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REVIEW ARTICLE

The concept of molecular machinery is useful for design of stimuli-responsive gene delivery systems in the mammalian cell

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Abstract Since the first generation of molecular machines including photoresponsive crown ethers and its analogues was reported by Shinkai et al., a huge number of molecular machines exhibiting dynamic chemical and physical functions have been designed and developed. On the other hand, non-viral vectors are desired to possess conflicting properties to associate with DNA until reaching the nucleus as their final destination and dissociate from DNA there. In other words, non-viral vectors should work as a sort of molecular machinery. To overcome this dilemma, recently, much attention is focused on the development of the intelligent vectors, also called as 'stimuli responsive vectors' working as molecular machines. In this review, stimulus responsive gene delivery systems in which some structural factors and/or physiological properties are regulated in response to extracellular signals such as redox, pH, ultrasound, light, temperature, etc. are introduced as a new generation of non-viral vectors. These extracellular signals such as ultrasound, light, and temperature can be potent stimuli capable of site-, timing-, and duration-specific gene expression.

Keywords Non-viral vector · Gene delivery · Stimuli-responsive systems · Molecular machinery · Photo-responsive systems · Transfection

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Introduction

Sequencing of the 22,000–23,000 genes in the human genome has recently reached completion [1]. Gene therapy is one of many applications of these newly sequenced genes. Gene therapy has been considered as a promising treatment not only for the recipe of diseases with genetic defects but also for cure and prevention of various acquired diseases such as infectious diseases [2] (acquired immune deficiency syndrome (AIDS) [3, 4]), cancer [5], and life-style related diseases [6, 7]). However, polyanionic substances like nucleic acids, in aqueous solution, are rapidly degraded by nucleases and exhibit poor uptake into cells. Therefore, vectors serve as the key for successful gene delivery. Vectors are divided into two classes; viral systems such as retrovirus, adenoviruses, adeno-associated viruses, and non-viral systems such as liposomes and polymers. Over the past few decades a great effort have been made to transfer therapeutic genes into living cells and produce proteins by using various vectors [8–13]. To date, although viral vectors have been mainly used as technologies that are being applied for gene therapy due to their higher transfection efficiency compared to non-viral vectors, its application is affected by safety concerns, including

virus replication and inflammatory reactions in addition to some drawbacks such as size limitation of transgenes and high cost. Especially, serious recurring issues have been reported in some patients due to immunogenicity and a random integration into the host chromosome (oncogenicity). In 1999, the death of the patient with ornithine transcarbamylase deficiency had been caused by adenovirus-mediated gene therapy [14, 15]. Also, children with severe combined immunodeficiency (SCID) caused by a mutation on the X chromosome, whom a French gene therapy team treated by retrovirus vectors, faced the insertional mutagenesis [16, 17]. Therefore, the necessity of much safer non-viral vectors leaves no room for doubt.

The non-viral delivery systems involve the use of plasmid DNA alone or supramolecular assembly composed of plasmid DNA and synthetic cationic molecules. One of major reasons for the low transfection activity is the presence of various barriers between the site of administration and gene expression of the target cells. In order to achieve high transfection efficiency by non-viral vectors, various biological barriers must be overcome: (A) cellular internalization, (B) escape from the endosomal compartment prior to trafficking to lysosomes, (C) resistance to enzymatic degradation by nucleases, (D) nuclear transport, and (E) release of plasmid DNA for the smooth transcription (Fig. 1) [2]. Cationic lipid/DNA complexes (lipoplex) and cationic polymer/DNA complexes (polyplex) can enhance gene translocation through cellular membrane and endosomal escape owing to stable and tight polyion-complexation. These complexes possessing a net positive charge have advantages not only to interact with negatively charged cellular membrane, resulting in cellular uptake by endocytosis, but also to protect DNA from hydrolysis by endogenous nucleases. However, this is also a disadvantage in the following processes, where the plasmid DNA is released within cytoplasm and/or nucleus. In order to initiate transcription and enhance gene expression, the polyion-complexes should dissociate to prevent the transcriptional inhibition by binding of cationic gene carriers. Therefore, non-viral vectors are desired to possess conflicting properties to associate with DNA until reaching the nucleus as the final destination and dissociate from DNA there. In other words, non-viral vectors should work as a molecular machinery to overcome the expected dilemma. More recently, much attention is focused on the development of the intelligent vectors, also called as ''stimuli responsive vectors'' [18–21].

On the other hand, the concept of molecular machinery was proposed by Feynman about half century ago [22]. Afterward, the first-generation molecular

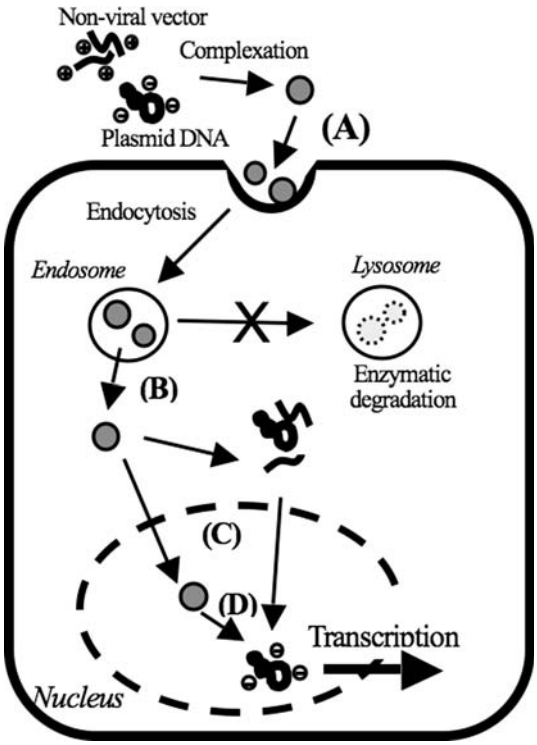


Fig. 1 Biological barriers in gene delivery and expression: (**A**) cellular internalization; (**B**) endosomal escape of gene to cytosol; (**C**) resistance to degradation by nuclease; (**D**) nuclear import; (**E**) release of gene

machine including photoresponsive crown ether was reported by Shinkai et al. [23]. The first example of such light-driven nano-machines consists of an azacrown ether bridged by an azobenzene group [24, 25]. The crown ether in *trans*-form has a stretched, oval-shaped ring structure, which shows the specific affinity with smaller metal ion Na^+, whereas the *cis*-form has a round-shaped ring structure, which shows the specific affinity with the larger metal ion K^+. One may conclude, therefore, that the nano-sized ring is manipulated by a nano-sized photo-expander (Fig. 2). Moreover, Shinkai et al. synthesized a photoresponsive bis(crown ether) with an azobenzene linkage [26, 27]. As the photo-isomerization between *trans*- and *cis*-form is reversible, this compound undergoes a butterfly-like motion (Fig. 3). The authors had expected that photo-isomerization-based switching might lead to a more efficient control of ion extraction, ion transport, and other effects. Thereafter, a huge number of molecular machines exhibiting dynamic chemical and physical functions have been designed and developed [28–39].

