Programmed Packaging for gene delivery

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Abstract

We report on the development of a Multifunctional Envelope-type Nano Device (MEND) based on our packaging concept “Programmed Packaging” to control not only intracellular trafficking but also the biodistribution of encapsulated compounds such as nucleic acids/proteins/peptides. Our strategy for achieving this is based on molecular mechanisms of cell biology such as endocytosis, vesicular trafficking, etc. In this review, we summarize the concept of programmed packaging and discuss some of our recent successful examples of using MENDs. Systematic evolution of ligands by exponential enrichment (SELEX) was applied as a new methodology for identifying a new ligand toward cell or mitochondria. The delivery of siRNA to tumors and the tumor vasculature was achieved using pH sensitive lipid (YSK05), which was newly designed and optimized under in vivo conditions. The efficient delivery of pDNA to immune cells such as dendritic cells has also been developed using the KALA ligand, which can be a breakthrough technology for DNA vaccine. Finally, ss-cleavable and pH-activated lipid-like surfactant (ssPalm) which is a lipid like material with pH-activatable and SS-cleavable properties is also introduced as a proof of our concept.

1 Introduction

In the 21st century, a paradigm-shift emerged in the field of drug discovery and development and the field of Nanomedicine is anticipated to have a major impact on addressing unmet medical needs such as cancer, central nervous diseases and immunological diseases, where the currently used low molecular weight molecules do not function. Antibody based therapy is a rapidly emerging area and promised to have significant impact as a Bio-medicine, mainly because they are so selective. However, antibodies typically function in the blood circulation and extracellular space. They cannot enter into cells to treat diseases. Nanomedicines are expected to be a breakthrough technology for delivering new medicines such as nucleic acids as well as proteins to the site of action inside a cell. Therefore it is important to control not only the biodistribution of new drugs but also the intracellular trafficking of these coumpounds, such as endosomal escape (in the case of siRNA, protein), transport in the cytosol, nuclear entry (in case of pDNA). To achieve this goal, sophisticated strategies will be needed to successfully develop such systems.

We have developed a multifunctional envelope-type nano device (MEND) to control intracellular trafficking as well as the biodistribution of nucleic acids/proteins/peptides based on a strategy called “Programmed Packaging” where 1) a program is designed to deliver drugs based on molecular mechanisms of transport in our body, 2) nano devices are designed to overcome each barrier, 3) these materials are assembled into nano-sized 3 dimensional structures so that they can exert their ability according to the program [1]. The MEND was developed originally to deliver pDNA into target cells such as tumor cells and immune cells by mimicking an envelope-type virus and its purpose was extended not only pDNA but also siRNA where the site of action is the cytosol. While it is difficult to achieve the efficiency of viral vectors even for the system used in in vitro conditions, we have been able to achieve a level that is equivalent to that of a viral vector when we transfect pDNA into immune cells such as a dendritic cells to develop a DNA vaccine. It has also been possible to develop a system which can deliver siRNA to tumor cells or tumor endothelial cells via an intravenous administration which requires stable blood circulation and efficient intracellular trafficking [2]. Recently developed breakthrough technologies in the area of tumor therapy based on active targeting using selective ligands are the focus in this review.

2 Original ligands for drug delivery system via active targeting strategies

2.1 Benefits of active targeting on drug delivery system (DDS)

Development of novel drug delivery system which can control and release of drugs are important issues on the field of medicine. Good drug delivery system can increase the efficacy of drugs and also reduce side effects. For this purpose, researchers tried to use nanoparticles as DDS and many findings were explored and nanoparticulate drug delivery systems were developed. In this situation, the most important discovery was the discovery of the enhanced permeability and retention (EPR) effect [3]. Tumor vascular is leaky due to angiogenesis on tumor tissue therefore nanoparticles, with sizes of around 100 nm can pass through tumor blood vessels. Generally, the duration of nanoparticles in the circulation is increased, the accumulation of nanoparticles are also increased. This approach takes advantage of the phenomenon of a tumor in a clever manner and is referred to as passive targeting. On the other hand, active targeting involves sending nanoparticles via specific devices that can bind to the targeted receptor. Small molecules, peptides, proteins and antibodies were used as the targeting ligands and can include folate, transferin etc. and have succeeded in enhancing the efficacy of DDS. Some nanoparticles using active targeting strategy have proceeded to phase I/II clinical trials and have shown positive results [4]. The active targeting strategy can increase the accumulation of drugs in the desired tissue and reduce side effects. Moreover, active targeted nanoparticles can escape multidrug resistance because efflux pumps are not able to remove nanoparticles that enter via receptor mediated endocytosis and such benefits have prompted researchers to investigate active targeting [5].

2.2 Nucleic acid aptamers and the choice of target molecules

Aptamers are molecules recently focused on the fields of biosensors, diagnoses and ligands. Nucleic acid aptamers are small ssDNA and RNA molecules, having specific 3-dimensional structures. They can bind to the target molecule via an induced-fit mechanism and show strong affinities and high selectivity. Aptamers can be considered to be a type of antibody but aptamers have some advantages compared to antibodies such as ease of chemical modification to enhance some functions, such as conferring stability toward nucleases, relatively low immunogenicity, small size and heat stability.

