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CITATION:

Yoshinaga, Naoto ...[et al]. Rational Designs at the Forefront of Mitochondria-Targeted Gene Delivery: Recent Progress and Future Perspectives. ACS Biomaterials Science & Engineering 2022, 8(2): 348-359

ISSUE DATE:

2022-02-14

URL:

http://hdl.handle.net/2433/278388

ACS Biomaterials SCIENCE & ENGINEERING

pubs.acs.org/journal/abseba

Perspective

Rational Designs at the Forefront of Mitochondria-Targeted Gene Delivery: Recent Progress and Future Perspectives

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Cite This: *ACS Biomater. Sci. Eng.* 2022, 8, 348−359

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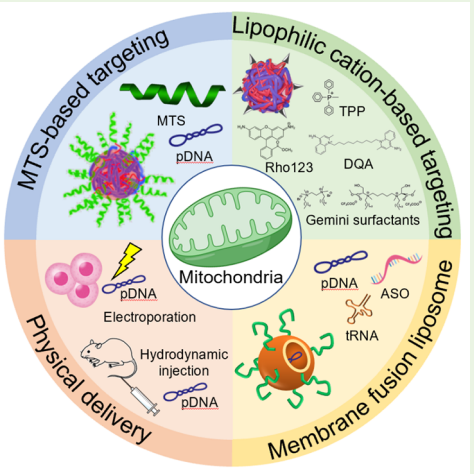
ABSTRACT: Mitochondria play an essential role in cellular metabolism and generate energy in cells. To support these functions, several proteins are encoded in the mitochondrial DNA (mtDNA). The mutation of mtDNA causes mitochondrial dysfunction and ultimately results in a variety of inherited diseases. To date, gene delivery systems targeting mitochondria have been developed to ameliorate mtDNA mutations. However, applications of these strategies in mitochondrial gene therapy are still being explored and optimized. Thus, from this perspective, we herein highlight recent mitochondria-targeting strategies for gene therapy and discuss future directions for effective mitochondria-targeted gene delivery.



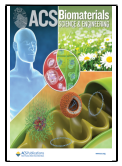
KEYWORDS: *mitochondria, gene therapy, organelle targeting, mitochondria-targeting peptides*

INTRODUCTION

Advances in drug delivery technologies have enabled the encapsulation of various cargos, such as small drugs, nucleic acids, and proteins, and the targeting of specific tissues and cell types to enhance delivery efficiency.[1−4] Moreover, this strategy has been further advanced in recent years to control the intracellular trafficking behaviors of delivery carriers to target specific organelles.[5−8] Appropriate organelle targeting can enhance therapeutic outcomes and minimize unfavorable side effects. Mitochondria are promising targets among subcellular organelles because they play an essential role in cell metabolism by producing adenosine triphosphate (ATP) as an energy source, controlling reactive oxygen species (ROS) and calcium ion levels, and regulating apoptosis.[9,10] These critical functions of mitochondria are supported by vital proteins encoded in not only nuclear DNA but also mitochondrial DNA (mtDNA).[11,12] The mtDNA in humans, which is multicopy, circular, and double-stranded, encodes 37 genes; 22 transfer RNAs (tRNAs), 13 proteins that are essential for the oxidative phosphorylation-induced synthesis of ATP, and 2 ribosomal RNAs (rRNAs).[13,14] As mtDNA, unlike nuclear DNA, is not packaged and protected by histone proteins but rather forms nucleoids with helicases[15] and is chronically exposed to ROS generated in the mitochondria, it is compromised by mutations, the risk of which increases over time.[9,16−18] Mitochondrial dysfunction caused by mtDNA mutation leads to a variety of inherited diseases, such as neurodegenerative diseases, diabetes, and cancer.[19−23] In addition to targeting human mitochondria for gene therapies, plant mitochondria are important target organelles in the agricultural field because plant mtDNA encodes genes responsible for cytoplasmic male sterility, which is a useful trait for breeding.[24] Therefore, the development of delivery strategies targeting mtDNA has been a research hotspot for enhancing benefits in various species.[25−33]

In the first study on the delivery of chemotherapeutics into the mitochondria, the antioxidant vitamin E was conjugated with triphenylphosphonium (TPP),[34] a lipophilic cation that interacts with a large negative mitochondrial inner membrane, ultimately allowing the chemotherapeutic drugs to passively accumulate in the mitochondria.[7,35−38] As shown in this first example, the approach of conjugating mitochondria-targeting

Received: September 1, 2021
Accepted: December 10, 2021
Published: January 3, 2022



https://doi.org/10.1021/acsbiomaterials.1c01114
ACS Biomater. Sci. Eng. 2022, 8, 348−359

molecules with small chemotherapeutic drugs leads to remarkable outcomes.[39−47] Additionally, the integration of peptides, including mitochondrial targeting sequences (MTSs), is a promising approach to mechanically guide small drugs into the mitochondria.[48−53] MTSs can be recognized by mitochondrial outer membrane proteins, resulting in the active targeting of mitochondria without unfavorable distribution in the crowded intracellular environment. For example, Schiller-Szeto peptides, tetrapeptides composed of hydrophobic/cationic amino acids that behave as antioxidants,[48] are known to accumulate in the mitochondria and exert efficient therapeutic effects.[54−56] Although the delivery of small compounds to mitochondria has been successfully achieved, the introduction of macromolecules, such as nucleic acids and proteins, into mitochondria remains challenging despite the promise of tremendous benefits, especially in regard to mitochondrial gene therapy. Thus, from this perspective, we discuss recent progress regarding the design of mitochondria-targeting nucleic acid delivery carriers and promising topics for future investigations.