This review focuses on recent developments in the area of non-viral vectors capable of control of active or

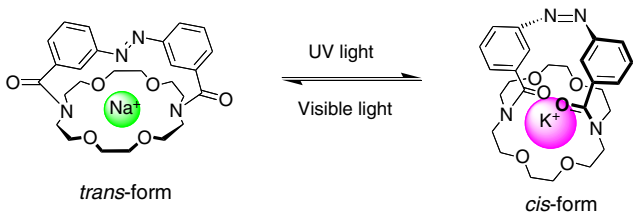


Fig. 2 The first example of molecular machinery to manipulate molecules. The nano-sized ring is manipulated by a nano-sized photo-expander

controlled DNA release by utilizing the concept of molecular machinery possessing photo- and other stimuli-responsive functions.

Photoresponsive vectors

Photochemical trasfection

As light is a very controllable stimulus, both temporally and spatially, photo-irradiation has been used to enhance the transfection efficiency by improving both the endosomal escape of exogenous DNA and the DNA release from complexes. For example, photochemical internalization (PCI) of drug and gene was proposed by Høgest et al. [40–46]. PCI is based on photochemical reactions initiated by a photosensitizer localized in endocytic vesicles such as endosome and lysosome, inducing rupture of these vesicles upon light irradiation. PCI potentially can be developed into a site- and timing-specific delivery system in vivo [47]. In photochemical transfection, amphiphilic photosensitizers such as porphyrins and phthalocyanines are initially excited to an excited state that change triplet ground-state molecular oxygen to highly reactive singlet oxygen (Fig. 4A). The singlet oxygen is a powerful oxidant that can oxidize various biomolecules such as unsaturated fatty acids, certain amino acids, and nucleic acids. Thus, activated oxygen damages and disrupts neighboring endocytic membranes (Fig. 4B).

Release of plasmid DNA from early endosomes seemed to be of importance because photochemical transfection is significantly inhibited by bafilomycinA1 that increases vesicular pH and interferes with endocytic transport upon the early stage [48, 49]. In contrast, photochemical transfection efficiency is not influenced by chloroquine or ammonium chloride that buffer and raise the pH of endosomes and lysosomes and affect intracellular trafficking [50]. Furthermore, photochemical transfection by a photosensitizer, aluminum phthalocyanine ($AlPcS_{2a}$) strongly improves transfection efficiency with cationic polymers (e.g.,

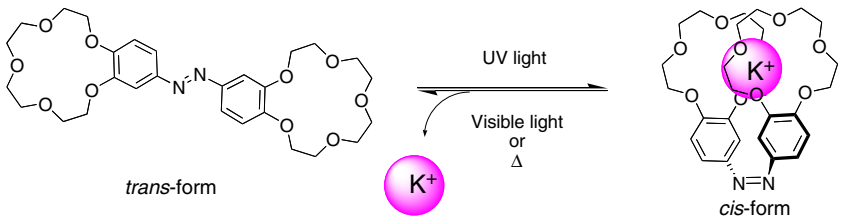


Fig. 3 Bis(crown ether) with a butterfly-like motion

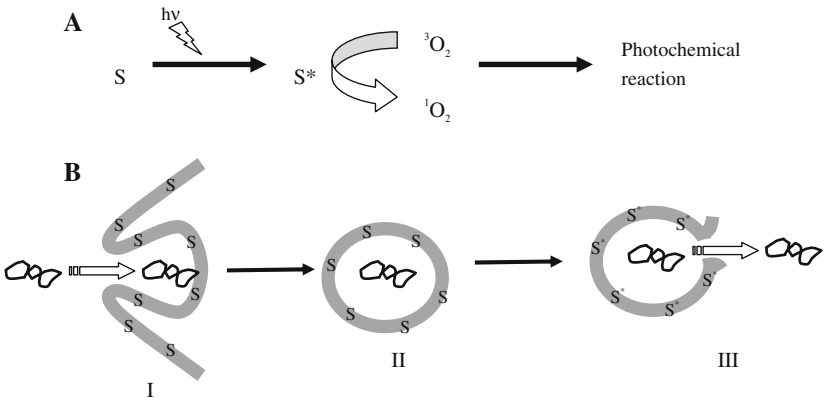


Fig. 4 Photo-induced cytosolic delivery by PCI. (**A**) Schematic representation of the photochemical reactions in PCI. S and S* indicate photosensitizer and excited one, respectively. (**B**) The mechanism of PCI. (I) The photosensitizer (S) localizes to the plasmamembrane, and the exogenous gene is internalized by endocytosis. (II) Photo-irradiation leads to chemical reactions to damage the endocytic membrane. (III) The rupture of endocytic membrane leads to escape of gene into cytosol

PLL and PEI), whereas the effect on that of cationic lipids (Lipofectin: DOTMA/DOPE = 1/1) is obviously negative. In human melanoma cells, the light irradiation increased the transfection efficiency with $AlPcS_{2a}$ and PLL by more than 20-fold, reaching transfection levels of about 50% of the surviving cells [51] (Fig. 5). Photochemical treatment also enhanced the transduction of polycation-complexed adenoviruses [52]. Prasmickaite et al. [53] investigated the role of the cell cycle for the efficiency of photochemical transfection. In aphidicolin-synchronized cell, photochemical transfection with PLL was dependent on the cell cycle: the transfection level was 4-fold higher when light exposure treatment was carried out during G2/M phase as compared to the G1/early-S phase. It seemed that photochemically liberated transgene close/during mitosis had the favorable opportunity to enter the nucleus. Photochemical transfection such as PCI has been developed for artificial gene delivery systems, however, this method has generally high toxicity.