Under such circumstances, many researchers are now attempting to identify such types of aptamers [6]. The procedure of the systematic evolution of ligands by exponential enrichment (SELEX) was independently investigated by two groups in 1990 [7,8]. Nucleic acids having random sequences were mixed with the target molecules and the bound nucleic acids were then collected. These nucleic acids were amplified and used for the next selection. After several rounds of selection, the nucleic acids are enriched and aptamers can be isolated. The choice of the target molecule is important in identifying and isolating an acceptable aptamer. When we attempt to use aptamers as ligands, we need to choose a suitable target molecule that is specifically expressed in large amounts on the target cells. For DDS purposes, the target molecule is located on the surface of cells or organelles and, therefore, can be a membrane associated protein. Sometimes it is difficult to mimic the higher order structure of a target protein only facing the outer membrane using a recombinant protein and apply regular SELEX. In contrast, when target cells are used as the target, these problems can be overcome. Actually, cell-based SELEX was explored in 2006 by Tan's group [9]. Cell-based SELEX have already shown several benefits such as the isolation of a new ligand along with a new receptor which is guaranteed to exist on the surface of a membrane as the natural higher order structure. Therefore we attempted to isolate a ligand that is specific for tumor endothelial cells and certain types of organelles.

2.3 Selection of aptamers for tumor endothelial cells

Angiogenesis-dependent tumor growth was first reported by Folkman in 1971 [10]. Tumor blood vessels provide nutrients and oxygen, and remove waste from the tumor tissue, resulting in tumor progression and are different from their normal counterparts in that they show leakiness and the thickness of the basement membrane is uneven. This suggests that tumor endothelial cells (TECs) may well express surface markers that are different from those found in normal cells. Tumor blood vessels contain tumor endothelial cells that might be genetically normal and stable, even though these endothelial cells are structurally and functionally abnormal. Preventing or inhibiting angiogenesis, which is associated with tumor progression and metastasis, is a challenging issue in combating cancer. Since tumor growth is dependent, to some extent, on the development of a neo-vascular supply, inhibiting angiogenesis by targeting tumor endothelial cells represents the ultimate goal in cancer therapy. Therefore a ligand for

targeting tumor endothelial cells would represent an ideal candidate for anti-angiogenesis therapy (**Figure 1A**). First, a library for the selection was chemically synthesized via the phosphoramidite method. The resulting library had a 40mer random sequence flanked by 21mer sequences for forward and reverse primers which can be amplified by PCR to regenerate the recovered library. The sequence was (5'-CGTAGAATTCATGAGGACGTT-N40- AGCTAAGCTTACCAGTGCGAT-3'). This ssDNA library was mixed with primary cultured mouse TECs, the unbound sequences removed and the bound ssDNAs collected. Trypsin which can be used to detach cells, can be used to remove the surface proteins on TEC and this was not desirable in the case of cell-based SELEX. We introduced temperature-sensitive cell culture dishes, referred to as RepCells, in order to overcome this problem. This permits cells to be detached on cooling and the surface proteins can be kept intact and this method allows for a greater recovered DNA libraries to be produced. The library was amplified by conventional PCR and proceeded to the next round of selection. Amplifying the random sequence by PCR was sometimes a problem, because the random sequence functions as a primer and an unexpected sequence can be produced. It is known that too much template can cause unexpected amplification, therefore the amount of produced library in each PCR cycle must be checked. The PCR amplified library was double stranded DNA but single stranded DNA is needed for the selection and the asymmetric PCR method was employed to generate ssDNA. Asymmetric PCR (also called Linear After The Exponential PCR (LATE PCR)) was conceived by Gyllensten [11]. This method contains two sequential PCR procedures. The first PCR was regular PCR and the second PCR was carried out without a reverse primer to produce only the desired strand. Counter selection was also applied with Skin-EC as the normal endothelial cell model and OS-RC-II as the tumor parenchymal cell model. The affinity of each cycle of the library was checked in FACS experiments and the entire SELEX procedure was finished when the binding affinity of the library reached a sufficient level.

2.4 Identification of aptamers for tumor endothelial cells

After 12 rounds of selection, a significant shift in fluorescent intensity was found on FACS. This indicates that the DNA library was enriched to a sufficient level. The DNA library was cloned and sequenced to determine the DNA sequence and 48

sequences were identified as aptamer candidates. The binding affinities of these aptamers were investigated by flow cytometry and an aptamer that can bind strongly to mTEC but not Skin-EC and OS-RC-II cells was identified. We refer to this aptamer as AraHH001. Mean fluorescent intensities were measured for various concentrations of the aptamer and the dissociation constant of this aptamer was determined to be 43.8±13.7 nM (**Figure 1B**). The findings indicate that this aptamer had a strong binding affinity and acceptable selectivity [12].

2.5 Organelle-based SELEX for mitochondria

After mitochondria were incorporated into eukaryotic cells, eukaryotic cells became dependent on the many functions on mitochondria and a symbiotic relationship eventually developed. Mitochondria host the citric acid cycle and oxidative phosphorylation and can supply energy to eukaryotic cells. Mitochondria have their own DNA called mitochondrial DNA (mtDNA) and prepare some proteins by transcribing and translating their DNA, thus mitochondria have their unique functions and machinery fir sustaining life. Therefore, the mutation of mtDNA or the down-regulation of protein expression can result in a loss of mitochondrial function, thus resulting in the development of illnesses called mitochodorial diseases, neurodegenerative diseases, type 2 diabetes etc. Investigations of drug delivery systems (DDS) that are specific to mitochondria is an important issue in terms of overcoming these diseases [13], therefore we investigated the use of aptamers that target mitochondria.

For the selection of mitochondria targeting aptamers, the same method can be applied for the selection of organelle targeting aptamers as shown in section 2.3 (**Figure 1A**). The isolation of mitochondria is an important step in this experiment because if the surface structure of the mitochondria is damaged, this mitochondria-based SELEX becomes meaningless. We isolated mitochondria from rat liver following a reported procedure and estimated that the mitochondria were intact based on the respiratory control index (RCI) which showed values higher than 4 [14]. We then prepared an RNA library. When we attempted to use aptamers as ligands, the stability of the apatmer is important because there are several nucleases and phosphodiesterases in the cytosol. Non-modified RNAs are usually very easily digested by nucleases and we employed 2'-F RNA which is used for aptamer selection and is generally considered to be more

stable to nuclease.