MITOCHONDRIAL GENE DELIVERY

As mentioned above, human mtDNA mutations are related to a variety of mitochondrial diseases and severely disrupt cellular metabolism. In particular, mutations in oxidative phosphorylation-related proteins inhibit ATP production.[23,57] Such mitochondrial dysfunction occurs when the ratio of mutated mtDNA to healthy wild-type mtDNA within one mitochondrion, called heteroplasmy, exceeds a certain threshold.[22] For mitochondrial disease therapies, the improvement of heteroplasmic conditions by supplying wild-type mtDNA, repairing mutated mtDNA by genome editing, or eliminating mutated mtDNA is an effective strategy (Figure 1). In the very early

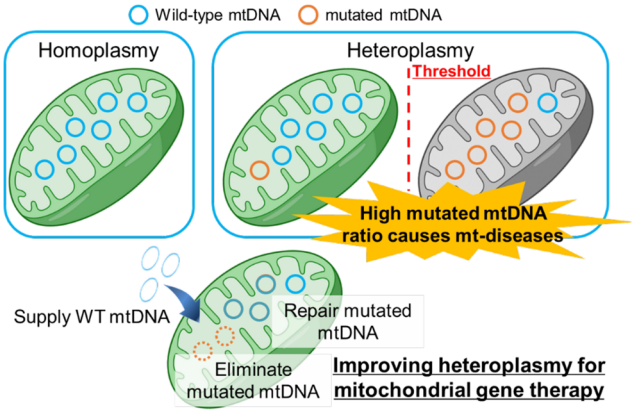


Figure 1. Schematic illustration of the mechanism by which mitochondrial heteroplasmy causes mitochondrial diseases.

stage of mitochondrial gene therapy, the cytoplasm of healthy cells was fused with mitochondria-defective cells in an *in vitro* model to supply intact mitochondria.[58] Via this approach, mitochondrial functions were recovered, indicating the importance of mtDNA gene therapy. Various strategies have thus far been developed for mitochondrial gene therapy. Below, we highlight recent promising achievements in mitochondrial gene therapy on the basis of the delivery of several nucleic acids and categorize the methods on the basis of how mitochondria are targeted (Figure 2 and Table 1).

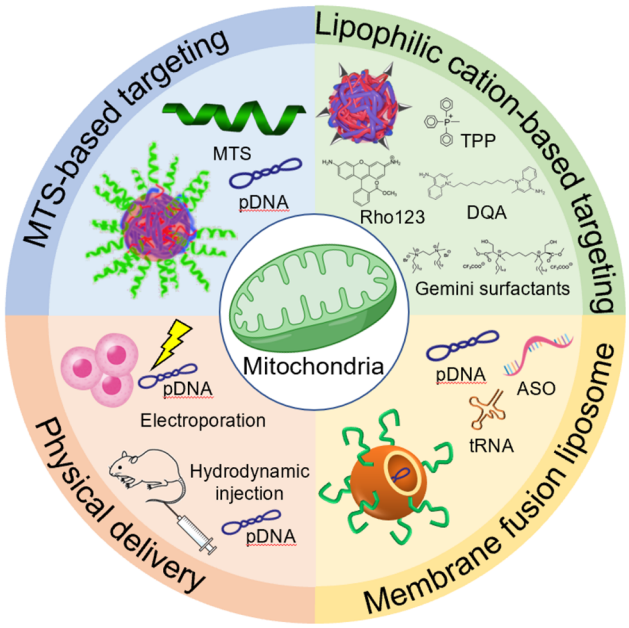


Figure 2. Overview of the mitochondria-targeting strategies. Four distinct approaches are capable of successfully delivering loaded nucleic acids into the mitochondria. (Blue) Mitochondrial targeting signals (MTSs) can guide delivery carriers to mitochondria via biological mechanisms. (Green) Lipophilic cations strongly interact with the mitochondrial inner membrane, which has a large negative membrane potential. (Yellow) Liposomes composed of mitochondrial membrane fusion lipids deliver loaded cargos to the mitochondrial intermembrane space or matrix. (Red) Loaded cargos introduced by the physical method are sometimes internalized in the mitochondria. Abbreviations: pDNA, plasmid DNA; tRNA, transfer RNA; ASO, antisense oligonucleotide; Rho123, rhodamine 123; DQA, 1,1′-(1,10-decamethylene-*bis*-[aminoquinaldinium])-chloride.

