

## A Method for Screening Mitochondrial Fusogenic Envelopes for Use in Mitochondrial Drug Delivery

Yuma Yamada and Hideyoshi Harashima

### Abstract

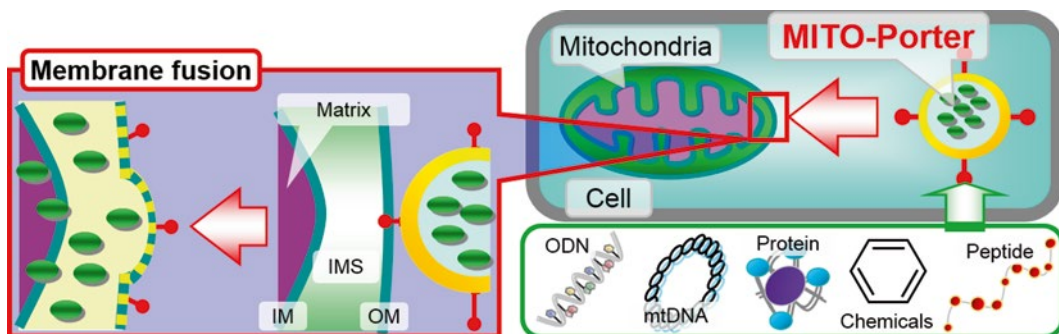
Various types of mitochondrial dysfunctions have been implicated in a variety of human diseases. This suggests that mitochondria would be promising therapeutic drug targets and mitochondrial therapy would be expected to be useful for the treatment of various diseases. We have already reported the development of a MITO-Porter, a liposome-based nano-carrier that delivers its cargo to mitochondria via a membrane-fusion mechanism. In our strategy for delivering cargos to mitochondria using a MITO-Porter, the carriers must fuse with the organelle membrane. Here we report on methodology for screening various types of lipid envelopes that have the potential for fusing with a mitochondrial membrane. The method involves monitoring the cancellation of fluorescence resonance energy transfer (FRET) and evaluating membrane fusion between the carriers and mitochondria in living cells by FRET analysis using a spectral imaging fluorescent microscopy system.

**Key words** Mitochondria, Mitochondrial drug delivery, Mitochondrial macromolecule delivery, MITO-Porter, Membrane fusion, Mitochondrial gene therapy, Mitochondrial medicine, Octaarginine

---

### 1 Introduction

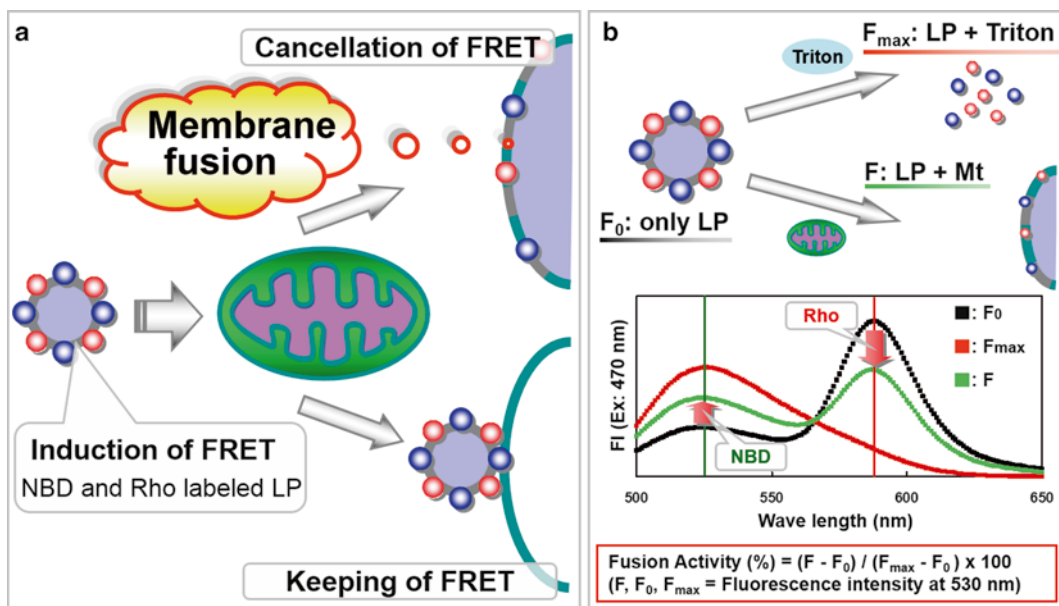
Various types of mitochondrial dysfunction have recently been implicated in a variety of human diseases. Therefore, this organelle is a promising therapeutic drug target, and mitochondrial therapy would be expected to be useful and productive for the treatment of various diseases. The targeted delivery of an engineered gene or gene product to the mitochondrion is an essential first step towards the therapeutic restoration of a missing cellular function. The conjugation of a mitochondrial-targeting signal (MTS) peptide to exogenous proteins and small linear DNAs was reported to aid their delivery to mitochondria [1–4], but this strategy is not viable for delivering pDNA. This is because large molecules such as pDNA and folded proteins do not readily pass through the mitochondrial membrane, thus making it nearly impossible for them to be easily delivered to mitochondria [5, 6].



