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Mitochondrial drug delivery and mitochondrial disease therapy
– An approach to liposome-based delivery targeted to mitochondria -

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Abstract

Recent progress in genetics and molecular biology has provided useful information regarding the molecular mechanisms associated with the mitochondrial diseases. Genetic approaches were initiated in the late 1980s to clarify the gene responsible for various mitochondrial diseases, and information concerning genetic mutations is currently used in the diagnosis of mitochondrial diseases. Moreover, it was also revealed that mitochondria play a central role in apoptosis, or programmed cell death, which is closely related to the loss of physiological functions of tissues. Therefore, drug therapies targeted to the mitochondria would be highly desirable. In spite of the huge amount of mechanism-based studies of mitochondrial diseases, effective therapies have not yet been established mainly because of the lack of an adequate delivery system. To date, numerous investigators have attempted to establish a mitochondrial drug delivery system. However, many problems remain to be overcome before a clinical application can be achieved. To fulfill a drug delivery targeted to mitochondria, we first need to establish a method to encapsulate various drugs, proteins, peptides, and genes into a drug carrier depending on their physical characteristics. Second, we need to target it to a specific cell. Finally, multi-processes of intracellular trafficking should be sophisticatedly regulated so as to release a drug carrier from the endosome to the cytosol, and thereafter to deliver to the mitochondria. In this review, we describe the current state of the development of mitochondrial drug delivery systems, and discuss the advantage and disadvantage of each system. Our current efforts to develop an efficient method for the packaging of macromolecules and regulating intracellular trafficking are also summarized. Furthermore, novel concept of “Regulation of intramitochondrial trafficking” is proposed herein as a future challenge to the development of a mitochondrial drug delivery system.
Keywords:

Mitochondrial drug delivery; Mitochondrial protein therapy; Mitochondrial gene therapy; Transferrin and GALA system; multifunctional envelope-type nano device (MEND); Regulation of intramitochondrial trafficking.
1. Introduction

Recently, it has been progressively evident that mitochondrial dysfunction is responsible for a variety of human disorders including neurodegenerative and neuromuscular diseases, obesity and diabetes, ischemia-reperfusion injury, cancer and inherited mitochondrial diseases (Wallace, 1999, Chinnery and Turnbull, 2000, Wallace, 2005b). Progress in genetics and molecular biology has revealed that mitochondria play a role in the homeostasis of the physiological functions including electron transfer, the regulation of apoptosis, calcium storage, all of which are vital for living cells (Scheffler, 2000). For example, diabetes, cancer, and inherited mitochondrial diseases are all caused by the dysfunction of energy transfer system, the loss of apoptosis regulation and mutations in mitochondrial DNA (Holt et al., 1988, Green and Reed, 1998, Wallace, 2005a). Therefore, mitochondria are promising organelles for drug targeting.

In contrast to a mechanism-based study concerning mitochondrial diseases, strategies to complement these losses of function are limited, mainly because of the lack of an adequate delivery system (Weissig and Torchilin, 2001, Weissig et al., 2004). The development of useful delivery systems is required for small drug molecules, including the coenzymes of the electron transfer system and antioxidants such as vitamin E, and macromolecules including mitochondrial protein and mitochondrial DNA (mtDNA). Numerous investigators have attempted to construct delivery systems targeted to mitochondria (Fig. 1). However, many problems remain to be overcome before this can be realized.

In the present review, the current state of the development of mitochondrial drug delivery systems is described along with our own efforts in the development of an efficient
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packaging method for macromolecules by a condensed particle in a lipid envelope structure (Kogure et al., 2004) and exploring the intracellular trafficking regulation system (Kakudo et al., 2004). Furthermore, the advantages and disadvantages of current systems are addressed, and sub-mitochondrial (or intramitochondrial) trafficking regulation is proposed as a future challenge for mitochondrial targeting. Our interest is contributing to mitochondrial disease therapy from the point of view of an efficient delivery system.
2. Therapeutic strategy for mitochondrial diseases using small drug molecules

Mitochondrial disease therapies have been addressed using a variety of small molecule drugs (Weissig et al., 2004). One of the most important function of mitochondria is the synthesis of ATP, in which various small molecules such as vitamins and coenzyme Q along with proteins function (Saraste, 1999). The dysfunction of this system can be attributed to quite a number of mitochondrial diseases. For example, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF), and chronic progressive external ophthalmoplegia (CPEO) are etiologically attributed to defects in the respiratory chain (Chinnery and Turnbull, 2000).