Mitochondrial Targeting Signal Peptides. The introduction of plasmid DNA (pDNA) into mitochondria is a straightforward approach to supply the essential mitochondrial proteins needed for mitochondrial gene therapy. However, as the mitochondrial outer membrane is only permeable to substances with low or medium molecular weights,[88−90] efficient delivery systems are required to pass through the barrier. The use of MTS peptides is one of the most powerful tools for mitochondria-targeting delivery. Most mitochondrial proteins are encoded in the nuclear genome[91] and mechanically transported to the mitochondria. Mitochondrial proteins typically possess MTSs at the N-terminus and exhibit amphiphilic α-helix structures that are flanked by cationic and hydrophobic residues on either side. The MTS sequences are recognized by negatively charged translocase of the mitochondrial outer membrane (TOM) complexes and transferred to the mitochondrial intermembrane space through the barrel-like TOM40 translocase. The translocase of the inner membrane (TIM), which is also a negatively charged complex, then recognizes the MTS and finally allows the entry of mitochondrial proteins into the mitochondrial matrix.[91−97] This mechanism has been extended to mitochondria-targeted delivery. Khan and Bennett designed an MTS-based gene delivery system, called protofection, that was based on a functional protein.[59] The protein was composed of three domains: (i) a protein transduction domain (PTD), whose sequence was similar to that of viral proteins, for enhancing cellular uptake, (ii) an MTS domain for mitochondrial

https://doi.org/10.1021/acsbiomaterials.1c01114
ACS Biomater. Sci. Eng. 2022, 8, 348−359

Table 1. Summary of Nucleic Acid Delivery Carriers Targeting Mitochondria[a]

mitochondria-targeting strategy	targeting material(s)	outcomes	ref
MTS	superoxidase dismutase-2 sequence	decreased the mutated mtDNA ratio by introducing WT mtDNA	59−63
	cytochrome c oxidase subunit IV sequence	reporter gene expression	64−67
	cytochrome c oxidase subunit VIII sequence	recovery of optic atrophy in mice with vision loss	68, 69
	computationally predicted sequence	reporter gene expression	70
	six-residue peptide ($\sigma\varphi\chi\beta\varphi\varphi$[b])	efficient mitochondrial localization	71
	tail-anchored membrane proteins	efficient mitochondrial localization	72
lipophilic cations	triphenylphosphonium	reporter gene and ND4 protein expression	73
	rhodamine 123	reporter gene expression	74, 75
	gemini surfactants	reporter gene expression	76
	dequalinium	reporter gene expression	77, 78
membrane fusion	DOPE and cell-penetrating peptides	reporter gene expression, target gene silencing by an ASO, and improvement of ATP production via the introduction of WT mitochondrial tRNA	79−84
electroporation		delivery of pDNA to the mitochondria	85
hydrodynamic injection		delivery of pDNA to the mitochondria	86, 87

[a]Abbreviations: WT, wild-type; ASO, antisense oligonucleotide; tRNA, transfer RNA; pDNA, plasmid DNA. [b]σ: hydrophilic; φ: hydrophobic; β: basic; χ: any amino acid.

targeting, (iii) a mitochondrial transcription factor A (TFAM) domain for pDNA complexation.[98] Wild-type mtDNA complexed with PTD-MTS-TFAM was successfully transported into the mitochondria, thereby increasing the respiration rate and mtDNA copy number in disease cell lines with reduced levels of mutated mtDNA.[60,61,63] Remarkably, the intravenous injection of the PTD-MTS-TFAM/mtDNA complex into the mouse tail vein enhanced the NADH ubiquinone oxidoreductase-driven respiration rate in the mitochondria of the brain and skeletal muscle.[61] It should be noted that the PTD-MTS-TFAM treatment without mtDNA loading induced the activation of mitochondrial biogenesis, probably due to the increased levels of TFAM,[60,61] which is involved in the formation of nucleoids with mtDNA.[99] The synergistic effect of increasing the levels of mtDNA and TFAM may have outstanding therapeutic effects. Moreover, the PTD-MTS-TFAM system enabled the delivery of a mutated mtDNA (Leber's hereditary optic neuropathy (LHON) mtDNA) into human neural progenitor cells for the preparation of a LHON model cell line.[62]

We also reported mitochondria-targeting micelles prepared from pDNA and MTS-conjugated peptides.[65] The MTS with a partial cytochrome c oxidase (cytocox) subunit IV sequence was conjugated with lysine-histidine (KH) repeats, which facilitate nanoparticle (NP) formation with pDNA through electrostatic interactions. The cytocox-KH-based micelles significantly enhanced the gene expression in the cultured cells without unfavorable cytotoxicity. Moreover, we successfully delivered loaded pDNA to the mitochondria in plants and led to efficient reporter gene expression,[64,66] indicating their utility for mitochondrial targeting beyond plant species. Very recently, Faria et al. screened the cationic sequences of cytocox-conjugated peptides to enhance the delivery of pDNA into mitochondria.[67] The screening revealed that KH repeats as the cationic sequence were more preferred for mitochondrial delivery than tryptophan- and arginine-rich amphipathic peptides.