More recently, Kataoka et al. [54] reported the first successful example in the PCI-mediated gene delivery in vivo. The system is a ternary complex composed of a core polyplex with cationic peptide enveloped in the anionic dendrimer phthalocyanine as the photosensitizer (Fig. 6A). In this system, following points consider to contribute to its high performance. (i) The photosensitizer is integrated into gene carrier as one component, because separate administration of photosensitizers might result in their diffused localization to the surrounding tissues in vivo. (ii) After internalization by the endocytosis, the photosensitizer could liberate the gene complex, otherwise, photoirradiation should cause damage to gene. (iii) The photosensitizer could localize the vesicle membrane under endosomal conditions to accomplish the photochemical rupture of the endosomal membrane (Fig. 6B). In animal experiments, subconjuctival injection of packaged DNA enveloped in a dendrimeric photosensitizer followed by laser irradiation resulted in transgene expression only in the laser-irradiated site. The new biomedical application can be potentially useful for the gene therapy of ophthalmic diseases.

Photoresponsive gene delivery systems with photochromic compounds

In addition to PCI using photosensitizers, the direct introduction of photosensitive unit into the non-viral vector has been also reported in order to construct the photoresponsive gene delivery system. Of the various non-viral vectors, dendritic polymers have been used as safe, non-immunogenic, and highly efficient non-viral vectors (Fig. 7) [55–61]. Nagasaki et al. synthesized a novel cationic L-lysine-modified polyazobenzene dendrimer (**Lys-G2**) as a synthetic vector for a mammalian cell (Fig. 8). UV irradiation after the incorporation of **Lys-G2**-complex with plasmid DNA into a cytoplasm caused a 50% increase in the transfection efficiency as compared with the case without UV irradiation; namely, *trans*-to-*cis* photo-isomerization had protonated amines serried and decreased the cationic density on the surface of the complex to suppress the cationic repulsion. The decreased charge density was also confirmed by zeta potential measurements. This work demonstrates that the transfection

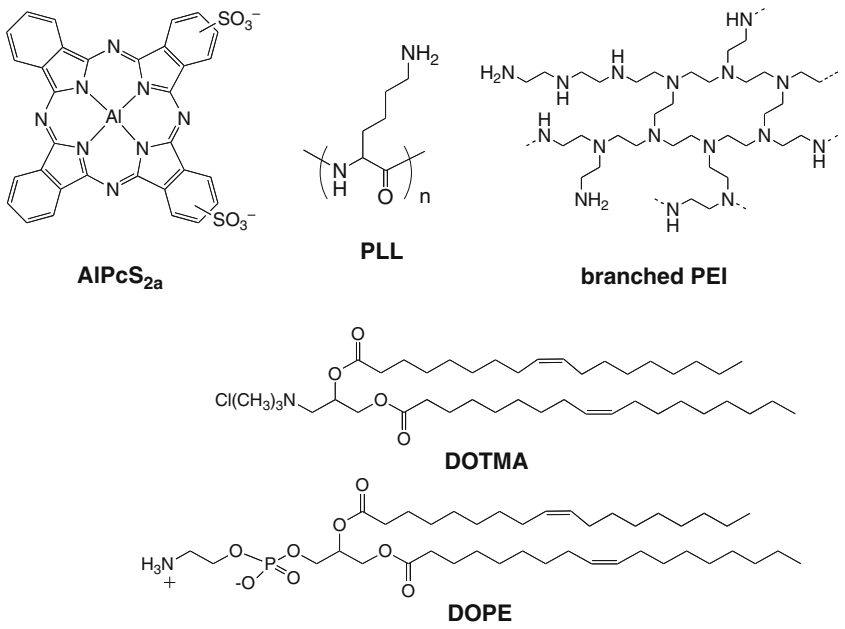


Fig. 5 Chemical structures of photosensitizers, polymeric, and amphiphilic vectors

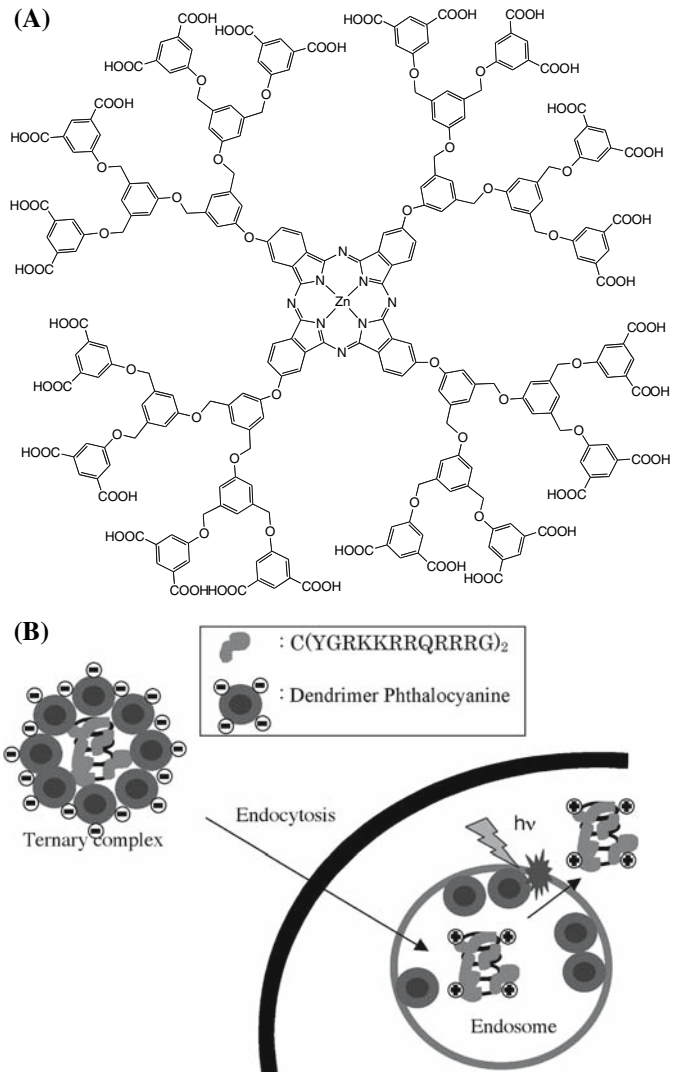


Fig. 6 Photochemical internalization by using dendritic photosensitizer. (**A**) Chemical structure of dendrimer phthalocyanine; (**B**) Schematic representation of mechanism of ight-induced gene transfer from packaged DNA enveloped in a dendritic photosensitizer