To overcome these diseases, coenzymes in the respiratory chain such as vitamin B₁ or B₂, succinic acid and coenzyme Q, and ATP itself were tested to compensate for mitochondria by oral administration. Although theses strategies resulted in positive therapeutic effects, to some extent, they were not sufficient to permit the loss of function to be completely recovered (Nishikawa et al., 1989, Suzuki et al., 1995, Tanaka et al., 1997). Without a drug carrier, the active control of tissue distribution, cell uptake and intracellular trafficking targeted to mitochondria are insufficient for therapeutic activity.

Smith and co-workers recently developed a procedure for selectively delivering an effective low molecular mass antioxidant (i.e. vitamin E) to mitochondria in an in vitro cell culture system (Smith et al., 1999). Reactive oxygen species produced in the mitochondria damage not only mtDNA but also nuclear DNA, and induce mutations and/or defects in DNA. Therefore, the delivery of vitamin E to mitochondria could protect DNA by removing reactive oxygen species from mitochondria. This delivery was carried out by covalently coupling vitamin E to a lipophilic triphenylphosphonium cation (TPP), driven by the large
potential (-150 to -180 mV) across the mitochondrial inner membrane (Fig. 1 A). TPP-modified vitamin E crossed lipid bilayers and accumulated within the mitochondrial matrix more effectively, compared with native vitamin E. They concluded that the approach is applicable to relatively large molecules as well as small molecules (Muratovska et al., 2001).

3. Protein delivery for mitochondrial disease therapy

3.1. Mitochondrial protein delivery via protein import machinery

Of the hundreds of proteins in mitochondria, only 13 gene products are encoded in mtDNA (Douglas and Takeda, 1985). Most of these proteins are encoded in the nuclear genome and are translated to the cytosol. They are then delivered to mitochondria by the use of presequences, a mitochondrial targeting signal peptide (MTS), that is located at the N-terminus of the precursor protein (Mayer et al., 1995, Schatz, 1996). These MTSs typically consist of 10-70 amino acids, which are removed in 1 or 2 proteolytic steps once delivered inside the mitochondria.

To realize this strategy, plasmid DNA (pDNA) encoding MTS-fused proteins have been transfected into cultured cells. The MTS sequence leads the cargo protein inside the mitochondria, and is then cleaved, allowing for the complete localization and function of the fused protein (Seaton and Vickery, 1992, Zhang et al., 1998) (Fig. 1 B (b)). Candidate proteins, applicable to this strategy include superoxide dismutase, the apoptosis-inducing protein and the anti-apoptosis protein for the protection of mtDNA and nuclear DNA from reactive oxygen species, cancer therapy and therapy for cardiomyopathy induced from excess apoptosis, respectively.
As a useful strategy using MTS, several reports have been reported concerning gene therapy with restriction enzymes targeted to mitochondria (Srivastava and Moraes, 2001, Tanaka et al., 2002). Tanaka and co-workers chose restriction endonuclease SmaI as a candidate protein. SmaI selectively digests mutant mtDNA derived from mitochondrial diseases caused by the 8993T→G mutation. They transiently the SmaI gene fused to a MTS in cybrids carrying the mutant mtDNA. The result demonstrated that mitochondria targeted by the SmaI showed specific elimination of the mutant mtDNA. These data indicated that this approach could be a novel strategy for gene therapy of a special form of mitochondrial diseases (Tanaka et al., 2002).

Although the strategy using MTS appears to be promising, it may not be applicable to some special cases. The first case is defects in protein import into mitochondria (i.e. X-linked human deafness-dystonia syndrome [Mohr-Tranebjaerg syndrome]) (Tranebjaerg et al., 1995), in which the delivery of an MTS-fused protein into mitochondria is perturbed. The second case is that the mitochondrial delivery of proteins encoded in mtDNA is needed. In this case, these proteins are generally too hydrophobic to maintain an unfolded conformation, which is required for import by MTS (Owen et al., 2000). Therefore, a novel strategy for delivering the proteins regardless of their physical characteristics is required to realize a therapy for human diseases that involve mitochondrial dysfunction.