**Fig. 1** MITO-Porter is surface-modified with a high density of R8, which can be internalized by cells via macropinocytosis. When the MITO-Porter reaches the cytosol, it binds to mitochondria via electrostatic interactions with R8. Encapsulated compounds are delivered to the intramitochondrial compartment via fusogenic lipids that fuse to the mitochondrial membrane. *OM* outer membrane, *IMS* intermembrane space, *IM* inner membrane [22]

To overcome these problems, we recently proposed an original and innovative strategy for overcoming the mitochondrial membrane via membrane fusion, namely, the development of a MITO-Porter system (Fig. 1) [7–9]. This MITO-Porter is potentially promising for use in delivering a wide variety of carrier-encapsulated molecules, regardless of size or physicochemical properties, to mitochondria. The first barrier to intracellular targeting is the plasma membrane. In a previous study, we showed that high-density octaarginine (R8)-modified liposomes are taken up mainly through macropinocytosis and are delivered to the cytosol while the aqueous phase marker is retained [10]. Therefore, we used R8 as a cytosol delivery device for the MITO-Porter. We also predicted that R8, which mimics TAT, might have mitochondrial-targeting activity [8, 11]. To deliver cargos to target organelles using our strategy, the liposomes must fuse with the organelle membrane. As previously reported, R8-modified envelopes composed of 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE), and 5-cholesten-3-ol 3-hemisuccinate (CHEMS) showed high transfection activities in dividing cells [12]; however, these lipids may not be the best lipid composition for use in targeting mitochondria.

Therefore, screening was initiated for lipid compositions that are adequate for fusion to the mitochondrial membranes. This was done by monitoring the cancellation of fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores, modified on the surface of liposomes [13–15]. Liposomal membranes were labeled with both 7-nitrobenz-2-oxa-1,3-diazole (NBD) (excitation at 460 nm and emission at 534 nm) and rhodamine (excitation at 550 nm and emission at 590 nm) so that energy would be transferred from NBD to rhodamine. Membrane fusion between the dual-labeled liposomes and the mitochondria would lead to the diffusion of NBD and rhodamine into the lipid



**Fig. 2** Schematic image (a) and protocol (b) for a FRET analysis using isolated mitochondria to evaluate the mitochondrial membrane fusion. LP dual-labeled liposome

membranes, which causes a reduction in energy transfer, resulting in an increase in fluorescence intensity at 530 nm (Fig. 2).

To date, we have successfully identified lipid compositions for the MITO-Porter that promote both its fusion with the mitochondrial membrane and the release of its cargo to the intramitochondrial compartment in living cells [7, 16]. In this chapter, we describe the method used for the screening of a series of lipid envelopes that are able to efficiently fuse with isolated rat liver mitochondria. This was achieved by varying the lipid composition of a panel of liposomes and monitoring membrane fusion by FRET analysis. In addition, membrane fusion between the MITO-Porter and mitochondria in living cells was evaluated by FRET analysis using a spectral imaging fluorescent microscopy system [17].

## 2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C) and commercially available reagent-grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated).

### 2.1 Lipids

- 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) (Avanti Polar lipids, Alabaster, AL, USA).
- 5-cholesten-3-ol 3-hemisuccinate (CHEMS) (Sigma, St. Louis, MO, USA).