After 7 rounds of selection, we were able to isolate the sequences that canbind to isolated mitochondria. We referred to these as mitomer1 and mitomer2. We checked the binding affinity of each of these toward isolated mitochondria. 6-carboxyfluorescein-modified mitomers were incubated with isolated mitochondria, and bound RNAs were collected and their fluorescence intensity measured. As a result, mitomer2 showed a higher binding affinity compared to mitomer1. We analysed the secondary structure of mitomer2 and the findings indicate that it contains a one stem-loop structure with a flanking single strand region. The secondary structure of RNA contributes binding capacity to the target and the removal of non-essential nucleotides increases the binding affinity of an aptamer. We designed a short-mitomer2 which contains only the stem-loop structure of mitomer2 and checked its affinity. The short-mitomer2 showed a significantly higher affinity than mitomer2 and a random sequence (**Figure 1C**). It was close to the same level as the D-arm which is a special sequence contained in *Leishmania* tRNA which a well known targeting device to mitochondria. We proved that the stem-loop structure of mitomer2 is essential for binding to mitochondria.

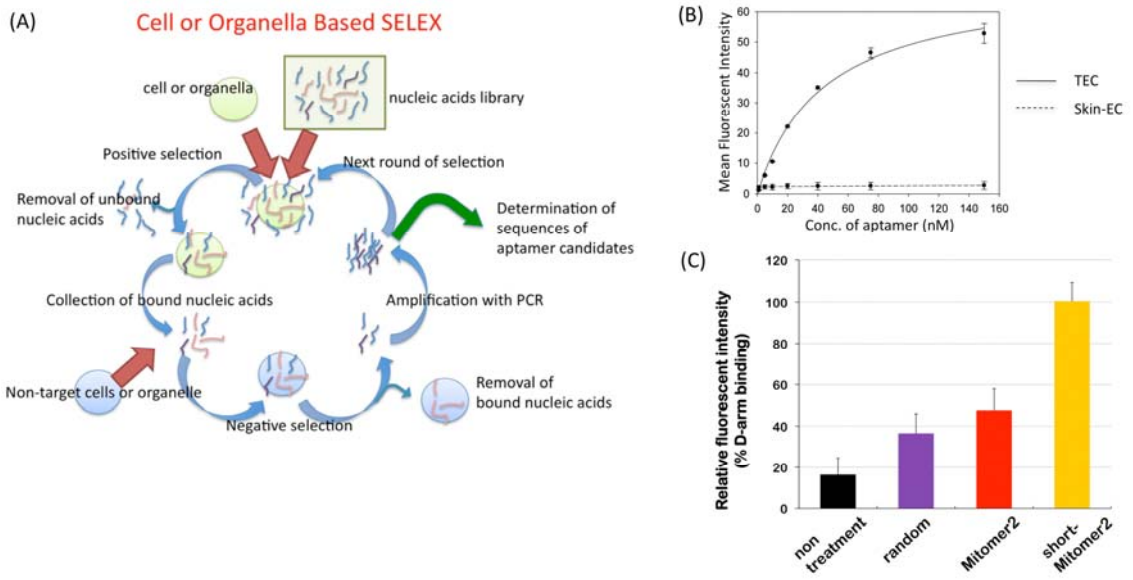

Figure 1. (A) Schematic representation of cell and organelle-based SELEX. (B) Dissociation constant of the AraHH001 aptamer toward TECs and Skin-ECs. (C) Binding affinities of Mitomers toward isolated mitochondria.

3 siRNA delivery to tumors and the tumor vasculature using a pH-sensitive lipid

Since it was reported that RNA interference (RNAi) can be induced without severe toxicity in mammalian cells using synthetic double-stranded RNA [15], a great effort has been made to develop an RNAi medicine that is directed against many diseases such as cancer and viral infections [16-18]. RNAi is an intrinsic pathway that is highly conserved among most eukaryotes, and is induced by endogenous microRNA (miRNA) and artificial short interfering RNA (siRNA) [19]. Above all, synthetic siRNA is one of the most attractive species for creating a new class of medicines. Either strand of double-stranded siRNA is assembled into an RNA-induced silencing complex (RISC), and the incorporated single-stranded siRNA (guide strand) subsequently cleaves the target mRNA whose sequence is complementary to RISC-loading siRNA strand via an activity of Argonaute 2, while the other siRNA strand (passenger strand) is degraded. Based on this proposed model, siRNA would be expected to knock down any gene in principle with a high efficiency and sequence-dependency. This fact furnishes us with a new strategy for using siRNA in the treatment of a wide variety of diseases, in which a responsible gene has been investigated.

To silence a gene of interest by RNAi, the siRNA must be delivered to the inside of the target cell, where the RNAi-associated machinery is located. However, siRNA itself cannot diffuse across the plasma membrane due to its high molecular weight and hydrophilicity, and intravenously administered siRNA is subject to rapid renal clearance and enzymatic degradation by ribonucleases. Therefore, a delivery vehicle or chemical modification is needed to stabilize the siRNA cargo in the bloodstream, deliver siRNA to the target cell in a target organ, circumvent endo/lysosomes degradation and increase cellular uptake [20, 21]. A large variety of delivery materials, including polymers [22], liposomes [23-25], micelles [26], peptides [27] and proteins [28], are currently under development, in attempts to overcome these obstacles associated with *in vivo* siRNA delivery.