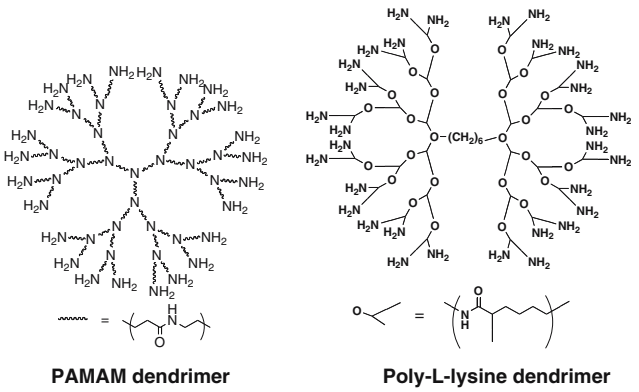


Fig. 7 Chemical structure of dendritic polyamines as synthetic vector

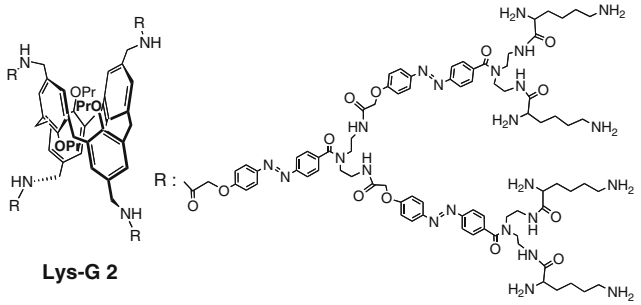


Fig. 8 Chemical structure of cationic L-lysine-modified polyazobenzene dendrimer (**Lys-G2**)

efficiency is controllable by UV irradiation using a cationic dendrimer, which possesses a photochromic polyazobenzene structure [62–64]. Furthermore, a novel photoresponsive cationic lipid (**KAON12**) having an azobenzene structure between hydrophilic and hydrophobic groups has been synthesized (Fig. 9) [65]. The self-assembling behavior of **KAON12** was directly examined by transmission electron microscopy. With

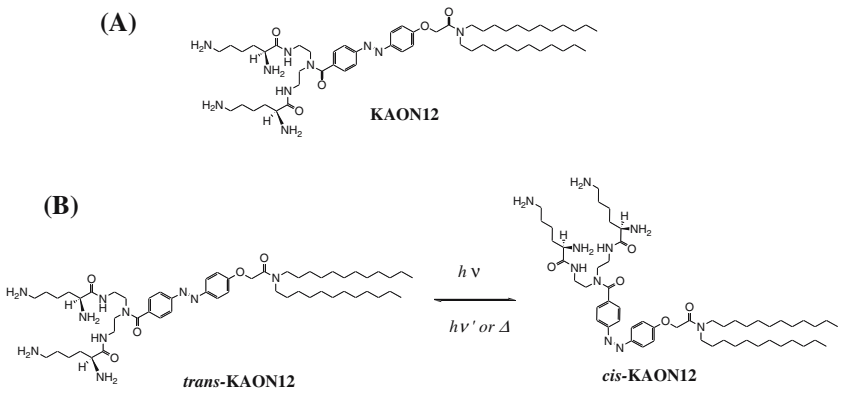


Fig. 9 (**A**) Chemical structures of azobenzene-based photo-responsive lipid (**KAON12**). (**B**) photo-isomerization of **KAON12**

trans-azobenzene, small unilamellar vesicles (SUVs) with a diameter of approximately 20 nm were observed (Fig. 10A). In contrast, with UV (365 nm) irradiation, the azobenzene structure underwent *trans*-to-*cis* isomerization, and the lamellar structure other than SUVs appeared (Fig. 10B), thus suggesting that the membrane fusion between vesicles occurred. The increase in the average diameter of self-assembling vesicles due to destabilization of photoresponsive membrane was confirmed with dynamic light scattering: it increased from 28.8 nm (all *trans*-form) to 209 nm (*cis/trans* =57/43 as photostationary state). When **KAON12** formed cell sized giant vesicles, the effect of photo-isomerization of azobenzene on the fluctuation of vesicle membrane was observed by phase-contrast images with microscopy (Fig. 11A) [66].

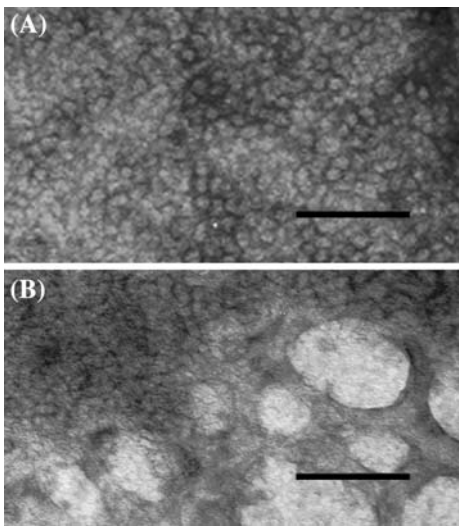


Fig. 10 Transmission electron micrograph of **KAON12** membrane before (**A**) and after (**B**) UV irradiation (365 nm, 3.5 $mWcm^{-2}$) for 5 min. The membrane is negatively stained with uranyl acetate. Each scale bar indicates 100 nm. Reprinted by permission from ref. [65] (Copyright© Chemical Society of Japan 2003)

The difference in the fluctuation behavior between *trans*- or *cis*-azobenzene isomers has been estimated with changes in the radius and its distribution (Fig. 11C). As shown in Fig. 11B, the radius along the contour r (θ) and the angle θ were determined by microscopic observation. Much greater fluctuation was confirmed after UV irradiation than that after visible light irradiation, indicating that a membrane consisting of *cis*-isomer exhibits surplus membrane area to encapsulate the inner aqueous solution. On the contrary, the small fluctuation on the periphery of a membrane consisting of *trans*-isomer suggests that the membrane area just fits the spherical surface of the inner liquid phase. Such a fluctuation behavior of giant vesicle membrane composed of photoresponsive lipids agrees with the results of the membrane fusion of SUVs (Fig. 10B). In a transfection experiment using the cationic lipid, all procedures were carried out in the dark. The transfection efficiencies were shown in Fig. 12 where Lipofectin, a commercially available cationic lipid gene carrier, was used for comparison. Even without UV irradiation, the transfection efficiency of **KAON12** was 2-fold higher than that of Lipofectin, and UV irradiation further improved the transfection efficiency of **KAON12**. After **KAON12**/DNA complex passes through the plasma membrane by endocytosis, *trans*-to-*cis* isomerization of azobenzene moiety would destabilize the vesicle membrane, thus accelerating membrane fusion of photo-responsive lipid vesicles (Fig. 13). This phenomenon facilitates the escape of plasmid DNA from endocytic vesicles, thus improving transfection efficiency with UV irradiation.