3. Cardiolipin (CL) (Sigma).
4. Phosphatidic acid (PA) (Sigma).
5. Phosphatidyl glycerol (PG) (Sigma).
6. Phosphatidyl inositol (PI) (Sigma).
7. Phosphatidyl serine (PS) (Sigma).
8. Sphingomyelin (SM) (Sigma).
9. Cardiolipin (CL) (Sigma).
10. Stearyl octaarginine (STR-R8) (Kurabo Industries Ltd, Osaka, Japan) [18].
11. 7-nitrobenz-2-oxa-1, 3-diazole-labeled DOPE (NBD-DOPE) (Avanti Polar lipids).
12. Rhodamine-labeled DOPE (Rho-DOPE) (Avanti Polar lipids).

## **2.2 Liposome Preparation**

1. Lipid stocks: dissolve 1 mM lipid in 100% ethanol (*see* **Notes 1–4**). Store at  $-20^{\circ}\text{C}$ .
2. Mitochondrial isolation buffer (MIB): 250 mM sucrose, 2 mM Tris-HCl, pH 7.4. Store at  $4^{\circ}\text{C}$ .
3. Bath-type sonicator (85 W) (Aiwa Co., Tokyo, Japan).
4. Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany).

## **2.3 FRET Analysis Using Isolated Mitochondria**

1. Prepare isolated mitochondria from livers of Wistar rats (6–8 weeks old), as reported by Shinohara et al. [19, 20] (*see* **Notes 5 and 6**). Store on ice.
2. For the FRET analysis, prepare a mitochondrial suspension in MIB (corresponding to the 0.9 mg of mitochondrial protein/mL) (*see* **Note 7**).
3. Triton sol.: 0.5% Triton X-100 (Sigma) in MIB (v/v).
4. Fluorescence spectrometer: FP750 instrument (JASCO, Tokyo, Japan).

## **2.4 Evaluation of Membrane Fusion in Living Cells Using Spectral Imaging Fluorescent Microscopy**

1. HeLa cells: HeLa human cervix carcinoma cells (RIKEN Cell Bank, Tsukuba, Japan).
2. DMEM: Dulbecco's Modified Eagle Medium (Invitrogen Corp., Carlsbad, CA, USA) (*see* **Note 8**).
3. FBS: inactivate fetal bovine serum (Invitrogen Corp.).
4. 35 mm glass-base dishes (IWAKI, Tokyo, Japan).
5. PBS (–): 137 mM NaCl, 2.68 mM KCl, 8.05 mM,  $\text{Na}_2\text{HPO}_4$ , 1.47 mM  $\text{KH}_2\text{PO}_4$  in water. Autoclave.
6. MitoFluor Red 589 [excitation at 588 nm and emission at 622 nm] (Molecular Probes; Eugene, OR, USA).
7. Confocal laser scanning microscopy (CLSM): LSM510 META (Carl Zeiss Co. Ltd., Jena, Germany).

**Table 1**  
**Lipid composition of dual-labeled liposome**

1 mM Lipid X <sup>a</sup>	112.5 $\mu$ L
1 mM Lipid Y <sup>b</sup>	25 $\mu$ L
0.1 mM NBD-DOPE	13.8 $\mu$ L
0.1 mM Rho-DOPE	6.9 $\mu$ L
CHCl <sub>3</sub>	125 $\mu$ L