3.2. Novel approaches for delivering therapeutic proteins by means of the protein transduction domain

Recent studies have revealed that the protein transduction domain (PTD) is a promising device for improving the delivery of various types of biologically active molecules.
One of the PTD derived from the human immunodeficiency virus-1 TAT protein consists of 11 amino acid including 6 arginine and 2 lysine residues. It is capable of rapidly translocating the cargo into cells both in vivo and in vitro. Fusion of PTD with proteins and peptides has been shown to facilitate the effective transduction of the fused cargos into cultured cells and animal tissues while preserving their biological activity (Schwarze et al., 2000, Futaki, 2002). In the past few years, a growing number of proteins of varying sizes and types have been successfully transduced into cells (Wadia and Dowdy, 2003, Khalil et al., 2006b). Furthermore, it has been revealed that TAT-fusion proteins are also able to overcome the nuclear membrane (Gupta et al., 2005, Khalil et al., 2006a). Along with the nuclear membrane, a recent study demonstrated the potential of TAT for delivering a cargo to mitochondria. (Embury et al., 2001).

Del Gaizo and co-workers constructed a TAT fusion protein that consisted of MTS derived from mitochondrial malate dehydrogenase and the green fluorescent protein (TAT-mMDH-GFP) (Fig. 1 C). By incubating TAT-mMDH-GFP with isolated mitochondria and intact cells, it accumulated in the mitochondria via an independent pathway of MTS (Del Gaizo et al., 2003, Del Gaizo and Payne, 2003). Assuming that TAT is able to non-specifically penetrate the mitochondrial membrane, this protein may re-distribute to the cytosol after it accumulates in mitochondria. To avoid this situation, TAT is fused with the N-terminus of the full length of mMDH. In this system, MTS is specifically cleaved at the matrix region, allowing GFP to be separated from TAT. As a result, GFP is effectively trapped within the matrix.

Asoh and co-workers showed that the PTD-fused FNK protein, constructed from Bcl-XL by the site-directed mutagenesis of three amino acids improves cytoprotective activity
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(Asoh et al., 2000), protected a certain type of cells from both necrotic and apoptotic cell death (Asoh et al., 2002, Asoh et al., 2005) (Fig. 1 C). Their results suggested that the PTD-fused FNK protein could pass through the plasma membrane cell and the mitochondrial membrane. These mitochondrial delivery strategies using PTD have certain advantages compared with MTS, which is dependent on protein import machinery via the TOM/TIM complex. For example, PTD was able to deliver drugs into mitochondria even if cell had a defect in protein import machinery. Moreover, it may be used to introduce macromolecules such as folded proteins and mtDNA into mitochondria, although a systematic comparison of size-dependant transduction has not been demonstrated.

Shokolenko and co-workers attempted the mitochondrial delivery of Exonuclease III protein (ExoIII) with a combination of PTD and MTS (Shokolenko et al., 2005) (Fig. 1 B (c)). They constructed a MTS-ExoIII-TAT-fusion protein, in which MTS and TAT were fused to Exo III at the N-terminus and C-terminus, respectively. The overexpression of Exo III caused the cancer cells to be more sensitive to oxidative stress, which results in a diminished cell survival (Shokolenko et al., 2003). The results showed that the transduced protein was effectively targeted to the mitochondrial matrix, where it diminished the repair of mtDNA following oxidative stress. This diminished repair rendered the cells more susceptible to the lethal effects of oxidative stress. In this strategy, PTD functions as a cytoplasmic delivery device and the mitochondrial targeting activity of MTS could compensate for the non-specific protein delivery of PTD, as a result, efficient cytoplasmic and mitochondrial delivery was achieved.

4. Approaches to mitochondrial gene therapy
4.1. Relationship between mitochondrial diseases and mutation and/or defect of mitochondrial DNA

Human mtDNA is a circular 16,569-base pair (bp) molecule that codes for 13 genes for oxidative phosphorylation and 2 rRNAs and 22 tRNAs are necessary for the expression of the gene product. A lack of exon regions means that mtDNA has a much higher information density than nuclear DNA. Therefore, mtDNA is more susceptible to mutation, compared with nuclear DNA (>20 fold), which results in a high frequency of mitochondrial diseases (Wallace, 1999, Kagawa et al., 2001).