Photoresponsive gene delivery system with photo-cleavable moiety

Another approach for photo-enhancement of transfection efficiency by using cationic lipids was achieved by the introduction of a photo-cleavable bond into the

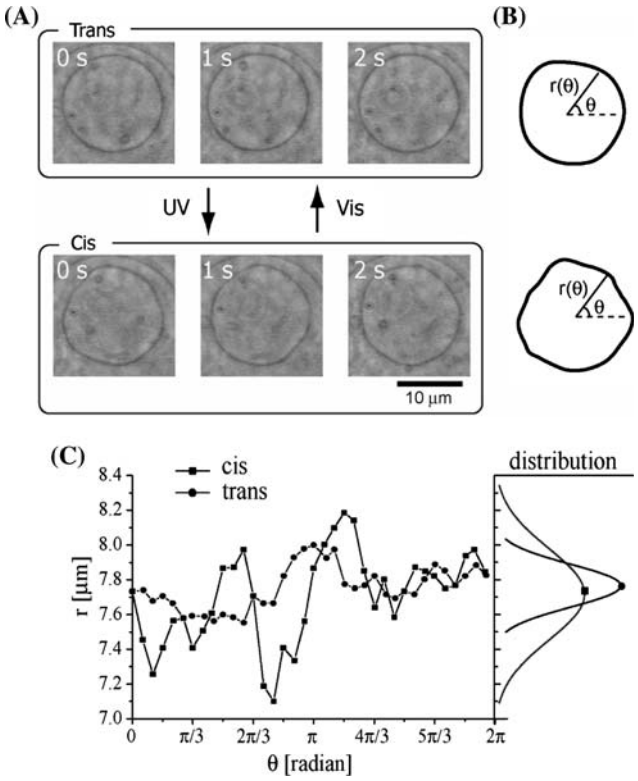


Fig. 11 (**A**) Phase-contrast images of the difference in the fluctuation behavior between *trans*- and *cis*-**KAON12** vesicles at 1 s intervals. Cell-sized vesicles were prepared from dioleylphosphatidylcholine (DOPC) and KAON12 through natural swelling where the lipid film was swollen with distilled water ([KAON12]/[DOPC] = 60 μM/100 μM). Ten microliters of the vesicle solution was placed on glass slip, covered with another small slip at a spacing of 50 μm and sealed. The vesicle morphology was observed with phase-contrast microscope (Nikon TE-300, Japan) and irradiated them through a dichroic unit, UV (365 nm) and green (546 nm), with an extra-high-pressure mercury lamp (100 W) for photoisomerization. The images were recorded on digital videotape at 30 frames/s. (**B**) Schematic representation of the difference in membrane fluctuation. (**C**) Spatial fluctuation in the radius around the counter together with its distribution profile. Reprinted by permission from ref. [66] (Copyright© American Chemical Society 2005)

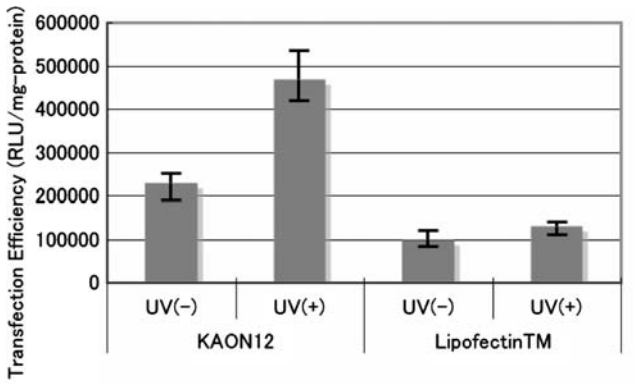


Fig. 12 Transfection Efficiency of DNA Complexes with cationic lipids. COS-1 cells were seeded at a density of 5×10^4 cells/well in 1 mL of the growth medium in 24-well plates and incubated for 20 h prior to transfection. DNA complex was formed using 1 mM *trans*-**KAON12** dispersion (7.7 μL) and plasmid DNA (pGL3-control, 1 μg). At the time of transfection, the culture medium was replaced with 200 μL/well of the fresh medium and 50 μL of complex solution was added into each well. After 3 h of an exposure time the medium containing the complexes was removed and 1 mL/well of fresh medium was supplied. The cells were further incubated for 48 h and the luciferase activity in cells was measured by Steady-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions using a Fluoroskan Ascent FL microplate luminometer (Thermo Labsystems). In order to investigate the effects of photo irradiation on transfection, after allowing cells to come in contact with complexes for three hours, UV (365 nm, 3.5 $mWcm^{-2}$) was irradiated for zero or ten minutes, and the results were compared. The measurement of transfection efficiencies was performed in triplicate

