Coenzyme Q₁₀ (CoQ₁₀), which is found in most eukaryotic cells, is primarily associated with the inner mitochondrial membrane.¹ It was first isolated from beef heart mitochondria in 1957 by Fredrick Crane.² In 1961, the important role of CoQ₁₀ as an electron carrier in the electron transport pathway was first proposed by Peter Mitchell, making it a subject of interest to medical and pharmaceutical researchers.³,⁴ Since then, substantial amounts of laboratory and animal data on CoQ₁₀ have been published. These findings indicate that, in addition to its function as an electron carrier, CoQ₁₀ acts as an antioxidant and can prevent damage to cellular components caused by free radicals and can also stabilize cell membranes.⁵,⁶ The effect of CoQ₁₀ for the treatment of a variety of medical conditions, including mitochondrial diseases,⁷ cancer,⁸ cardiovascular diseases⁹ and neurodegenerative diseases⁹ has attracted the interest of researchers. Even though the therapeutic effect of CoQ₁₀ has still not been verified, it is generally considered to be safe and is available as an over-the-counter dietary supplement in most countries.¹⁰

However, only a very small percentage of orally administered crystalline CoQ₁₀ is taken up in the blood stream, making the treatment of CoQ₁₀ deficiencies difficult. Even though the absorption levels of CoQ₁₀ are increased in the presence of lipids, temporarily boosting the blood concentration, no overall uptake into tissues could be observed.¹¹ Due to the hydrophobic nature of CoQ₁₀, the use of a liposomal carrier for the delivery might be possible. Since CoQ₁₀ is largely located in the inner mitochondrial membrane, a mitochondrial-targeted delivery system will be needed. We previously reported on the preparation of a MITO-Porter, a mitochondrial-targeted liposome,¹²,¹³ and the fact that it can be used to deliver some mitochondrial functional molecules, including CoQ₁₀, to mitochondria and that excellent pharmaceutical effects, both in vitro and in vivo were observed.¹³,¹⁴ Because of its highly mitochondrial fusogenic lipid components, it is taken up by mitochondria via membrane fusion, thus permitting its cargo to be released into the mitochondrial membrane. CoQ₁₀ can then exert its pharmaceutical effect on the mitochondrial membrane, where endogenous CoQ₁₀ functions as an important functional molecule.

Earlier research showed that CoQ₁₀ can be encapsulated in liposomes by using methods such as the lipid film hydration method, the reverse-phase evaporation (REV) method or the ethanol dissolution method.¹⁴–¹⁸ However, while it has been reported that the method used to prepare such liposomes had an influence on CoQ₁₀ encapsulation efficiency, attempts to optimize CoQ₁₀ encapsulation by varying the initial CoQ₁₀ concentration have not been reported. In addition, a comparison of the characteristics of CoQ₁₀ encapsulated liposomes among different packaging methods have not been reported, which is important information, in terms of establishing the optimal method for packaging CoQ₁₀ in liposomes.

In this study, three liposome preparation methods, namely, the lipid film hydration method (Fig. 1A), the REV method (Fig. 1B) and the ethanol dilution method (Fig. 1C) were compared, with regard to CoQ₁₀ encapsulation efficiency and the drug/lipid ratio of the carrier. As CoQ₁₀ is mainly located in the inner mitochondrial membrane, a mitochondria targeted liposome, a MITO-Porter, was used as a model liposome. After preparation of the carrier, physicochemical properties...
such as size and its distribution—the polydispersity index (PDI)—, the surface charge (ζ-potential) and the concentrations of encapsulated CoQ10 were determined. The structure of CoQ10-MITO-Porter was examined by transmission electron microscopy (TEM). After optimizing the preparation method, the intracellular trafficking of the carrier in patient-derived mitochondrial disease cells was analyzed using confocal laser scanning microscopy (CLSM).