Genetic mutations that are responsible for the mtDNA disease can be categorized four groups; missense mutations, protein synthesis mutations, insertion-deletion mutations and copy number mutations. Examples of a missense mutation include Leber’s hereditary optic neuropathy (LHON) (Newman, 1993) and neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) (Holt et al., 1990). Synthetic mutations are mutations in the encoding region of tRNA, which include MELAS (Goto et al., 1990), MERRF (Shoffner et al., 1990), and maternally inherited cardiomyopathy (CM) (Taniike et al., 1992). Deletions in mtDNA have been found in the majority of CPEO and Kearns-Sayre syndrome (KSS) (Shanske et al., 1990). A marked decrease in the number of mtDNA molecules is associated with a familial mitochondrial myopathy (Otsuka et al., 1990). Since various diseases are caused by mutations in mtDNA as described above, a mitochondrial gene delivery leading to the repair of a mutation in mtDNA and/or complement normal mtDNA would be expected to serve as a novel strategy for curing a mitochondrial disease.

4.2. Approach to repairing mitochondrial DNA by oligodeoxynucleotides and
peptide nucleic acid

The loci of mutations in mtDNA have been reported in many mitochondrial diseases. For example, the major point mutations found in MERRF, MELAS, and CM are localized at bp 8344 (A to G; tRNA$^{Lys}$) (Shoffner et al., 1990), bp 3243 (A to G; tRNA$^{Lue}$ (UUR)) (Goto et al., 1990) and bp 4269 (A to G; tRNA$^{Ile}$) (Taniike et al., 1992), respectively. Accordingly, the precise correction of mutations in mtDNA is a sophisticated strategy for genetic disorders. Chen and co-workers demonstrated that, not only nucleus, but also mitochondria contain the machinery required for the repair of genomic mutations using oligodeoxynucleotides (ODN). Therefore, the delivery of therapeutic ODN to the mitochondrial matrix is promising for the treatment of such mitochondrial diseases (Chen et al., 2001).

To date, it has been reported that DNA is introduced into isolated mitochondria by covalently linking the MTS to either ODN or double stranded DNA (Vestweber and Schatz, 1989, Seibel et al., 1995) (Fig. 1 B (a)). Seibel and co-workers showed that these conjugates were imported into mitochondria through the outer membrane and the inner membrane via the TOM/TIM complex. The reported length of DNA applicable to this strategy is from 17 bp to 322 bp (Seibel et al., 1995).

A similar strategy has also been developed for the mitochondrial delivery of peptide nucleic acid (PNA) (Fig. 1 B (a)). With the help of the membrane permeability toxin as a device the cytoplasmic delivery, MTS-conjugated PNA was imported into mitochondria. (Fig. 1 B (a)). Their method would be expected to provide a viable strategy for the genetic modification of mitochondria in cultured cells, animals, and patients (Flierl et al., 2003).
4.3. Delivery of a circular DNA to mitochondria for mitochondrial gene therapy

4.3.1. Utilities and problems for delivery of mtDNA to mitochondria

In an ideal situation, all of the mtDNA is completely lacking in mutations. The cells in this situation are denoted as homoplasmy. However, in certain situations, cells have both mutant and wild-type mtDNA. This situation is denoted as heteroplasmy. In the case of mitochondrial related diseases, when the percentage of mutant mtDNA exceeds a certain threshold level, mitochondrial dysfunction becomes clinically apparent (Holt et al., 1990, Kagawa et al., 2001). Accordingly, the delivery of a large number of wild-type mtDNA into the mitochondrial matrix in diseased cells would decrease the percent of mutated mtDNA, resulting in the suppression of mitochondrial disease.

As described above, it has been reported that the conjugation of MTS permits the delivery of an exogenous protein and an ODN into mitochondria (Vestweber and Schatz, 1989, Seaton and Vickery, 1992, Mayer et al., 1995, Seibel et al., 1995, Schatz, 1996, Zhang et al., 1998, Flierl et al., 2003). However, mitochondrial delivery is severely limited by the size of the cargoes (Endo et al., 1995). Therefore, an alternative strategy for delivering macromolecules such as mtDNA and pDNA is needed for the mitochondrial gene therapy.

