuli causes transcriptional activation of activator protein 1 (AP-1) and nuclear factor-kappaB (NF-κB)[95–98]; thus, inflammatory responses are objectives of evaluation for safety. Analyses at both the *in vivo* and *in vitro* level are preferable for elucidating the safety of nucleic acid and gene medicines.

Both the development of delivery systems and the development of useful evaluation methods are important issues. Currently, we have developed a novel tissue clearing method that preserves lipid structures and has sufficient clearing efficiency (unpublished work). Our tissue clearing method was applicable to the simultaneous imaging of blood vessels, transgene expression and oxidative stress. Combination of tissue clearing with liver perfusion would be valuable to elucidate the hepatic disposition of nucleic acid and gene medicines. Novel evaluation methods may accelerate the development of efficient and safe delivery systems.

To elucidate accurate intracellular trafficking of nucleic acid and gene medicines, the application of super-resolution microscopy[99,100] may be useful. Super-resolution microscopy overcomes the diffraction limit of light and provides imaging at high spatial resolution (up to 20 nm in XY plane). Since the diffraction limit of light is half of light's wavelength, the particle size of nanoparticles for nucleic acid and gene delivery (around 100 nm) is generally below the diffraction limit (*ca.* 200 nm, dependent on wavelength). There are several principles for super resolution microscopy, including structured illumination microscopy (SIM),[101] stimulated emission depletion microscopy (STED),[102] and stochastic optical reconstruc-

tion microscopy (STORM).[103] The resolution of SIM, STED, and STORM is 2, 10, and 10 times higher than diffraction limit (*ca.* 200 nm), respectively. The merits of super resolution microscopy compared with electron microscopy include multicolor and live cell imaging. However, a multicolor setting is difficult in super resolution microscopy since there are limited types of fluorescent dyes.[99] Also, the temporal resolution of super resolution microscopy (second level) is much slower than confocal microscopy (millisecond level). Consequently, Carl Zeiss Microscopy GmbH launched a super resolution-like microscope, Airyscan,[104] which has 1.7 times higher resolution than the conventional confocal system. The frame rate of Airyscan (up to 13 frames per second (fps)) is slower than confocal systems (up to 420 fps in Nikon A1R$^+$ confocal microscope), but may be enough for visualization of the intracellular trafficking of nucleic acid and gene medicines. The tissue clearing method SeeDB2 has been applicable for super resolution microscopy SIM, STED, and Airyscan.[85] As for *in vivo* live imaging, the bio-distribution of microbubbles and liposomal nanobubbles can be visualized using Contrast Pulse Sequencing.[105] In case of microbubbles and liposomal nanobubbles, theranostics (simultaneous diagnosis and therapeutics) is possible and useful.[106] In addition, intravital confocal laser scanning microscopy[107] or two-photon excitation microscopy[108] will elucidate the *in vivo* disposition and intracellular trafficking of nucleic acid and gene medicines. However, the *in vivo* behaviors of nucleic acid and gene medicines remain to be elucidated.

6. Summary

In the current review, we summarized delivery processes of nucleic acid and gene medicines, the delivery advantages of external stimuli, and evaluation methods used to elucidate the fate of nucleic acid and gene medicines. We focused on evaluation methods for bio-distribution and intracellular disposition as essential processes for the successful delivery of nucleic acids and genes. In particular, we focused on methods of visualization of the behavior of nucleic acid and gene medicines upon administration. Recent progress in visualization, including tissue optical clearing and super resolution microscopy, promised to be valuable in elucidating the accurate behavior in the body of nucleic acid and gene medicines, and should accelerate the development of effective and safe nucleic acid and gene delivery systems.

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Conflict of Interest The authors declare no conflict of interest.

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