MATERIALS AND METHODS

Chemicals and Materials 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), sphingomyelin (SM), and DOPE-N-(7-nitro-2-1,3-benzoxadiazole-4-yl) (NBD-DOPE) were purchased from Avanti Polar lipids (Alabaster, AL, U.S.A.). 1,2-Dimyristoyl-sn-glycerol, methoxy polyethylene glycol 2000 (DMG-PEG 2000) was obtained from the NOF Corporation (Tokyo, Japan). Stearylated R8 (STR-R8)19 was obtained from KURABO Industries (Osaka, Japan). STR-S2 (stearyl-Dmt-Dmt-Arg-FK-Dmt-Arg-FK-NH2, Dmt=2, 6-dimethyltyrosine)20 was obtained from Toray Research Center (Tokyo, Japan). CoQ10 was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were commercially available reagent-grade products.

Construction of CoQ10-MITO-Porter CoQ10 was added to the lipid components in concentrations of 5, 10, 15, 20 and 25 mol% of the total lipid. A CoQ10-free carrier was also prepared. To investigate the recovery of the lipids, the carriers were labeled with NBD-DOPE (0.1 mol% of the total lipids) and the lipid concentration and drug/lipid ratio was calculated. STR-R8 or STR-S2 solutions (5 mol% total lipid) were added to the suspension to attach the peptide to the surface for intracellular trafficking analysis. To investigate physicochemical properties such as size and the concentration of encapsulated CoQ10 and TEM analysis, the liposomes were used before being modified with the peptides.

Preparation of CoQ10-Liposomes by Lipid Film Hydration Method

Liposomes were prepared by previously described methodology.12 Lipid stock solutions prepared in chloroform and CoQ10 and DMG-PEG 2000 stock solutions prepared in ethanol were stored at −20°C until used. A lipid film was formed on the bottom of a glass tube by evaporating a chloroform/ethanol solution containing 2.75 mol% lipids [DOPE/SM/DMG-PEG2000 (9/2/0.33, molar ratio)]. After the film was formed, 250 µL of 10 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) was added, followed by incubation for 15 min at room temperature and sonication for 1 min in a bath-type sonicator (85 W, Aiwa, Co., Tokyo, Japan). To remove excess drug, the solution was centrifuged twice at (4°C, 20000 × g, 10 min).

Preparation of CoQ10-Liposomes by REV Method

Liposomes were prepared by a previously described method.21 Stock lipid solutions in chloroform, and CoQ10 and DMG-PEG 2000 stock solutions in ethanol were stored at −20°C until used. Components containing 2.75 mm lipids [DOPE/SM/DMG-PEG2000 (9/2/0.33, molar ratio)] were added to a glass tube. Diisopropyl ether and 10 mm HEPES buffer (pH 7.4) were then added, followed by sonication for 15 s with a probe-type sonicator. After removing the organic solvent with a stream of N2 gas, the liposome solution was sonicated for 30 s in a bath-type sonicator. To remove excess drug, the solution was centrifuged twice at (4°C, 20000 × g, 10 min) twice.

Preparation of CoQ10-Liposomes by Ethanol Dilution Method

These liposomes were prepared by a previously described method.14 Lipid, CoQ10 and DMG-PEG2000 stock solutions were prepared in ethanol and stored at −20°C until used. A 100% (v/v) EtOH solution containing 2.75 mm lipids
[DOPE/SM/DMG-PEG2000 (9/2/0.33, molar ratio)] was prepared. The ethanol solution of the lipid was mixed with 10 mM HEPES buffer (pH 7.4) under strong agitation at a concentration of 90% (v/v) EtOH. The resulting suspension was then added to 10 mM HEPES buffer (pH 7.4) under strong agitation to a final concentration of ca. 5% (v/v) EtOH, followed by ultrafiltration through an Amicon system (Millipore, Billerica, MA, U.S.A.) to remove the remaining ethanol. To remove excess drug, the solution was centrifuged at (4°C, 20000×g, 10 min) twice.