4.3.2. Strategy for DNA delivery using mitochondriotropic lipoplex

Weissig and co-workers attempted to deliver pDNA to mitochondria using DQAsomes, which are mitochondriotropic and cationic ‘bola-lipid’-based vesicles (Weissig et al., 2001, D'Souza et al., 2003) (Fig. 1 D). They previously demonstrated that DQAsomes-DNA complexes (DQAplexes) selectively release pDNA when they come into contact with isolated mitochondria derived from rat liver (Weissig et al., 2001). In addition,
they demonstrated that DQAplexes appear to be able to escape from endosomes without losing their pDNA, and specifically release pDNA at the proximal region of mitochondria (D'Souza et al., 2003). More recently, they tried to deliver pDNA into mitochondrial matrix by means of a more optimized system based on their carrier (D'Souza et al., 2005). In the future, it would be expected that mitochondrial gene therapy will be realized when the effective delivery of DNA into the mitochondrial matrix is accomplished based on such novel approaches.

5. Challenges for the development of multifunctional drug carrier for mitochondrial delivery system

5.1. Drug carrier design for achievement of mitochondrial drug therapy

To realize a therapy for a mitochondrial disease, considerable efforts have focused on studies of drug delivery systems that are targeted to mitochondria as well as investigations of the molecular mechanism of mitochondrial diseases. In spite of great efforts, a universal strategy for therapies for mitochondrial diseases has not been established. To achieve this, a break through regarding the following issues for an establishment of mitochondrial drug delivery system must be made (Fig. 2). First, we must design an efficient and simple method for packaging various types of cargos including chemicals, proteins, peptides, ODN and circular DNA into a drug carrier depending on their physical characteristics. Second, we must establish targeting system that can deliver encapsulated carriers to specific cells. Third, we must regulate intracellular trafficking including endosomal escape and subsequent mitochondrial targeting.

Ultimately, it is necessary to program all of the devices to target the cells and to
regulate intracellular trafficking into one drug carrier so as to exhibit the function of each device at the appropriate site and timing. (Kamiya et al., 2003). This section focuses on our current trials in exploring the development of a liposome-based carrier system for the design of a vector suited for the packaging and transport of a drug to the cytosol in living cells (Kakudo et al., 2004, Kogure et al., 2004). We also describe a novel system targeted to mitochondria by a combination of accumulated knowledge of mitochondrial targeting and our cytoplasmic delivery system.

5.2. Development of efficient packaging method for macromolecules

The efficient packaging of therapeutic drugs and macromolecules into a drug carrier is an important factor in developing a successful delivery system (Fig. 2). In the case of doxorubicin (an anticancer drug), it is well-known that efficient packaging could lead to an increase in pharmacological activity (Oku et al., 1994). To cure various types of mitochondrial diseases, a huge variety of molecules including ODN, pDNA and folded proteins should be delivered into the mitochondria in living cells. However, the lack of an efficient method for the packaging of macromolecules prevents gene therapy and protein therapy.

We recently established a multifunctional envelope-type nano device (MEND), which permits the efficient and simple packaging of pDNA (Kamiya et al., 2003, Kogure et al., 2004). The MEND consists of a condensed core of pDNA and a lipid envelope structure. The MEND structure has various advantages. First, the condensed pDNA particle is efficiently encapsulated in the lipid envelope via electrostatic interactions between the condensed core and the lipid envelope. Second, various functional devices such as
polyethyleneglycol to prolong blood circulation, a ligand for targeting and a pH-sensitive fusogenic peptide to facilitate endosomal escape is easily incorporated. Furthermore, the size of the MEND can be controlled to 100-300 nm, which is an advantage in terms of cellular uptake. Therefore, this packaging concept would be expected to solve many of the problems (i.e. low encapsulation efficiency and controlled release in the cytosol). Subsequently, we succeeded in developing an ODN-MEND, which consists of a condensed core of ODN and a lipid envelope structure (Yamada et al., 2005a). The calculated encapsulation efficiency of the ODN-MEND was 380%, due to the fact that ODN-MEND significantly enhances the encapsulation efficiency of ODN by lipid by forming condensed ODN particles. The encapsulation efficiency of the ODN-MEND was much higher than those for other methods reported as efficient ODN packaging methods (Yamada et al., 2005a).