lipid. As a photoresponsive gene carrier, new cationic lipids having an *o*-nitrobenzyl moiety as a photo-cleavable spacer between its hydrophilic and hydrophobic region were synthesized and evaluated [67] (Fig. 14). To estimate whether cationic, photo-cleavable lipids can protect DNA from nuclease, the protection ability and its photo-regulation with **KNBN12** and **RNB12** were investigated (Fig. 15). These lipids were dissolved in chloroform/methanol (50/50) solution, and through the use of an evaporator, a thin membrane was prepared along the wall of a glass tube. Then, through the use of a voltex, the thin membrane was subjected to ultrasound at 50 °C and dispersed in Tris buffer (pH 7.5) to attempt lipid assembly. This lipid assembly was utilized to make the DNA complexes (lipoplexes). After the DNase I treatments of DNA lipoplex of **KNBN12** and **RNB12** without UV irradiation (lanes 4 and 6), the intact supercoiled plasmid DNA bands were still appeared, although some nicking and decomposition of plasmid DNA occurred under recovering conditions with SDS (lane 7). In contrast to case without UV irradiation, intact bands completely disappeared with UV irradiation, although degradation was not completed (lanes 3 and 5). Both **KNBN12** and **RNB12** exhibited high DNA protection, but 5 min UV irradiation (365 nm, 3.5 $mWcm^{-2}$) decreased this ability. These results demonstrate that photo-irradiation lowers the affinity of cationic lipids towards plasmid DNA and makes it easier for the nuclease to access to DNA. The hypothesis proposed by Szoka et al. [68] suggests that dissociation of internalized DNA from cationic lipid carriers is an important process for successful nuclear import and following transcription of a transgene. In the case of such photo-cleavable lipids, not only the photo-enhanced DNA release but also photo-stimulated endosomal escape could contribute to the

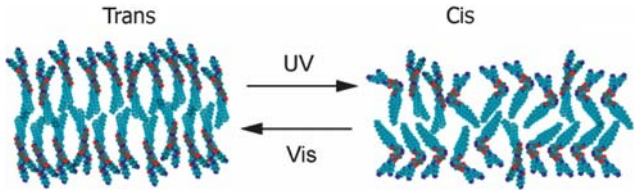


Fig. 13 Schematic representation of membrane destabilization by photo-isomerization of azobenzene

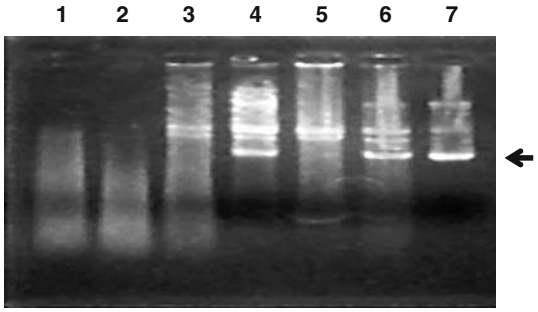


Fig. 15 Complexes that protect plasmid DNA (pGL3-control) against DNase I digestion. Lane 1, DNA (UV+); Lane 2, DNA (UV–); Lane 3, DNA recovered from **KNBN12** complex (UV+); Lane 4, DNA recovered from **KNBN12** complex (UV–); Lane 5, DNA recovered from **RNBN12** complex (UV+); Lane 6, DNA recovered from **RNBN12** complex (UV–); Lane 7, undigested DNA. Band marked by arrow means intact super coiled plasmid. Reprinted by permission from ref. [67] (Copyright© American Chemical Society 2005)

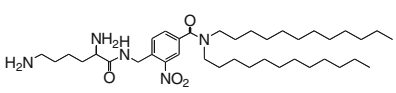
enhancement of transfection efficiency. Actually, when COS-1 cells were transfected with FITC-labeled plasmid DNA complex, UV irradiation was performed to estimate the effect on the intracellular distribution of transgene by the confocal fluorescence microscopy (Fig. 16). At 3 h post-transfection, before UV irradiation weak green fluorescence (FITC-labeled plasmid DNA) was observed as dotted vesicles at same site for red fluorescence (rhodamine-labeled phospholipid) indicating endocytic vesicles in the cytoplasm (Fig. 16A). On the other hand, after UV irradiation strong green fluorescence had exuded into cytoplasm from dotted vesicles, thus facilitating the endosomal escape of FITC-labeled DNA (Fig. 16B). In the endocytic vesicles, since FITC-labeled DNA still complexes with the lysine-attached lipid carriers, fluorescence of FITC can be quenched by electron transfer from amino groups of lysine residues. Therefore, weak fluorescence of FITC-labeled DNA was intensified after UV-stimulated endosomal escape (Fig. 17). Transfection was carried out for 3 h using luciferase-coding plasmid DNA, and the activity of reporter protein, luciferase, in cell lysate was measured 48 h later to assess transfection efficiency (Fig. 18). To investigate the effects of photo-irradiation on transfection, after allowing cells to come in contact with lipoplexes for 3 h, UV irradiation (365 nm, 3.5 $mWcm^{-2}$) for 0 min or 10 min was carried out. Compared with Lipofectin, the transfection efficiencies of **KNBN12** and **RNBN12** were 13- and 3-fold greater without UV irradiation and more than 19- and 10-fold greater with UV irradiation, respectively. Also, since the UV irradiation did not influence the transfection efficiency of **KBN12** (an analogue without a photo-cleavable structure), the decomposition of lipid carriers must be involved in the improved transfection process. Lower activity of **RNBN12** with respect to **KNBN12** was attributed to less DNA release due to the higher basicity of **RNBN12**. Whatever, a new transfection technique using cationic lipids having a photo-cleavable spacer was demonstrated to become a useful tool for not only a gene but also a drug delivery system.

Other stimuli-responsive vectors

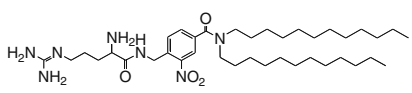
Redox-responsive vectors

A disulfide bond is a covalent linkage that is obtained from the oxidation of two sulfhydryl groups. Two advantages that render the disulfide bond attractive in gene delivery systems are their reversibility and their relative stability in extracellular environment. There is the high gradient of redox potential between intracellular and extracellular space. Such environment is attributed to the difference of the concentration of glutathione (GSH: L-γ-glutamyl-L-cysteinyl-glycine),

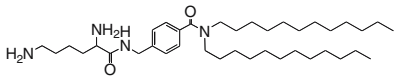
Fig. 14 Chemical structures of photo-cleavable lipids for gene carriers and their reference compound