To achieve an efficient siRNA delivery system, we focused on a pH-sensitive lipid. pH-sensitivity has several advantages in *in vivo* siRNA delivery [21]; in that serum protein absorption in the circulation is avoided, it is positively charged specifically in acidic endosomes, and therefore would be less toxic [21]. Because of these virtues associated with pH-sensitive carriers, several groups have reported on some original pH

sensitive systems [29-32]. To date, one of the most effective systems is lipid nano particles (LNPs), which consist of a hydrophilic moiety containing a tertiary amino group, a linker and two hydrophobic dialkene groups [33]. They suggested that liposomal carriers become internalized into the cytosol of target cells from endosomes via membrane fusion by the transition of an inverse hexagonal (H_{II}) phase structure [33, 34]. We previously reported that the membrane fusogenic peptide, GALA, was useful for siRNA delivery [35-37]. Since GALA contains Glu-Ala-Leu-Ala repeats in its sequence, the structure changes from a random coil to an alpha helix through protonation of the glutamine residues in response to acidification in early endosomes. To achieve a more effective knockdown, we designed YSK05 with the intention of emphasizing the cone shape of the cationic lipid (IUPAC name of YSK05; 1-methyl-4,4-bis[9Z,12Z]-octadeca-9,12-dien-1-yloxy) piperidine). As shown in **Figure 2A**, the hydrophobic moiety of YSK05 was directly conjugated with a piperidine linker via an acetal bond, which resulted in a 109.5° angle between the two hydrophobic moieties. Since the cone shape tends to be the H_{II} phase (**Figure 2B**), the MEND composed of YSK05 (YSK05-MEND) exhibited a highly fusogenic activity specifically at an acidic pH (pH 5.5), and therefore would beexpected to achieve the efficient cytosolic delivery of siRNA (**Figure 2C**). Actually, the YSK05-MEND showed a significant gene silencing compared with a commercially available transfection reagent, as evidenced by a dual-luciferase reporter assay. We developed a tumor-targeting siRNA delivery system via the enhanced permeability and retention (EPR) effect, which involves the passive accumulation of a long-circulating macromolecule in tumor tissue [38]. We reported that the PEGylated YSK05-MEND retained encapsulated siRNA intact in the circulation and PEGylated YSK05-MEND suppressed a cancer-related gene via RNAi-mediated silencing using 5’ rapid amplification and cDNA ends (5’-RACE)-PCR [39]. Likewise, the liver can be targeted by passive accumulation because neutral liposomes can accumulate in the liver through the fenestrae of liver endothelial cells and can be internalized into hepatocytes via a low density lipoprotein receptor through absorption of apolipoprotein E in the bloodstream [40]. In addition to tumor tissue, we have developed liver-targeting.

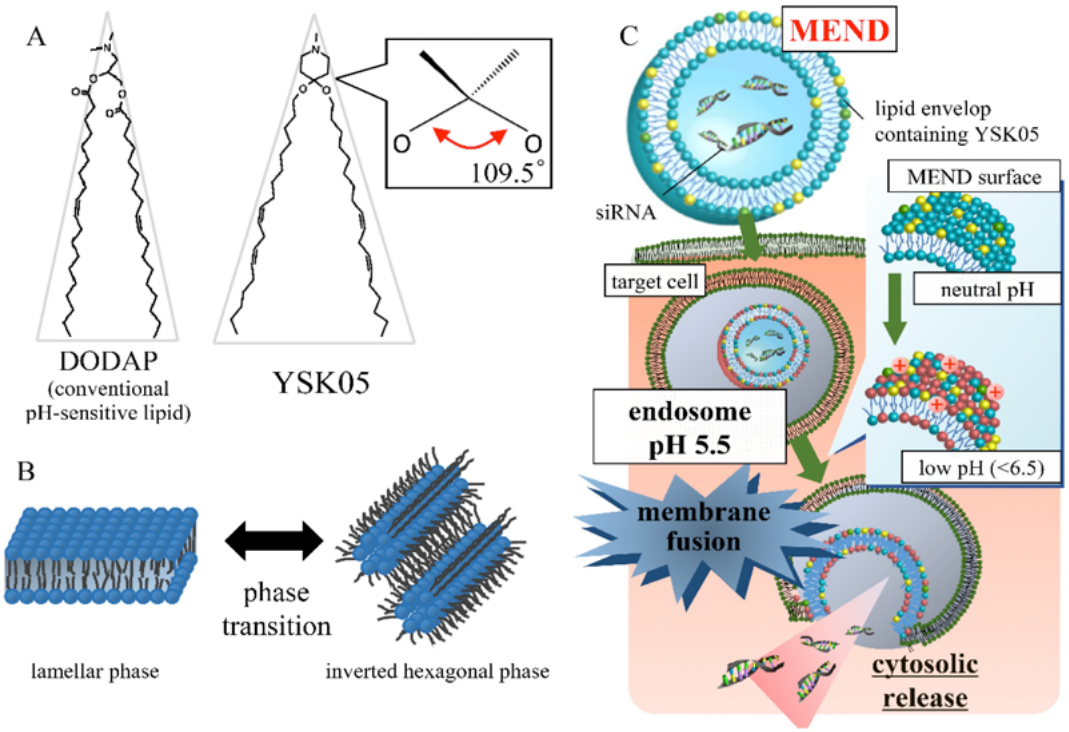

Figure 2. The illustration of pH-dependent membrane fusion and siRNA cytosolic delivery. A) The structure of YSK05. B) The image of phase transition to inverse hexagonal.