<sup>a</sup>Lipid X is DOPE or EPC

<sup>b</sup>Lipid Y is CL, PI, PG, PS, PA, CHEMS, SM, or Chol

### 3 Methods

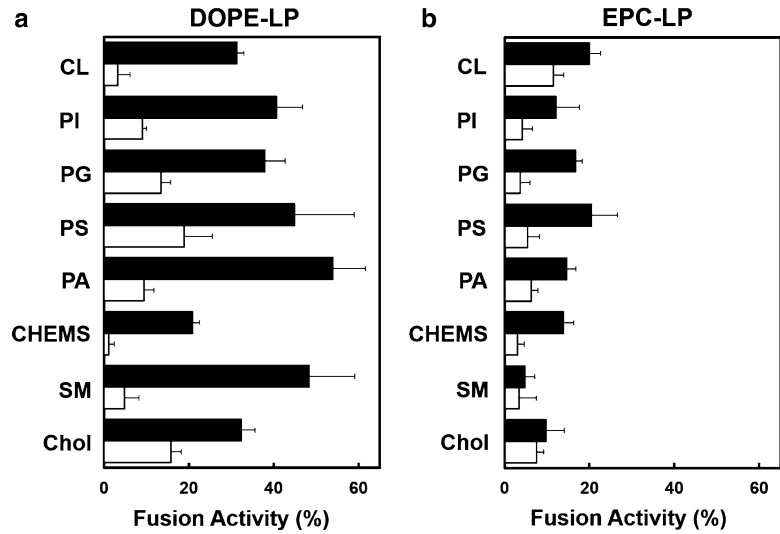
All procedures should be carried out at room temperature, unless otherwise specified.

#### 3.1 Preparation of Dual-Labeled Liposome for the Screening Assay by FRET

1. Add lipid stocks and chloroform to a glass tube following lipid composition list as shown in Table 1 (*see Note 9*).
2. Evaporate the organic solvent by means of a vacuum pump to form lipid films on the bottom of the glass tube (*see Notes 10 and 11*).
3. Add 0.25 mL of MIB to the dried lipid film on the bottom of a glass tube, and then incubate the suspension for 10–15 min at room temperature to achieve hydration (*see Note 12*).
4. Sonicate the suspensions using a bath-type sonicator for 15–30 s to generate empty vesicles (lipid concentration of liposomes, 0.55 mM) (*see Note 13*).
5. Measure the particle diameters of the liposomes using a quasi-elastic light-scattering method, and determine the  $\zeta$ -potentials by electrophoretically using laser Doppler velocimetry (Zetasizer Nano ZS) (*see Notes 14–16*).

#### 3.2 FRET Analysis Using Isolated Mitochondria to Evaluate Fusion with the Mitochondrial Membrane

1. Add a 10- $\mu$ L aliquot of dual-labeled liposomes to a mitochondrial suspension in 90  $\mu$ L of MIB to prepare sample [*F*].
2. As a control without mitochondria, prepare sample [*F*<sub>0</sub>] by adding a 10- $\mu$ L aliquot of dual-labeled liposomes into 90  $\mu$ L of MIB.
3. Add a 10- $\mu$ L aliquot of dual-labeled liposomes into 90  $\mu$ L of Triton sol. [*F*<sub>max</sub>], to define the maximum fluorescence of liposomes dissolved.
4. Incubate these samples for 30 min at 25°C in the dark, after mixing on a vortex mixer.
5. After incubation, measure the fluorescence intensity (excitation at 470 nm and emission at 530 nm) using fluorescence spectrometer to assess the energy transfer (*see Note 17*).



**Fig. 3** The screening of fusogenic lipid compositions with the mitochondrial membrane using isolated mitochondria. Fusion activities (%) of DOPE-contained liposomes (**a**) and EPC-contained liposomes (**b**) were calculated in terms of reduction of FRET. Data are presented as the mean of triplicate runs. *Closed bars* R8-LP, *open bars* unmodified liposome [7, 22]

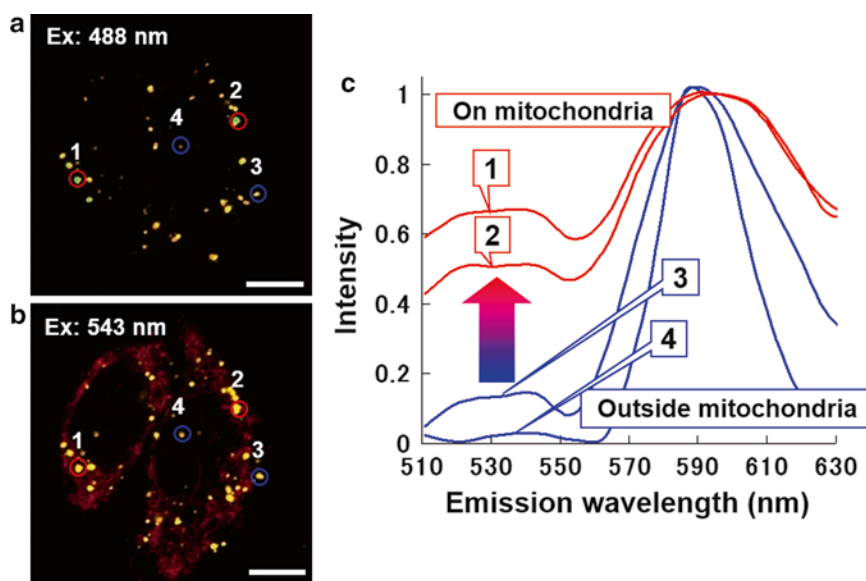
6. Estimate the fusion activity (%) by the reduction in the level of energy transfer as a function of membrane fusion (Fig. 3), which is calculated as follows:

$$Fusion\ activity\ (\%) = \frac{(F - F_0)}{(F_{max} - F_0)} \times 100$$

where  $F$ ,  $F_0$ , and  $F_{max}$  represent the fluorescence intensity of dual-labeled liposome after incubation with and without mitochondria and the maximum fluorescence intensity after a treatment with Triton X-100, respectively (*see Note 18*).

**3.3 Evaluation of Membrane Fusion in Living Cells Using Spectral Imaging Fluorescent Microscopy**

1. Culture HeLa cells ( $4 \times 10^4$  cells/dish) in 35 mm glass-base dishes with 2 mL of DMEM, which contained 10% FBS, under 5% CO<sub>2</sub>/air at 37°C for 24 h.
2. Wash the cells with ice-cold PBS (–), and incubate with the dual-labeled liposomes in 1 mL of serum-free DMEM (final lipid concentration, 5.4 μM) (*see Note 19*).
3. Wash the cells with ice-cold PBS (–) after a 1-h incubation under 5% CO<sub>2</sub>/air at 37°C, and further incubate the cells in DMEM containing 10% serum for 1 h in the absence of the carriers.



**Fig. 4** (a) Spectral image figures were observed in the case of excitation at 488 nm to evaluate membrane fusion by FRET. (b) Spectral image figures were observed in the case of excitation at 543 nm to identify the localization of liposomes (yellow color) and mitochondria (red color). (c) Fluorescence spectra of ROI were selected from Fig. 4a, and the emission was normalized to the Rho-DOPE peak fluorescence intensity. Scale bars, 10  $\mu\text{m}$  [7]

4. After the incubation, replace with fresh DMEM containing MitoFluor Red 589 (final concentration, 100 nM), and incubate further for 20 min to permit the mitochondria to be stained.
5. After the incubation, wash the cells with ice-cold PBS (–) and then observe them by CLSM (LSM510 META) using an oil-immersion objective lens (Plan-Apochromat 63x/NA=1.4).
6. To evaluate membrane fusion between the carriers and mitochondria, excite the NBD-DOPE in carriers within cells by light (488 nm) derived from an argon laser. Analyze spectroscopically the emitted light filtered through a dichroic mirror (HFT488) (510–630 nm) as shown in Fig. 4a (see Note 20).
7. Select fluorescence spectra of the ROI (region of interest) in several signals of Fig. 4a, and normalize the emission at each wavelength by the Rho-DOPE peak fluorescence intensity (emission at 590 nm), as shown in Fig. 4c (see Note 21).
8. To identify the localization of the carriers on the mitochondria, obtain spectral images ranging from 570 to 630 nm with a He/Ne laser (543 nm) using a dichroic mirror (HFT UV/488/543/633) as shown in Fig. 4b (see Notes 22 and 23).