KNBN12



RNBN12



KBN12

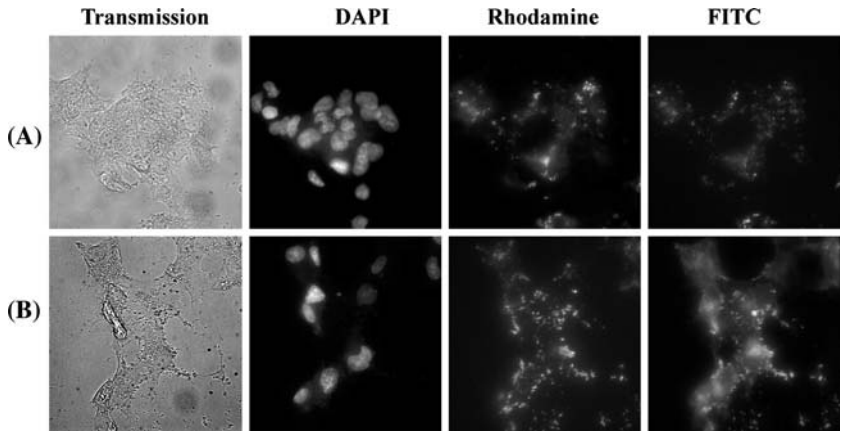


Fig. 16 Microscopic images showing COS-1 cells incubated with a complex of FITC-labeled pGL3-control DNA and rhodamine-PE-labeled KNBN12 aggregate for 3 h at 37 °C. The cationic KNBN12 aggregate was labeled using 1 mol% fluorescent rhodamine-modified phosphatidylethanolamine (rhodamine-PE). Lipid/DNA (1 mg of FITC-labeled pGL3-control) complex was prepared at charge ratio of 5. On glass-bottom dish, COS-1 cells (1×10^4 cells) were transfected with the lipid/DNA complex for 3 h. Nuclei are post-stained with DAPI. Intracellular trafficking of fluorescently labeled plasmid DNA (FITC) and carrier lipid (rhodamine) were fixed before (**A**) and after (**B**) UV irradiation (3.5 mWcm^{-2}, 10 min)

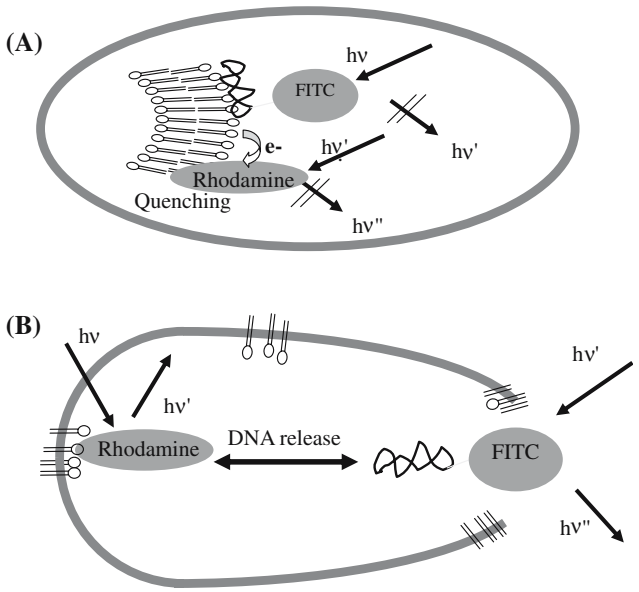


Fig. 17 Schematic representation of fluorescence phenomena of endocytic internalized lipoplexes before (**A**) and after (**B**) photo-induced endosomal escape. (**A**) In the endocytic vesicles, fluorescence of FITC is quenched by electron transfer from amino groups of lysine residues since FITC-labeled DNA still complexes with the lysine-attached lipid carriers. (**B**) After destabilization of photo-responsive membrane and following DNA release from the lipoplex, FITC-labeled DNA breaks out into cytoplasm. Then, fluorescent FITC is allowed by the dissociation of FITC-labeled DNA from cationic lipid carrier

the most abundant non-protein thiol source and reductant in mammalian cells. The concentration of GSH in the intravascular space is about 10 μM and that of GSH within the cell is millimolar level [69]. The intracellular reduction of disulfide bonds in cationic non-viral vector could lead not only the decrease of DNA affinity but also the release of DNA. Therefore, non-viral vectors containing disulfide bond can act as redox-resposive vectors.

In fact, the reversible disulfide cross-linkers are concerned in self-assembly and disorganization of viruses [70], and this strategy can be widely exploited in controlled release of DNA in the target cells. Various lipids, polymers, and peptides were introduced sulfhydryl groups using different linkers and the supramolecular assemblies with plasmid DNA to attain DNA release and higher transfection efficiencies [71–83].

pH-responsive vectors

Since endosomal escape is critical for many biomolecular drugs, in order to avoid degradation by lysosomal enzymes, delivery systems responding to gradient of pH have been exploited widely not only for drug delivery but also for gene delivery. pH gradients exist among various cell types, organs, tissues, and intracellular organelles. Decrease of pH is related to many physiological and pathological situations such as endosome processing, tumor growth, and inflammation [84, 85]. Intracellular pathway contains non-specific endocytosis, phagocytosis, pinocytosis, or receptor-mediated endocytosis. Generally, non-viral gene delivery systems transfer across the plasma membrane by endocytosis and were delivered to lysosomes for degradation. The pH decrease of the endosomes from cell uptake to lysosomes is 1–2 pH units and the time course is about 10–30 min [86]. Therefore, this strategy requires quick response to endogenous stimulation and

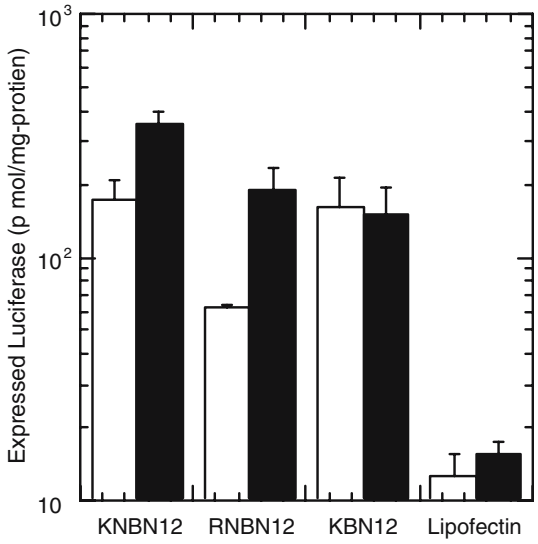


Fig. 18 Transfection Efficiency of DNA Complexes with cationic lipids. Open bars indicate transfection efficiency in the dark conditions. Closed bars indicate that under UV irradiation. DNA complex was formed using 1 mM lipid dispersion (7.7 μL) and pGL3-control (1 μg). After that, transfection was carried out for three hours using COS-1 cells in a 24-well plate, and the activity of luciferase in cell lysate was measured 48 hours later to assess transfection efficiency. Relative light units were measured by using Fluoroskan Acent FL luminometer (Thermo Labsystems). The protein concentrations of the cell lysates were measured as described in the protocol of NanoOrange Protein Quantitation Kit using bovine serum albumin as a standard. The expressed luciferase is standardized for total protein content of the cell lysate. In order to investigate the effects of photo irradiation on transfection, after allowing cells to come in contact with complexes for three hours, UV (365 nm, 3.5 $mWcm^{-2}$) was irradiated for zero or ten minutes, and the results were compared. The measurement of transfection efficiencies was performed in triplicate. Reprinted by permission from ref. [67] (Copyright© American Chemical Society 2005)