The MTS-based mitochondria-targeting strategy was expanded to viral gene delivery. Yu et al. developed an MTS-conjugated adeno-associated virus (AAV) loaded with pDNA encoding NADH ubiquinone oxidoreductase subunit 4 (pND4),[68] a mitochondrial inner membrane protein that is defective in LHON disease. During the AAV transduction process, three capsid proteins on the AAV surface contribute to cellular entry and intracellular trafficking. The authors replaced the capsid protein with an MTS, whose sequence was based on cytocox subunit VIII (COX8). The MTS modification of the AAV vector enhanced the accumulation of pND4 in the mitochondria, leading to the recovery of optic atrophy in mice with vision loss. Furthermore, the authors performed a next-generation sequencing study, revealing that the pND4 delivered to the mitochondria using MTS-AAV was episomal and thus indicating that the MTS-AAV strategy for pDNA insertion did not cause wild-type mtDNA mutations.[69] However, AAVs can only encapsulate pDNA cargo up to 5 kbp. Therefore, further investigations on the use of MTSs with other virus vectors, such as adenovirus and lentiviral vectors, are required.

The feasibility of MTSs for mitochondrial targeting motivated researchers to design more promising MTS motifs for mitochondrial delivery and to elucidate a structural basis for the mechanism underlying TOM complex recognition. To identify effective MTSs, Chin et al. compared 31 MTS motifs by observing the cytosolic transfer of each MTS-conjugated protein expressed in the nucleus.[100] From the screening, the MTS motif of a mitochondrial inner membrane protein (4-hydroxybenzoate polyprenyltransferase) in *Zea mays* tended to yield a high colocalization ratio with the mitochondria in HeLa cells. Consequently, the authors constructed a pDNA encoding the optimized MTS-fused mitochondrial ATP6, which is related to ATP synthesis and known to be defective in several mitochondrial diseases. Transfection of the constructed pDNA into the mitochondrial ATP6-mutated cell line critically improved ATP production, indicating the good functionality

https://doi.org/10.1021/acsbiomaterials.1c01114
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of the optimized MTS motif. MacMillan et al. evaluated the computationally predicted MTS motifs for plant mitochondrial targeting using TargetP,[70] which can predict the subcellular locations of proteins.[101] TargetP prediction using selection criteria provided 748 candidates from 785 615 plant proteins. Thirty-one of these peptides were randomly selected for labeling with FITC and then experimentally examined on the basis of the quantification of the colocalization ratio with mitochondria using confocal laser scanning microscopy. Five peptides were successfully directed to the mitochondria in protoplasts. These five peptides were then mixed with a pDNA encoding a reporter gene and then transfected into protoplasts and microspores of triticale. All five peptides were shown to form complexes with pDNA and to effectively promote reporter gene expression in the mitochondria.

For the identification of critical MTS sequences, Obita and et al. deduced the consensus motifs for the recognition site of TOM20.[71] They investigated the possibility of a six-residue amphiphilic peptide ($\sigma\varphi\chi\beta\varphi\varphi$; σ: hydrophilic; φ: hydrophobic; β: basic; χ: any amino acid) that forms a helical structure that is responsible for TOM20 recognition by estimating the relative affinities of the cytosolic domain of TOM20 with a variety of peptide sequences derived from aldehyde dehydrogenase (ALDH). In another approach, Wattenberg et al. focused on tail-anchored proteins located on the mitochondrial outer membrane.[72] Unlike normal mitochondrial peptides, including MTSs in the N-terminus, tail-anchored proteins contain a functional domain at the N-terminus and an anchoring domain at the C-terminus, and the translocation signal to the specific organelle is included in the C-terminus.[102] To identify the important parameters responsible for the mitochondrial translocation of tail-anchored proteins, the authors designed artificial proteins on the basis of the mitochondrial outer membrane protein monoamine oxidase A. The screening indicated that the hydrophobicity/hydrophilicity balance and a helical structure with polar amino acids on both sides are crucial for the mitochondrial targeting of tail-anchored proteins and that a precise sequence is not required for targeting.

Lipophilic Cation-Based Mitochondrial Targeting. The conjugation of lipophilic cations, such as TPP, as ligand molecules is a solid approach for mitochondria-targeted gene delivery as well as for the delivery of small drugs because lipophilic cations facilitate the electrostatic interaction between the delivery carrier and the mitochondria. Faria and co-workers conjugated TPP with the high-molecular weight agent poly(ethylene glycol)−poly(ethylenimine) (PEG−PEI) through amide coupling (PEG−PEI−TPP),[73] which enhanced both the internalization of pDNA into the mitochondria and cellular uptake. Finally, the PEG−PEI−TPP NPs significantly enhanced the expression of the reporter gene and ND4 protein.

A group at the University of Beira Interior developed rhodamine 123 (Rho123)-modified gene delivery carriers for mitochondrial targeting.[74,75] To construct Rho123-labeled NPs loaded with pDNA, the authors utilized a coprecipitation method, which was induced by the interaction between Ca^{2+} and pDNA in the presence of CO_3^{2-}. Interestingly, the size of the NPs was manipulated by the incorporation of cellulose or gelatin as a stabilizer. On the basis of confocal laser microscopy and fluorescence intensity observations in the extracted mitochondria, the NPs dominantly accumulated in the mitochondria and effectively promoted reporter gene expression.