---

## 4 Notes

1. Dissolve the lipid by sonication using bath-type sonicator, when the lipid is not readily soluble in the solvent.
2. Dissolve the lipid in 100% ethanol/chloroform (1:1, v/v) when PA is used, and store in a glass tube (not a plastic tube).
3. Store lipid stock solutions in tubes wrapped with aluminum foil in the case of fluorescent-labeled lipids (NBD-DOPE and Rho-DOPE).
4. Incubate lipid stocks at room temperature before use.
5. Isolated mitochondria should be freshly prepared each time and should be used as soon as possible.
6. When mitochondria are isolated by this procedure, the purity of the mitochondrial preparation was found to be greater than 90% as judged by electron microscopy observations, and the respiration control index of the mitochondria was determined to be 4.5–6 using a Clark oxygen electrode (YSI 5331; Yellow Springs Instrument Co., Yellow Springs, OH, USA), indicating that the mitochondria were intact and actively respiring. Please *see* refs. 19, 20.
7. Determine the concentrations of mitochondrial proteins using a BCA protein assay kit (Pierce; Rockford, IL, USA), after treatment with a detergent such as sodium dodecyl sulfate to lyse the mitochondria.
8. Use phenol red-free DMEM (Invitrogen Corp.) when the cells are observed.
9. We previously successfully prepared dual-labeled liposomes with various compositions of lipids for screening the fusogenic membrane with mitochondria [7].
10. Mix the lipid solution lightly by vortex mixing and wrap the glass tubes with aluminum foil, before removing the organic solvent by vacuum evaporation.
11. Check that a thin lipid film is formed. If you need to optimize the lipid film, after adding 125  $\mu\text{L}$  of 100% ethanol/chloroform (1:1, v/v) to the glass tubes, remove it by vacuum evaporation.
12. Mix the lipid suspension by vortex mixer before sonication.
13. In the case where the liposomes are modified with R8, add an STR-R8 solution (10 mol% of lipids) to the resulting suspension (liposomes) and then incubate for 30 min at room temperature after gentle mixing.
14. Overall, the  $\zeta$ -potentials of the neutral lipid-containing liposomes, the anionic lipid-containing liposomes, and the R8-modified liposomes should be approximately 0 mV, –50 mV, and +50 mV, respectively [7].



15. The  $\zeta$ -potentials of the R8-modified liposomes were positively charged, indicating that the surface of the carrier was modified with R8 [7].
16. We find that it is best to prepare the liposomes fresh each time.
17. FRET analysis is frequently used to detect protein interactions in living cells [21]. In this study, we applied this method to evaluate membrane fusion as shown in Fig. 2.
18. As shown in Fig. 3, R8-modified liposomes, which are composed of DOPE/SM/STR-R8 (9:2:1) or DOPE/PA/STR-8 (9:2:1), show a high fusogenic activity with mitochondria.
19. The MITO-Porter used in this experiment is composed of DOPE/SM/STR-R8 (9:2:1, molar ratios). It has been reported that high-density R8-modified liposomes are taken up mainly through macropinocytosis and delivered to the cytosol [10].
20. Through the spectra ranges used in the analysis, background fluorescence derived from the emission from Rho-DOPE and MitoFluor Red 589 was negligible when they were excited at 488 nm (data not shown).
21. In the non-fusogenic state, the emission energy of NBD (donor) is transferred to the excitation energy of rhodamine (acceptor). As a result, the fluorescence intensity of NBD at 530 nm was minimized (lines 3 and 4 in Fig. 4c). Lipid mixing by membrane fusion between the dual-labeled liposomes and mitochondrial membranes results in the diffusion of the donor and acceptor; this results in a reduction in FRET and an increase in the fluorescence intensity at 530 nm (lines 1 and 2 in Fig. 4c).
22. Carriers (yellow) and mitochondria (red) can be clearly distinguished, since the emission spectral peak of MitoFluor Red 589 was detected at the far-red region (emission at 622 nm) compared with that of Rho-DOPE (emission at 590 nm), as shown in Fig. 4b.
23. In Fig. 4c, the spectra present on and outside the mitochondria appear as red or blue lines, respectively. The fluorescence intensity at 530 nm increased significantly, indicating a reduction in energy transfer due to fusion between the liposomal and mitochondrial membranes on the mitochondria. On the other hand, no such increase at 530 nm is evident outside the mitochondria.

---

## Acknowledgements

This work was supported, in part, by the Grant-in-Aid for Young Scientists (A) and Grant-in-Aid for Challenging Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japanese Government (MEXT). We also thank Dr. Milton Feather for his helpful advice in writing the manuscript.