In contrast to tumors or the liver, other organs do not support passive accumulation. Therefore, active targeting is basically required to target other organs. We attempted to develop an active targeting system to the vasculature in various organs, as the vasculature is known to be an attractive target, specifically in cancer, because tumor growth is highly dependent on a supply of oxygen and nutrients supplied from tumor vessels [41]. Folkman reported this theory in 1971, and proposed that, if the blood supply to cancer cells could be interrupted, the tumor would be expected to shrink and ultimately die [10]. Since this report, many groups have studied “anti-angiogenic therapy”, and consequently several anti-angiogenic medicines including the anti-vascular endothelial growth factor (VEGF) antibody (Bevacizumab) are currently available for use in the treatment of some types of cancers [42]. However, as VEGF-related angiogenesis is a ubiquitous pathway for homeostasis maintenance in coagulation and fibrillogenesis, tumor-specific anti-angiogenesis therapy would be needed [42, 43].

To target the tumor vasculature, we modified the YSK05-MEND with cyclic RGD (cRGD) conjugated with the head of PEG-lipid via an amide bond (RGD-MEND) [44]. It is well known that cRGD is a specific ligand to integrin $\alpha_V\beta_3$, which is highly expressed in tumor vascular endothelial cells (TECs) and in some types of cancers. cRGD is currently being used in cancer imaging and drug delivery carriers [45]. In an *in vitro* study, RGD-MEND was taken up by HUVEC cells (integrin $\alpha_V\beta_3$ positive) and induced gene silencing in a dose-dependent manner. However, in HEK293T cells (integrin $\alpha_V\beta_3$), no such improvement was observed. Then, to evaluate the *in vivo* delivery of the RGD-MEND, renal cell carcinoma (RCCs)-bearing mice were intravenously injected with RGD-MEND encapsulating siRNA against *Cd31*, which is specifically expressed in endothelial cells. The expression of *Cd31* was reduced by 50% by the injection at 4.0 mg/kg siRNA, and 5' RACE-PCR clarified that this suppression was mediated by RNAi. This appears to be the first report to demonstrate direct evidence for RNAi-mediated gene silencing in TECs. The systemically administered fluorescence-labeled RGD-MEND was specifically co-localized in TECs, not cancer cells, as evidenced by con-focal laser scanning microscopy and flow cytometry, while the PEGylated YSK05-MEND had diffused into tumor tissue. As a result, the RGD-MEND failed to reduce gene expression in the cancer cells. The pharmacokinetics of RGD-MEND and *Cd31* expression in other normal organs after injection was determined, in order to investigate the organ specificity of the siRNA delivered by the RGD-MEND. Although the accumulation of the MEND in lung and spleen was slightly increased by RGD-modification, the RGD-MEND failed to inhibit endothelial cell gene expression in the lungs, spleen and liver. This result indicates that the RGD-MEND was able to deliver siRNA to TECs with a high degree of specificity, and thus that there is little possibility of an off-target effect, unlike small molecule inhibitors. We evaluated the therapeutic effect of the RGD-MEND encapsulating the VEGF receptor 2 (*VEGFR2*), which is a dominant receptor in angiogenesis. When VEGFR2 is inhibited by an antibody, tumor growth is significantly inhibited in some types of cancers [46]. When the RGD-MEND was administered into the tail vein of RCCs-bearing mice 3 times on every other day at a dose of 3.0 mg/kg, tumor growth rate became slower. Calculating vessel density by pixel counting revealed that tumor angiogenesis was moderately inhibited. Moreover, no severe change in body weight and no obvious hepatic damage was observed. Taken together, RGD-MEND appears to be

tumor-specific, and therefore a safe carrier to TECs. We recently reported that a large RGD modified carrier (>300 nm) can efficiently target the tumor vasculature due to multivalent association between the carrier and its receptor [47]. Based on these new findings, an siRNA-encapsulating RGD-MEND would be an alternative strategy for anti-angiogenic therapy instead of a small molecule inhibitor and an antibody.

4 Control of the intracellular trafficking for pDNA delivery

For the delivery of pDNA, the nuclear membrane, along with the endosomal membrane is a crucial barrier that must be overcome to maximize transfection efficacy. It is generally considered that the endogenous proteins that are inherently sorted to the nucleus possess a nuclear localization signal (NLS). Further biological analysis clarified that cytoplasmic receptors (importin families [48, 49] or others [50]) recognized the NLS or the 3-demensional structure of the protein, and then form a complex that can pass through the nuclear pore complex (NPC) [51]. One of the typical NLS is derived from the SV40 T-antigen (NLS_{SV40}): PKKKRKV [52]. In an initial trial, the NLS was directly conjugated to DNA. However, nuclear delivery efficacy is not enhanced drastically, even when 1 or 2 NLS was chemically conjugated to the linearized DNA [53, 54]. Thus, modification of the pDNA with proteins (i.e. importins) via avidin-biotin interactions represents an alternative strategy [55, 56]. The other strategy is the compaction of pDNA with NLS-conjugated cationic polymers [57, 58] or the cationically charged NLS itself [59-63]. Although transgene expression was enhanced in the latter case, these strategies may not be ideal due to the difficulties associated with controlling the topology and the density of the NLS on the surface of the particle. Thus, as a third strategy, we modified the NLS or sugars (i.e. maltotripse) that can target nucleus on the envelope structure of the MEND by inserting a lipid derivative of a NLS [64, 65]. This design permits the the NLS peptide to be spontaneously oriented in an outward direction from the particle surface.