Gemini surfactants, composed of two hydrophobic tails and two hydrophilic head groups linked by a spacer, have been widely used as effective nuclear transfection reagents on the basis of low critical micelle concentrations, potentially reducing cytotoxicity due to the small amount of surfactant required to condense pDNA.[103−105] Cationic head gemini surfactants can enable the targeting of mitochondria. NPs prepared from serine-derived bis-quat gemini surfactants and pDNA exhibited a high transfection efficiency in mitochondria, and approximately 40% of the cultured cells showed positive reporter gene expression.[76] Intriguingly, the NPs were internalized through endocytosis and direct membrane translocation. These two routes facilitated cellular uptake, followed by localization to the mitochondria.

Dequalinium (DQA: 1,1′-(1,10-decamethylene-*bis*-[aminoquinaldinium])-chloride)-based NPs complexed with pDNA, called DQAsomes, are also known as promising mitochondria-targeting carriers.[106,107] DQA is an FDA-approved cationic lipid and can form liposome-like structures with pDNA through electrostatic interactions.[108] DQAsomes loaded with a pDNA encoding GFP induced mitochondrial gene expression in cultured cells.[77] However, the transfection efficiency of DQAsomes in the mitochondria was limited to only 5%. To address this issue, Choi and co-workers mixed DQA with other lipids, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE), to further improve the functionalization of DQAsomes.[78] The combined use of DQA and DOTAP/DOPE improved the cellular uptake and endosomal escape ability, further enhancing the mitochondrial gene expression beyond that achieved with DQAsomes.

Lipophilic cation-based carriers have been used to successfully deliver loaded pDNA into the mitochondria and to induce reporter gene expression, but few carriers have successfully delivered functional proteins specific to the mitochondria. To consolidate lipophilic cation-based strategies, mitochondrial function assays after functional gene transfection must be evaluated.

Mitochondrial Fusion Lipid-Based Delivery. Another lipid-based delivery carrier, MITO-Porter, represents an exciting strategy for mitochondrial targeting.[30,109−112] MITO-Porter is a liposomal carrier composed of DOPE, sphingomyelin (SM), and the cell-penetrating peptide (CPP) octaarginine (R8). MITO-Porter can deliver loaded cargoes to mitochondria through a membrane fusion mechanism induced by DOPE, and cellular uptake is accelerated by R8 on the surface. This membrane fusion system is capable of delivering a variety of cargos to mitochondria.[30] To enhance the efficiency of exogenous pDNA transfection into mitochondria using MITO-Porter, Yamada et al. explored the use of another CPP, the KALA peptide with lysine−leucine−alanine repeats, to fine-tune the intracellular trafficking process.[81] The transfection efficiency of the KALA-modified MITO-Porter construct (KALA-MITO-Porter) was approximately 10-fold higher than that of the R8-modified MITO-Porter construct; however, the replacement of R8 with KALA unfortunately increased the cytotoxicity, which was most likely due to the destabilization of the mitochondrial membrane induced by the KALA peptide. The authors further enhanced the functionality of KALA-MITO-Porter by integrating an additional mitochondrial RNA aptamer (RP) ligand to enhance cellular uptake and

https://doi.org/10.1021/acsbiomaterials.1c01114
ACS Biomater. Sci. Eng. 2022, 8, 348−359

Table 2. Summary of mtDNA Editing Technologies[a]

gene editing approaches	mitochondria-targeting strategy	target	outcomes	ref
meganuclease	MTS conjugation	mouse liver and skeletal muscle	decreasing the level of mutated mtDNA *in vivo*	142
RE	MTS conjugation	Leigh's disease cells	decreasing the level of mtDNA mutation	128
	MTS conjugation	mouse oocytes and embryos	preventing the transmission of mtDNA	133
ZFN	MTS and NES conjugation	mtDNA mutated cells	target site cleavage	129
	MTS conjugation	heteroplasmic cells	cleaving a target site and decreasing the level of mtDNA mutation	130
	MTS and NES conjugation	cybrid cell model	decreasing the level of mtDNA mutation and restoring mitochondrial respiratory functions	132
		mitochondrial disease model mice	decreasing the level of mtDNA mutation	139
TALEN	MTS conjugation	mitochondrial disease patient's derived cells	decreasing the level of mtDNA mutation	131
	MTS conjugation	mtDNA mutated iPSC	decreasing the level of mtDNA mutation	135
	MTS conjugation	mitochondrial disease patient's derived cells	decreasing the level of mtDNA mutation	134, 136
	MTS conjugation	mitochondrial disease patient-specific iPSCs	decreasing the level of mtDNA mutation and restoring mitochondrial respiratory functions	137
	MTS conjugation	heteroplasmic mtDNA model mouse	decreasing the level of mtDNA mutation and improving the wild-type tRNA level	138
	MTS conjugation	CMS varieties of rice and rapeseed	revealing the genes responsible for CMS	140
	MTS conjugation	*Arabidopsis thaliana*	inducing breaks at target mtDNA sites	141
	MTS conjugation	cultured cells, mouse, and protoplast	C·G to T·A conversion	143−145
CRISPR	MTS conjugation	culturing cells	inducing breaks at target mtDNA sites	146
	MTS conjugation for Cas9 and d-loop structure for gRNA	culturing cells and zebrafish	knock-in of a DNA fragment at the mitochondrial target site	147
	MTS conjugation for Cas9 and RP loop for gRNA	culturing cells	inducing breaks at target mtDNA sites	148