The MEND is capable of serving as a useful carrier for the efficient delivery of pDNA and ODN by introducing various functional devices, as described below. We first constructed a MEND encapsulating anti-luciferase siRNA plasmid condensed, and evaluated its silencing effect in vitro (Moriguchi et al., 2005). In the co-transfection experiment using the siRNA plasmid and a luciferase-encoding plasmid, luciferase activity was reduced to 4%. Furthermore, optimization of the condenser improved the RNAi effect. As a result, approximately a 70% silencing effect was achieved even in luciferase transformed cells (Moriguchi et al., 2005). We next constructed an ODN-MEND, in which the encapsulated ODN is condensed by an optimal polycation, and then evaluated the anti-sense effects. As a result, we succeeded in developing an ODN-MEND, which exhibits a high antisense effect (Nakamura et al., 2006). Considering that ODN functions in the cytoplasmic region, the effective pharmacological activity of ODN indicated that the MEND is potent in delivering
encapsulated macromolecules to the cytosol. We are presently constructing a novel type of MEND which is useful for the delivery of therapeutic proteins and peptides (Yamada et al., unpublished results).

5.3. Selective drug delivery and efficient cytoplasmic introduction using multifunctional device modified liposomes

5.3.1. Efficient cytoplasmic delivery using transferring-modified liposomes equipped with a pH sensitive fusogenic peptide

We recently developed transferrin-modified liposomes (Tf-L) to target tumor cells which up-regulate Tf receptors (Kakudo et al., 2004). Tf-L is potent in targeting cells, which express transferring receptors such as tumor cells, via receptor-mediated endocytosis. However the encapsulated fluorescent marker is trapped in the endosomal/lysosomal compartments because they lack the ability to escape from the endosomes. To enhance endosomal escape, a pH-sensitive fusogenic peptide, GALA was introduced in the preparation of Tf-L. When the GALA peptide was encapsulated in the liposomes along with an aqueous phase marker (sulforhodamine B; Rho), the encapsulated marker remained in the endosome/lysosome fraction and subsequently degraded. In contrast, when GALA was modified with cholesterol and displayed on the surface of the liposome (Tf-GALA-L), encapsulated Rho was efficiently released to the cytosol. Therefore, the control of the topology of GALA is critical for the function GALA (Kakudo et al., 2004).

5.3.2. Mitochondrial delivery of mastoparan with transferring and GALA system for selective cancer therapy
To apply the Tf-GALA-L to the delivery of a functional peptide, mastoparan (MP) was encapsulated. MP is a 14-amino acid amphipathic peptide from wasp venom (Zimmerberg and Parsegian, 1986) and is known to induce a mitochondrial permeability transition (PT) in a concentration between 5 – 100 μM by forming a permeability transition pore (Pfeiffer et al., 1995). Therefore, the selective delivery of MP to tumor cells could be a useful strategy for cancer treatment.

In previous study, the cellular response to MP encapsulated Tf-GALA-L was compared with that of free MP (Yamada et al., 2005b). In the case of free MP, cytochrome c was released not only in the cytosol but also to the medium, indicating that the plasma membrane along with mitochondria was damaged by MP. In contrast, in the case of MP encapsulated in Tf-GALA-L, it was internalized to target tumor cells via a Tf receptor-mediated endocytosis, and the MP was subsequently delivered to the cytosol, leaving the plasma membrane in an undamaged state. In fact, cytochrome c was observed only in the cytosol fraction. These results indicate the clear advantages of Tf-GALA-L for cancer therapy (Yamada et al., 2005b). Similarly, if other types of mitochondriotropic molecules such as MTS- or PTD- conjugated/fused ODN, peptides and proteins were to be encapsulated into Tf-GALA-L, the selective targeting of cells and intracellular trafficking (i.e. endosomal release and mitochondrial targeting) should be intelligently regulated. In our laboratory, improvements in the delivery system via the use of Tf-GALA-L are currently in progress.