One of the targets that can be linked to gene-based medication is dendritic cells for DNA vaccine technology. While the corresponding particle exhibited a high level of gene expression in dividing cells [66, 67], nearly background level of transfection activity was observed by the R8-modified MEND (R8-MEND) in JAWS II cells derived from murine dendritic cells. Furthermore, some efforts directed to the use of the NLS

failed to enhance the transfection efficacy in JAWS II cells or primary mouse bone marrow-derived dendritic cells (BMDCs). As an alternate approach, we attempted to overcome the nuclear membrane as well as the endosomal membrane via step-wise membrane fusion [68, 69]. In this strategy, we developed a tetra-lamellar MEND (T-MEND), in which the pDNA/polycation complex particle was coated with nucleus-fusogenic lipid (composed of caldiolipin and DOPE) and an endosome-fusogenic lipid (composed of phosphatidic acid and DOPE). As a result, a two-orders of magnitude higher transfection activity was achieved. To upgrade the function of the T-MEND, a KALA peptide that forms an α-helical structure in a neutral pH environment [70] was modified on the inner (nuclear membrane-fusogenic) or outer (endosomal membrane-fusogenic) envelopes of T-MEND (KALA/T-MEND) as an inducer of the membrane fusion at physiological pH in cytoplasm [71]. For the surface modification of the STR-KALA onto the particle, a stearylated moiety was conjugated on the KALA peptide as an anchor to the lipid envelope. With additional attempts to decrease the charge ratio (+/-) in order to enhance the intra-nuclear release of pDNA, transfection efficacy was further increased to a level sufficient for antigen presentation in JAWS II cells [71].

Furthermore, we found, unexpectedly, that the KALA-modified R8-MEND (R8/KALA-MEND) particle had adjuvant activity. As a comprehensive analysis of the biological response of the dendritic cells to the R8/KALA-MEND, variations in the gene expression profile in host cells was assessed using a whole-genome oligonucleotide microarray [72]. At 6 h after transfection with the R8/KALA-MEND, approximately 800 out of 21995 genes analyzed were up-regulated or down-regulated in JAWS II cells, more than >3-fold in comparison with those in control cells that had been transfected with the KALA-unmodified R8-MEND. Further analysis of the pathway in the category of transcription-related genes revealed that R8/KALA-MEND stimulated the expression of immune-responsive transcription factors (i.e. NF-kB and STAT). As a result, mRNA expression of an inflammatory cytokine and a type I interferon, as well as their relative transcription factors (i.e. interferon regulatory factor 7: IRF7) were drastically enhanced. Generally, dendritic cell function is supressed to avoid auto-immune responses. The KALA-modified particle has the ability to turn the “switch” on for immune stimulation. However, the issue of what is a functional “switch” in dendritic cells, and also which molecule is a key component in the

KALA-mofified MEND particle actually activate signal transduction remains to be clarified. Very recent studies indicated that TBK1 [73], and upper stream STING [74, 75] play a key role in the phosphorylation of IRF3, and subsequent IFNβ expression in response to a viral infection. Thus, it is possible that the pDNA molecules delivered by KALA-modified MEND are recognized by a cytoplasmic sensor, thus triggering immune-stimulative signaling. This hypothesis is supported by the fact that the gene expression of cytoplasmic DNA sensors (i.e. gamma-interferon-inducible protein; IFI16) are elevated in response to transfection with the KALA-modified MEND. Further analysis is currently underway concerning this issue in our laboratory.

In summary, we successfully developed a nuclear delivery system based on the two pathways; an NPC-dependent pathway and an NPC-independent (fusion) pathway. However, the application of each strategy is still limited to specified situations or in particular cells. For instance, the transfection activity of the T-MEND or the KALA-T-MEND were comparable to that for the conventional R8-MEND system in dividing HeLa cells, while these systems are highly potent in inducing transgene expression in dendritic cells. A breakthrough technology that can be commonly applicable to any types of cells is still awaited.

5 Lipid nanoparticles prepared with pH-activated and SS-cleavable lipid-like materials (ssPalm) as a novel platform for the delivery of pDNA

Many of the previous carriers were designed to carry a high level of positive charges, by virtue of the extensive condensation of pDNA with cationic liposomes and polycations under the assumption that a cationic charge is a crucial driving force for the first cellular contact with heparan sulfate proteoglycan (HSPG) [76], and the subsequent cellular uptake process. Nonetheless, viral vectors such as adenovirus, retrovirus, vaccinia virus and adeno-associated viruses have accounted for more than 65 % of the clinical trials, mainly because transfection activities using such viral vectors are typically higher than that of non-viral ones [77]. To understand the crucial rate-limiting step in the transfection efficacy in non-viral vectors, we previously compared intracellular trafficking between adenovirus and Lipofectamine Plus® (lipoplex), as typical viral and non-viral gene carriers [78, 79]. This information would enable us to clarify which of the intracellular process should be improved to maximize the

transfection activity of a non-viral gene carrier. Comparing the dose-response curves for the transfection activity in terms of the number of gene copies, we found that the lipoplex needed 3-4 orders of magnitude more gene copies to exhibit a transfection activity comparable to that for adenovirus in dividing cells (i.e. A549 cells and HeLa cells) [78, 79]. Further quantification of the amount of nucleus-delivered pDNA or adenovirus genomes provided information related to the contribution of intracellular trafficking and subsequent post-nuclear events to the overall difference in transgene expression. To our surprise, intracellular trafficking failed to explain the difference in transfection efficiency: the nuclear delivery efficacy of adenovirus, denoted as transfection activity divided by the amount of nuclear DNA was higher than lipoplex by nearly 3-fold. This, in turn, means that the post-nuclear delivery processes (i.e. transcription and translation) are dominantly responsible for the 3- or 4-orders of magnitude difference in transfection efficiency [78, 79]. Similar results have also been reported for other types of non-viral vectors [80, 81].

To understand the mechanism responsible for the poor transcription in non-viral vectors, intra-nuclear DNA was visualized using in situ hybridization, a method that can theoretically detect the DNA molecules that are released or dissociated from the gene carriers (lipoplex or adenovirus) [78]. The findings showed that nuclear pDNA was poorly stained in the nucleus while adenoviral genome was clearly detected. Moreover, adenoviral DNA was specifically located in the euchromatin region, while the pDNA delivered by lipoplex was randomly distributed in the nucleus. Collectively, these data indicate that poor decondensation and poor targeting activity of DNA to the euchromatin region are responsible for the less efficient nuclear transcription in the case of cationic vectors (**Figure 3**; left panel). Regarding translation, we found that the *in vitro* translation reaction was drastically hampered in the presence of cationic lipoplex (Lipofectamine Plus; LFN), whereas only a slight inhibition was observed in the case of adenovirus [78]. These data indicate that electrostatic interactions between LFN and mRNA is one of the crucial reasons for the poor translation efficiency of cationic vectors (**Figure 3**; left panel).