## References

1. Flierl A, Jackson C, Cottrell B, Murdock D, Seibel P, Wallace DC (2003) Targeted delivery of DNA to the mitochondrial compartment via import sequence-conjugated peptide nucleic acid. *Mol Ther* 7:550–557
2. Vestweber D, Schatz G (1989) DNA-protein conjugates can enter mitochondria via the protein import pathway. *Nature* 338:170–172
3. Schatz G (1987) 17th Sir Hans Krebs lecture. Signals guiding proteins to their correct locations in mitochondria. *Eur J Biochem* 165:1–6
4. Seibel M, Bachmann C, Schmiedel J, Wilken N, Wilde F, Reichmann H, Isaya G, Seibel P, Pfeiler D (1999) Processing of artificial peptide-DNA-conjugates by the mitochondrial intermediate peptidase (MIP). *Biol Chem* 380:961–967
5. Endo T, Nakayama Y, Nakai M (1995) Avidin fusion protein as a tool to generate a stable translocation intermediate spanning the mitochondrial membranes. *J Biochem* 118:753–759
6. Wiedemann N, Frazier AE, Pfanner N (2004) The protein import machinery of mitochondria. *J Biol Chem* 279:14473–14476
7. Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y, Yamashita K, Kobayashi H, Kikuchi H, Harashima H (2008) MITO-Porter: a liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. *Biochim Biophys Acta* 1778:423–432
8. Yamada Y, Harashima H (2008) Mitochondrial drug delivery systems for macromolecule and their therapeutic application to mitochondrial diseases. *Adv Drug Deliv Rev* 60:1439–1462
9. Kogure K, Akita H, Yamada Y, Harashima H (2008) Multifunctional envelope-type nano device (MEND) as a non-viral gene delivery system. *Adv Drug Deliv Rev* 60:559–571
10. Khalil IA, Kogure K, Futaki S, Harashima H (2006) High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. *J Biol Chem* 281:3544–3551
11. Del Gaizo V, Payne RM (2003) A novel TAT-mitochondrial signal sequence fusion protein is processed, stays in mitochondria, and crosses the placenta. *Mol Ther* 7:720–730
12. Khalil IA, Kogure K, Futaki S, Hama S, Akita H, Ueno M, Kishida H, Kudoh M, Mishina Y, Kataoka K et al (2007) Octaarginine-modified multifunctional envelope-type nanoparticles for gene delivery. *Gene Ther* 14:682–689
13. Struck DK, Hoekstra D, Pagano RE (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* 20:4093–4099
14. Maier O, Oberle V, Hoekstra D (2002) Fluorescent lipid probes: some properties and applications (a review). *Chem Phys Lipids* 116:3–18
15. Akita H, Kudo A, Minoura A, Yamaguti M, Khalil IA, Moriguchi R, Masuda T, Danev R, Nagayama K, Kogure K et al (2009) Multilayered nanoparticles for penetrating the endosome and nuclear membrane via a step-wise membrane fusion process. *Biomaterials* 30:2940–2949
16. Yamada Y, Akita H, Harashima H (2012) Multifunctional envelope-type nano device (MEND) for organelle targeting via a stepwise membrane fusion process. *Methods Enzymol* 509:301–326
17. Haraguchi T, Shimi T, Koujin T, Hashiguchi N, Hiraoka Y (2002) Spectral imaging fluorescence microscopy. *Genes Cells* 7:881–887
18. Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, Harashima H, Sugiura Y (2001) Stearoylated arginine-rich peptides: a new class of transfection systems. *Bioconjug Chem* 12:1005–1011
19. Shinohara Y, Almofti MR, Yamamoto T, Ishida T, Kita F, Kanzaki H, Ohnishi M, Yamashita K, Shimizu S, Terada H (2002) Permeability transition-independent release of mitochondrial cytochrome c induced by valinomycin. *Eur J Biochem* 269:5224–5230
20. Shinohara Y, Sagawa I, Ichihara J, Yamamoto K, Terao K, Terada H (1997) Source of ATP for hexokinase-catalyzed glucose phosphorylation in tumor cells: dependence on the rate of oxidative phosphorylation relative to that of extra-mitochondrial ATP generation. *Biochim Biophys Acta* 1319:319–330
21. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca<sup>2+</sup> based on green fluorescent proteins and calmodulin. *Nature* 388:882–887
22. Yamada Y, Harashima H (2012) Targeting mitochondria: innovation from mitochondrial drug delivery system (DDS) to mitochondrial medicine. *Yakugaku Zasshi* 132:1111–1118

Drug Delivery System

Jain, K.K. (Ed.)

2014, XI, 280 p. 78 illus., 51 illus. in color., Hardcover

ISBN: 978-1-4939-0362-7

A product of Humana Press