[a]Abbreviations: NES, nuclear export signal; CMS, cytoplasmic male sterility; iPSC, induced pluripotent stem cell.

mitochondria-targeting activity.[80] The RP modification dramatically improved the transfection efficiency of the KALA-MITO-Porter construct loaded with pDNA into the mitochondria.[82] Additionally, the administration of RP/KALA-MITO-Porter did not alter the biomarker levels in mouse serum, suggesting its good biocompatibility for *in vivo* applications. However, as mammalian mitochondria, in which mitochondrial tRNA (tRNA) is supplied by mtDNA, do not import RNA molecules from the cytoplasm, unlike yeast and plant mitochondria, some researchers have questioned the mechanism by which mammalian mitochondria accomplish this cellular process.[113−115] Some studies have claimed that RNA is internalized into mammalian mitochondria from the cytoplasm.[116−120] Thus, further investigation into the mechanism by which nucleic acids enter mammalian mitochondria is necessary.

MITO-Porter technology has been expanded to other nucleic acid delivery methods for mitochondrial gene therapy. Antisense RNA oligonucleotides (ASOs) and small interfering RNAs can knock down mitochondrial RNA through effective mitochondrial targeting, thereby reducing the amount of mutated mRNA and regulating transcription in mitochondria. To demonstrate RNA knockdown in the mitochondria, a COX2-targeting ASO with a D-arm sequence, which is an important signal recognized by *Leishmania* mitochondria,[121] was designed and loaded into the MITO-Porter system.[79] D-arm conjugation was shown to facilitate the transportation of the ASO into the mitochondrial matrix after the fusion of MITO-Porter to the mitochondrial membrane. The targeted mitochondrial *COX2* mRNA was suppressed after D-arm-ASO introduction, but the efficiency was limited, probably due to the low packaging efficiency of D-arm-ASO. To enhance the knockdown efficiency, D-arm-ASO was first complexed with PEI and then loaded into MITO-Porter,[83] which significantly enhanced the knockdown efficiency by up to 40%. However, the high dosage of MITO-Porter utilized in the knockdown experiment was demonstrated to be very cytotoxic.

tRNAs are also a good candidate for mitochondrial gene therapy because mutations in the regions of mtDNA encoding tRNAs are known to cause some mitochondrial diseases. Thus, researchers delivered wild-type tRNA into disease cell types using the MITO-Porter system to decrease the heteroplasmic rate.[84] Transfection of MITO-Porter loaded with wild-type tRNA dramatically decreased the ratio of mutated tRNA by 75% compared to that in the untreated disease cells, thereby improving ATP production and the cellular respiration rate. On the other hand, the expression of wild-type mitochondrial tRNA with a mitochondria-targeting RP sequence in cell nuclei did not improve the cellular heteroplasmic rate. These results indicate the utility of the MITO-Porter system for mitochondrial targeting and highlight the fact that the mechanism by which RNA is transported from the cytoplasm to mitochondria remains unknown.

Physical Approaches to Mitochondrial Gene Delivery. Physical approaches not involving delivery carriers have been used to introduce exogenous nucleic acids into cells.[122−124] Collombet et al. introduced a 7.2 kbp pDNA into the mitochondria of cultured cells using electroporation without disrupting the mitochondria.[85] Southern blot and immunoelectron microscopy analyses revealed that approximately half of the transfected pDNA reached the mitochondrial intermembrane space or matrix. While the mitochondrial transfection

https://doi.org/10.1021/acsbiomaterials.1c01114
ACS Biomater. Sci. Eng. 2022, 8, 348−359

efficiency of pDNA was high in cultured cells, targeting mitochondria using *in vivo* electroporation is difficult.

The hydrodynamic injection technique, which enhances the permeability of endothelial cells and makes the surrounding cell membrane unstable,[125] is known to be an effective method for *in vivo* transfection. Yasuzaki et al. successfully delivered pDNA into the mitochondria of skeletal muscle cells via limb vein hydrodynamic injection.[86] They further improved the efficiency of hydrodynamic injection into mitochondria by complexing pDNA with the cationic protein protamine.[87] The main advantage of physical approaches is that they typically do not require carrier components, which are cationic materials and sometimes damage cells and tissues.[126,127] However, actively targeting mitochondria by physical methods is quite difficult because pDNA randomly enters cells and is accidentally transported into the mitochondrial matrix. Another disadvantage of physical approaches is that cells and tissues can be damaged even without the use of delivery carriers.