Based on these drawbacks, we focused on the development of a pDNA-encapsulating nanoparticle (LNP) which was designed to be neutral at physiological (cytoplasmic) pH to avoid electrostatic interactions with mRNA, and to be degradable for the effective release of pDNA in response to the cytoplasmic environment (**Figure 3**; right panel).

The key molecule for the development of this particle is an SS-cleavable Proton-Activated Lipid-like Material (ssPalm) that mounts dual sensing motifs that are responsive to the intracellular environment; positively charged tertiary amines responsible for an acidic compartment (endosome/lysosome) for membrane destabilization, and disulfide bonding that can be cleaved in a reducing environment (cytosol) (**Figure 4**). As a 1st generation ssPalm, we developed ssPalmM, in which myristic acid was used as a hydrophobic scaffold [82]. The most significant achievement of this study is that a neutral MEND prepared with ssPalmM ($MEND_{ssPalmM}$) exhibited a high gene expression in the presence of serum. The expression was comparable to the level for a highly cationic MEND prepared with a conventionally used cationic lipid (2-dioleyl-3-trimethylammonium propane; $MEND_{DOTAP}$) even though the cellular uptake of the $MEND_{ssPalmM}$ was much less than that of the $MEND_{DOTAP}$. Analysis of intracellular trafficking by means of confocal laser scanning microscopy (CLSM) revealed that the high transfection efficiencies can be attributed to the efficient endosomal escape and decapsulation of pDNA (collapse of the envelope structure) in the cytoplasm, most likely triggered by the cleavage of SS bonds in the ssPalm. Furthermore, cytotoxicity was extremely low; the amount of protein remained constant, even when the dose was increased by 10-fold over that used under conventional transfection conditions, while drastic, dose-dependent cytotoxicity was observed in cationic carries (i.e. Lipofectamine Plus® or LNP_{DOTAP}). Also, the pDNA encapsulated in the $MEND_{ssPalmM}$ is stable for periods of up to at least 24 h, while naked DNA and pDNA/protamine core particles were rapidly degraded within 1 h. These data strongly indicate that encapsulation into the lipid envelope can protect the encapsulated cargo from enzymatic degradation [82].

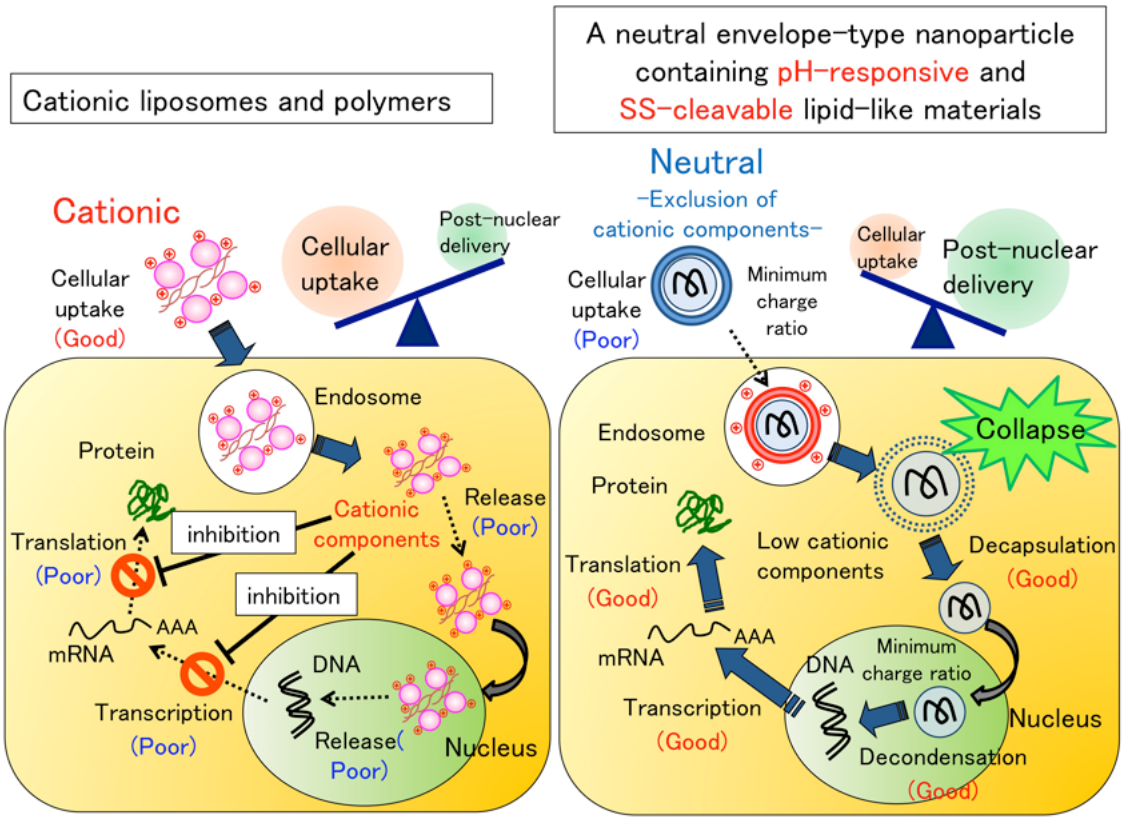

Figure 3. Schematic diagram illustrating the rate-limiting step and the mechanism responsible for the poor transfection efficacy in the conventional cationic gene carriers (left panel) and the design/concept to overcome these drawbacks (right panel).