release of DNA prior to trafficking into the lysosomal compartments. Especially, pH of endosomes among inflammatory sites and solid tumors is only 0.4–0.8 units more acidic than that of plasma. Delivery system for these target tissues requires to response to such small stimulation. pH-responsive vectors are divided into a few types, such as vectors with buffering effect, vectors exploiting membrane-destabilization peptide, vector exploiting pH-sensitive polymer, and vector exploiting pH-sensitive linkage.

PEI, polyamidoamine dendrimers, and imidazole containing polycations to exhibit buffering effects in the endosomal compartment are known as vectors exploiting pH gradients. These polymers are believed to have the proton sponge effect to escape from the endosomes according to the hypothesis [56, 87–89]. The effect occurs with the following mechanism. (i) After cell uptake, pH of the endosome decreases. (ii) Protonation of these polymers causes increased influx of protons and counter-ions into endocytic vesicles. (iii) Increasing osmotic pressure causes collapse of the endosome [89]. These polymers are used widely as transfection agents for the study of gene therapy.

Another type of pH-sensitive vectors are carriers with membrane destabilization peptide such as GALA [55, 90–92], hemagglutinin [93–95] and others [96, 97]. For example, GALA (WEAALAEALAEALAEALEALAALAAGGSC) is an acidic peptide of 30 amino acids. Conformation of this peptide changes from random coil to hydrophobic amphiphilic α-helical structures under the acidic conditions. Because protonation of the glutamic acids decreases water-solubility, this peptide exercises membrane destabilization activities. GALA improved transfection activity and was used widely in drug delivery and gene delivery.

Ultrasound-responsive vectors

Ultrasound that has been widely used for diagnostic imaging applications [98, 99] is also now being adopted in various drug delivery and therapeutic applications. Recently, ultrasound-facilitated gene delivery has been numerously reported for not only in vitro but also in vivo [100–108]. Maruyama et al. [109] developed novel polyethyleneglycol (PEG) modified liposomes (Bubble liposomes) containing perfluoropropane, which is an ultrasound imaging gas. Ultrasound induced cavitation in Bubble liposomes and enhaced gene delivery. Bubble liposomes could deliver plasmid DNA to many cell types without cytotoxicity. Additionally, in vivo gene delivery, Bubble liposomes were more effective delivery into femoral artery than lipofection method.

Thermo-responsive vectors

Another potent non-viral vector bearing a stimulus-response is temperature-responsive vector based on the polymer carrier exploiting temperature-induced phase transition [110–117]. Temperature is one of the safest external stimulus and controllable temporally and spatially. Hyperthermia therapy against solid tumors has been widely carried out to heat the target site with microwave [118]. On the other hand, poly (*N*-isopropylacrylamide) (pNIPAM) is a polymer that undergoes a coil-to-globule phase transition at 32 °C. Such a transition is rapid, highly non-linear and reversible in response to small changes of temperature, which makes this signal responsive system very promising in design of drug delivery systems. The phase transition temperature of pNIPAM is a lower critical solution temperature (LCST). Below the LCST,

pNIPAM is water-soluble, while above the LCST, it undergoes a reversible phase transition to form an insoluble and hydrophobic aggregate. Incorporation of pNIPAM into cross-linked polymer generates a matrix that can exhibit thermally reversible shrinkage or collapse above the LCST.

Intracellular signal-responsive vectors

Recently, many biodegradable gene delivery systems were reported [119–130]. Especially, Katayama et al. [131–138] reported a novel strategy that can discriminate normal and target cells in gene delivery by focusing on differences of in intracellular signals. A drug capsule and a gene carrier system are designed so that they can respond to certain intracellular kinase or protease, respectively. For example, the cationic polymer (PAK) was a graft-type polymer with an oligopeptide that is a substrate for cyclic AMP-dependent protein kinase (PKA) and could regulate gene-expression in a cell-free system. In the unstimulated NIH 3T3 cells, transfection of the PAK–DNA complex showed no expression of the delivered gene. This indicated that PAK formed a stable complex with DNA in the normal cells to totally suppress gene expression. On the other hand, significant expression was observed when the PAK–DNA complex was delivered to forskolin-treated cells. Thus, activated PKA disintegrated the complexes even in living cells, resulting in efficient gene expression.

Conclusions

The effective gene delivery system is an essential element for successful achievement of not only the gene therapy but also exhaustive analyses of genes at post-genome era. The non-viral vector has to act as a molecular machinery like a virus infecting into host cells and amplifying efficiently in order to overcome extracellular and intracellular barriers and has to lead a smooth mRNA transcription at nuclei as a final destination. In this review, stimulus responsive gene delivery systems in which some structural factors and/or physiological properties are regulated responding extracellular signals such as light, redox, pH, ultrasound, temperature, etc. are introduced as a new generation of non-viral vectors. Among these extracellular signals, light is the most potent stimulus capable of site-, timing-, and duration-specific gene expression. On the other hand, intracellular signals such as redox and pH allow cells to express exogenous genes on intact conditions. More remarkable intracellular signals other than redox and pH fluctuations are endogenous proteins.

Recent progress on development of artificial multi-component vectors aimed at providing solutions to membrane crossing, endosomal escape and navigation through the nuclear pore [139–144]. The combination of these nature-inspired and chemically originated approaches is bringing us continually closer to the concept of constructing an artificial virus capable of delivering viable nucleic acid-based pharmaceuticals to defined cells as molecular machines that can work even in vivo.

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