MITOCHONDRIAL GENOME EDITING

Another important aspect of mitochondrial gene therapy is the genomic editing of mtDNA, which can further our understanding of mitochondrial biogenesis and the mechanisms underlying mitochondrial disorders. Insights obtained from numerous studies on nuclear genome editing by the restriction endonuclease (RE), zinc-finger nuclease (ZFN), and transcription activator-like effector nuclease (TALEN) methods have contributed to the development of mtDNA editing technologies. As these editing systems are based on the interactions between editing proteins and DNA, MTS conjugation with these editors can accelerate the import into mitochondria. In some fascinating studies on meganucleases, RE, ZFN, and TALEN, MTS-conjugated editors expressed in the nucleus were transported into mitochondria and then induced breaks at the mtDNA target sites in cultured cells, plants, and disease model mice (Table 2),[128−142] which helped to eliminate mutated mtDNA to improve heteroplasmic conditions and identify the genes responsible for cytoplasmic male sterility in plants. Moreover, Mok et al. successfully converted C·G to T·A in mtDNA with high targeting specificity and editing accuracy by using an MTS- and TALE-conjugated double-stranded DNA deaminase toxin A (DddA),[143] which was used to construct a disease model with mtDNA mutations at a specific sequence. Additionally, the technology was expanded to mouse and plant mitochondria and chloroplast genome editing.[144,145] MTS-conjugated editing was also applied to the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system[146−148] in which ribonucleoprotein (RNP) complexes with a guide RNA (gRNA) to induce a break at a target sequence. However, as mentioned above, as the mechanism by which RNA is transported into mammalian mitochondria from the cytoplasm remains unclear, further mechanistic analysis is required to evaluate the effectiveness of these MTS-CRISPR systems.

CHALLENGES AND FUTURE DIRECTIONS

The mutation of mtDNA severely damages cells and results in various inherited mitochondrial diseases even though mammalian mtDNA encodes only 37 genes. Mitochondrial dysfunction is induced when the ratio of mutated mtDNA exceeds a certain threshold. Thus, the amelioration of mtDNA mutations is an important approach in the gene therapy field. As shown in Figure 1, the replacement of wild-type mtDNA or essential proteins encoded in the mutated region and the repair or elimination of mutated mtDNA are considerable therapeutic strategies for mitochondrial diseases. Among the methods established thus far, the expression of MTS-fused proteins, especially genome editors, in cell nuclei was shown to be effective *in vivo*, but continuous expression of genome editors may cause unfavorable cytotoxicity in host cells. The direct *in vivo* delivery of genes into mitochondria should be safer and thus overcome this limitation. For secured *in vivo* delivery, carriers must have other essential abilities in addition to targeting mitochondria, including a high colloidal stability to protect loaded genes from nuclease degradation.[3] Furthermore, as mitochondrial dysfunction caused by mitochondrial diseases unfortunately affects various organs, carriers must be capable of delivering loaded genes to multiple organs without being rapidly cleared by the liver or spleen.[149−151] For preferable biodistribution, MTS-presenting NPs may not be suitable because MTSs, which are composed of cationic and hydrophobic residues, potentially interact with biomacromolecules and form aggregates in the physiological environment. Thus, more precise and sophisticated designs of molecular NP components are required to optimize biodistribution. To accommodate mitochondrial gene therapy *in vivo*, the expansion of mitochondrial targeting seems to be more achievable with peptidic and polymeric NPs harboring MTSs due to their advantageous ability to incorporate a wide variety of functional molecules and thereby overcome biological barriers en route to the target sites. For example, shielding MTS-conjugated NPs with biocompatible polymers without a size increase through a cleavable linkage protects the MTS during the delivery process and enables its retainment after cellular uptake at the target site. Moreover, mitochondrial gene therapies have thus far aimed to deliver loaded genes into the mitochondria, but the release of loaded genes after reaching the mitochondria to promote a smooth transition to the transcription process will be necessary in the future. From this viewpoint, peptidic and polymeric NPs appear to be promising because the cationic strands are easily functionalized by modification of the cationic strand. For example, they can be modified to promote ROS[152−156] and ATP[157−162] responsiveness and may thus be useful to further improve mitochondrial gene therapies in the future. Additionally, the integration of other functions into the MTS itself should be an attractive direction for MTS-based mitochondrial targeting. The currently available artificial MTSs were designed to enhance the mitochondria-targeting function and to elucidate details regarding the MTS-based delivery mechanism. From the viewpoint of functionality, the development of dual- or multifunctional peptides, such as mitochondria-targeting and cell-penetrating peptides, may dramatically improve the efficiency of mitochondrial delivery. Actually, the identification of peptides with this capability among tremendous numbers of candidate sequences is experimentally possible, but rapid advances in computational science can also contribute to the design of sophisticated peptides. For the design of multifunctional peptides, the mechanism underlying MTS-mediated mitochondrial entry must be further elucidated.