As a 2nd generation ssPalm molecule, a fat-soluble vitamin (i.e. retinoic acid) was used as a hydrophobic scaffold (ssPalmA) instead of the conventional hydrocarbon chain [83]. Gene expression was drastically improved when cells were transfected with the MEND prepared with ssPalmA ($MEND_{ssPalmA}$) in comparison with $MEND_{ssPalmM}$. Microscopic observations revealed that the substantial improvement in transfection efficacy in $LNP_{ssPalmA}$ can be first attributed to the fortuitously found function of ssPalmA, which functions as a powerful destabilizer of endosomes/lysosomes. We surprisingly found that the staining of the late endosome/lysosome compartment by an acidic pH-indicator (Lysotracker Green) was greatly diminished when cells were transfected with ssPalmA. In addition, particles reached the nuclear periphery in the form of intact particles. Moreover, nuclear accumulation and transgene expression of the ssPalmA was competitively inhibited by exposure to an excess amount of native vitamin A (retinoic acid) and this inhibition was dose-dependent. Collectively, these

data provide evidence to show that the ssPalmA functions as a pilot towards the nuclear periphery in traveling though the cytoplasm. Further development of gene carriers for DNA vaccines is currently ongoing and the use of combinations of ssPalm and KALA are being explored.

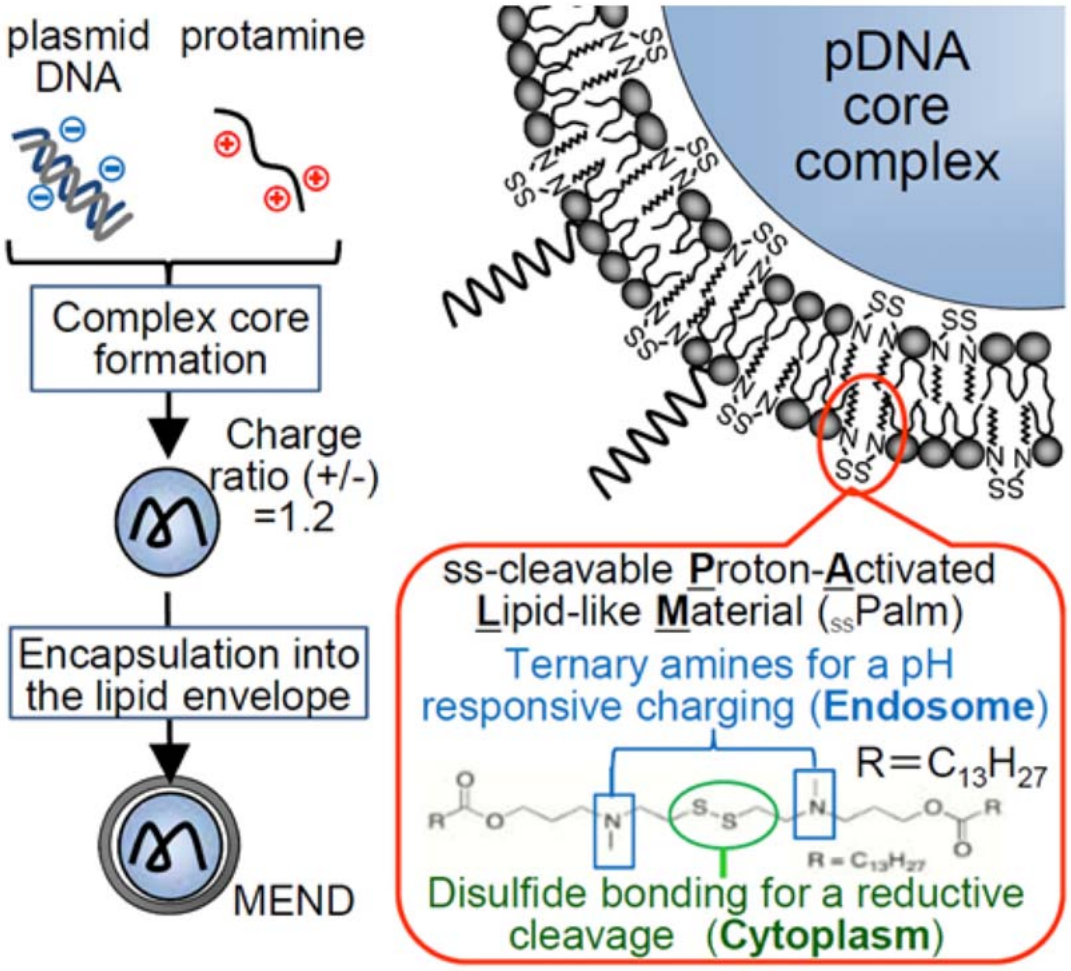

Figure 4. Schematic diagram illustrating the structure of the $MEND_{ssPalmM}$

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Figure Legends

Figure 1.

(A) Schematic representation of cell and organelle-based SELEX. (B) Dissociation constant of the AraHH001 aptamer toward TECs and Skin-ECs. (C) Binding affinities of Mitomers toward isolated mitochondria.

Figure 2.

The illustration of pH-dependent membrane fusion and siRNA cytosolic delivery. A) The structure of YSK05. B) The image of phase transition to inverse hexagonal.

Figure 3.

Schematic diagram illustrating the rate-limiting step and the mechanism responsible for the poor transfection efficacy in the conventional cationic gene carriers (left panel) and the design/concept to overcome these drawbacks (right panel).

Figure 4.

Schematic diagram illustrating the structure of the $MEND_{ssPalmM}$