6. “Regulation of intramitochondrial trafficking”, a future challenge for the mitochondrial delivery system
The delivery of drugs to mitochondria after their internalization into cells is considered to be an important step in mitochondrial disease therapy, as described above. Ultimately, each drug must be sorted to the appropriate region of a mitochondrion depending on the pharmacodynamic action of the molecule (Fig. 3). For example, apoptosis-inducing and apoptosis-inhibiting drugs should be delivered to the outer membrane, where the main events related to apoptosis are triggered. These delivery systems will be useful for cancer therapy and therapy for cardiac infarction caused by excessive apoptosis. In the case of respiratory chain related proteins and coenzymes, they should be delivered to the intermembrane space and the inner membrane region, where key components of the electron transfer system are found. Normal mtDNA and therapeutic ODN should be delivered to the mitochondrial matrix for mitochondrial gene therapy, where mtDNA is pooled. Therefore, the development of the novel drug carrier based on the novel concept of “Regulation of intramitochondrial trafficking” will be needed for the more efficient therapy of mitochondrial diseases in the future (Fig. 3).

7. Conclusion

In this review, recent reports concerning the molecular pathology of mitochondrial diseases and mitochondrial drug delivery systems were summarized. Furthermore, the utilities and problems to be overcome were highlighted. As an ideal drug carrier, an efficient delivery system using MEND was proposed. We also proposed the concept of “Regulation of intramitochondrial trafficking”, in which therapeutic molecules are sorted to an appropriate region of a mitochondrion depending on their pharmacodynamic action. Regarding progress in various research fields (i.e. genetics, molecular biology, pathology and etc…), a variety of
candidate cargos are proposed, intended to treat mitochondrial dysfunctions. Maintaining a great effort to establish a delivery system will be highly valuable in terms of realizing a therapy for mitochondrial diseases.
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Legends

Figure 1. **Current approaches for achieving a successful mitochondrial drug delivery system.** (A) Uptake into mitochondria through the lipid bilayer can be driven by the large membrane potential across the mitochondrial membrane. The attachment of a lipophilic cation such as triphenyl phosphonium (TPP) leads to the uptake of an attached, small neutral molecule. (B) The attachment of a mitochondrial targeting signal peptide (MTS) to ODN or PNA allows mitochondrial delivery via TOM/TIM complex (a). After the recognition of MTS, the protein is temporary unfolded with the function of chaperone proteins. Once the MTS-fused protein is delivered inside the mitochondria, the signal peptide is cleaved by a peptide processing protease, and the protein is then refolded to the mature form (b, c). (C) A protein conjugated with protein transduction domain peptide (PTD) is delivered to mitochondria without passing through the classical protein import pathway. This strategy allows a large macromolecule to be delivered via an unknown mechanism. (D) DQAsomes-DNA complexes selectively release DNA, when the carriers gain access to mitochondria. DNAs released at proximal region of mitochondria could then be taken up by mitochondria.

Figure 2. **Problem to be overcome for the development of a mitochondrial drug delivery system.** To establish a potent mitochondrial drug delivery system, the drug must be encapsulated in a drug carrier depending on the physical characteristics (1). Thereafter, intracellular trafficking of the carrier including endosomal escape (2) and subsequent mitochondrial targeting (3) must be regulated.
Figure 3. Novel concept for mitochondrial diseases’ therapy, “Regulation of intramitochondrial trafficking” For drug therapy, regulation of both the bio distribution and intracellular trafficking are important factors. In addition, we propose “Regulation of intramitochondrial trafficking” to effectively achieve a mitochondrial disease therapy. Sorting of a drug to the appropriate region in a mitochondrion can increase therapeutic effect and decrease undesired side-effects of that drug.
Figure 1
Figure 2
Figure 3

Regulation of Intracellular trafficking

Sorting to specific region in mitochondrion

Delivery to outer membrane
(A) Apoptosis inducing drugs for cancer therapy
(B) Apoptosis suppressing drugs for myocardial infarction and ischemic brain injury induced by excess apoptosis

Delivery to intermembrane space and inner membrane
(C) Respiratory chain related molecules for the treatment of dysfunction of electron transfer system
(D) Anti-oxidants drugs for anti-oxidant drug's therapy

Delivery to matrix
(E) Therapeutic ODN for gene therapy by gene correction
(F) mtDNA for gene substitution therapy

Regulation of Systemic distribution

Tissue-targeting

Drug administration
Mitochondrial diseases are induced in various tissues throughout body. Therefore, drug should be delivered to the proper organ e.g., brain (ischemic brain injury), heart (myocardial infarction), and etc.