The establishment of mitochondrial genome editing technology is also an important direction for research on mitochondrial gene delivery. Current strategies involve the

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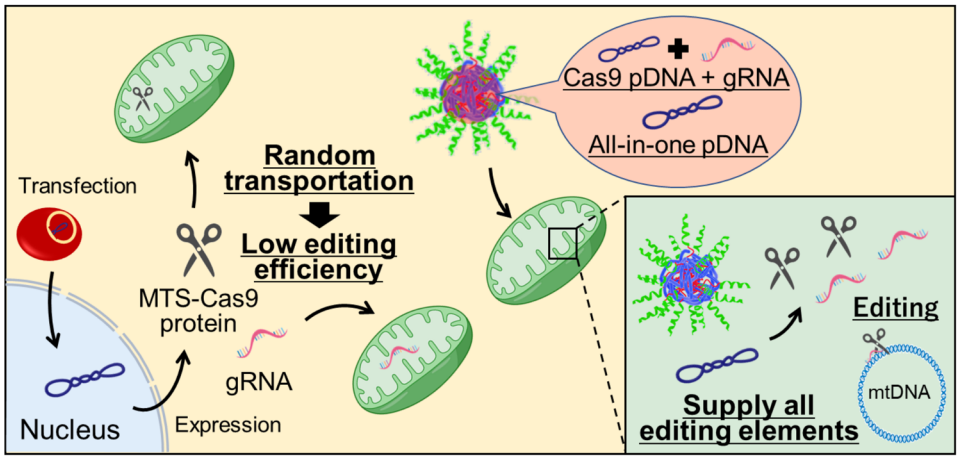

Figure 3. Schematic illustration of mitochondrial CRISPR/Cas9-based genome editing strategies. The current systems introduce a pDNA encoding Cas9 and gRNA and expect them to accumulate in the mitochondria. Random transportation may lead to limited mtDNA editing. On the other hand, the direct delivery of gene editing components to the mitochondria can improve the editing efficiency.

expression of MTS-fused gene editors in cell nuclei, thereby eliminating the mutated mtDNA. In addition to the genomic editing of mutated mtDNA, CRISPR activation (CRISPRa) combined with an endonuclease-deficient dCas9 fused to a transcriptional activator[163] may also be a good therapeutic approach for ameliorating heteroplasmic mtDNA. The use of dual- and multi-gRNAs allows the simultaneous upregulation of several target genes, which enables the supply of wild-type mitochondrial transcripts even under heteroplasmic conditions. To gain effective CRISPR-mediated effects, the introduction of gRNAs into the mitochondria with the normal CRISPR system is a major hurdle. Even though the expression of MTS-Cas9 and gRNA in the nucleus may be achievable, there has been no established CRISPR-based mtDNA editing technology, which indicates that a simple design using MTS is not enough to deliver all CRISPR-based editing tools in the mitochondria. Thus, both Cas9 and gRNA should possess mitochondria-targeting activity for CRISPR-mediated mtDNA editing. However, regardless of whether or not RNA molecules are introduced into mammalian mitochondria from the cytoplasm, the chance of both the Cas9 protein and the gRNA accumulating in the same mitochondrion is probably limited because these components are randomly directed to mitochondria. Furthermore, the use of multi-gRNAs makes the collection of all components into one mitochondrion difficult. However, carrier systems could be further improved to directly deliver genome editing elements to mitochondria, thereby enabling the formation of RNPs in one mitochondrion. An "all-in-one" pDNA encoding all CRISPRa components or a carrier encapsulating both gRNAs and dCas9-encoding pDNA could be delivered to mitochondria to supply all necessary CRISPRa components (Figure 3). NPs composed of PEG-poly(amino acid) derivatives were recently reported to encapsulate both the *Cas9*-encoding mRNA and gRNA with high loading efficiency, and the NP preparation process was not complicated.[164] The easy-loading property of polymeric (peptidic) NPs may motivate researchers to develop a cutting-edge mitochondrial genome editing system.

DNA insertion into mtDNA is an important approach to mitochondrial gene delivery beyond disease treatment. Mitochondria lack a nonhomologous end-joining repair system, and homologous recombinant repair is thus the dominant pathway, suggesting that DNA insertion into mtDNA is promising. However, mammalian mtDNA has few noncoding regions, and the insertion of the desired DNA without disrupting the function of the mtDNA may thus be difficult. On the other hand, plant mtDNA, which is larger in size and has many noncoding regions, may be more suitable for DNA insertion. Since plant mtDNA encodes genes responsible for cytosolic male sterility, the insertion of genes related to male sterility into plant mitochondria ameliorates the concern of the transgene being spread into the environment through pollen. For genetic recombinants, effective gene delivery carriers are, of course, necessary for the introduction of functional DNA into mtDNA despite advances in editing technologies. As mentioned above, codelivery approaches based on peptidic or polymeric materials are very useful for the delivery of editors and DNA into one mitochondrion.

In conclusion, current technologies involve the expression of functional proteins encoded in mitochondria. Future developments should focus on carrier designs to overcome the barrier of the long journey to mitochondria *in vivo* and on the genomic editing of mtDNA. When these limitations are finally overcome, the mechanisms of mitochondrial biogenesis will be elucidated in detail, and innovative therapeutic approaches for inherited mitochondrial diseases will be developed. Moreover, because the delivery of genes into mitochondria is a key phenomenon across species, the successful development of a mitochondrial gene delivery strategy would advance various research fields and thus enrich the lives of humans.

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https://doi.org/10.1021/acsbiomaterials.1c01114
ACS Biomater. Sci. Eng. 2022, 8, 348−359

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by Japan Science and Technology Agency Exploratory Research for Advanced Technology (grant JPMJER1602 to K.N.).

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