**Characterization of Prepared Carriers**

Particle diameter and PDI, indicators of the particle-size distribution, were measured using a dynamic light scattering (DLS) method (Zetasizer Nano ZS; Malvern Instruments, Worcestershire, U.K.). Samples were prepared in 10 mM HEPES buffer at 25°C and the values of particle diameters are shown in the form of volume distribution. The ζ-potentials of the samples were also determined in 10 mM HEPES buffer at 25°C using a Zetasizer Nano ZS.

**Estimation of CoQ₁₀ Amount Encapsulated in MITO-Porter**

Fluids associated with the liposome suspension were removed with a stream of N₂ gas. The sample was resuspended in EtOH, followed by centrifugation (4°C, 20000×g, 10 min). The supernatant was collected and the UV absorbance measured at 275 nm. The CoQ₁₀ recovery rate was calculated as follows: (CoQ₁₀ amount of sample)/(initial CoQ₁₀ amount).

**Estimation of Lipid Concentrations Based on NBD-DOPE Fluorescence Measurements and Calculation of the Drug Lipid/Ratio**

Liposomes were labeled with 0.1 mol% NBD-DOPE and an initial amount of 0 and 15 mol% CoQ₁₀ were prepared by the REV method, as described before. Carriers were post-modified with either 5 mol% R8 or 5 mol% S2-peptides to construct with R8-MITO-Porter or S2-MITO-Porter. Cells were seeded in 35 mm glass-bottom dishes (IWAKI, Osaka, Japan) 24 h prior to the experiment (2 mL, 8×10⁴ cells/mL, incubation at 37°C, 5% CO₂). After washing the cells twice with 1 mL DMEM (FBS−), the cells were incubated with DMEM not containing FBS and in the presence of the MITO-Porters (10 nm lipid) for 1 h. The medium was removed and 1 mL of DMEM (FBS+) was added. After further incubation for 1 h and 40 min, the cells were stained with MitoTracker Deep Red (1 mL, final conc. 100 nM) 20 min prior to observation. Cells were washed with DMEM (phenol red −) and 1 mL of fresh DMEM (phenol red −) was added, before CLSM images were obtained using a FV10i-LIV (Olympus Corporation, Tokyo, Japan), water-immersion objective lens (UPlanSApo 60x/NA=1.2) and a dichroic mirror (DM405/473/559/635).

**RESULTS AND DISCUSSION**

**Preparation of CoQ₁₀ Encapsulating Liposomes and Their Physiochemical Properties**

The preparation of the MITO-Porter involved two steps: the first is preparing mitochondrial fusogenic liposomes that contain encapsulated drugs, the second involves modifying the liposome with a functional peptide, such as R8, for cellular uptake and to target the mitochondria. Since liposomes that are prepared using different methods can result in significant differences in drug encapsulation efficiency and the physiochemical characteristics of the carrier, liposomes prepared using the three methods as shown in Fig. 1 were compared. All of the liposomes had the same composition (DOPE/SM/DMG-PEG2000 in the molar ratio 9/2/0.33), with increasing initial amount of CoQ₁₀ (0–25 mol%), and were prepared using the lipid film hydration method, the REV method or the ethanol dilution method.

The characteristics of liposomes in relation to the initial CoQ₁₀ concentration are shown in Table S1. It was observed that the method used for preparing the liposomes influenced their size, with the largest liposomes being obtained when the REV method was used, and the smallest liposomes when the ethanol dilution method was used (Fig. 2). Liposome size increased with increasing initial amount of CoQ₁₀, whereas the largest increase was observed when the REV method was used. This suggests that CoQ₁₀ changes the structure of the liposomes when it becomes incorporated into the lipid bilayer. It was therefore essential to investigate the structure of the liposomes further with regard to the final CoQ₁₀ concentration.

**Evaluation of the Recovery Ratio of CoQ₁₀ among the Different Preparation Methods**

The final CoQ₁₀ (µmol) concentration in relation to the initial CoQ₁₀ concentration and the drug recovery are shown in Fig. 3 and Table S2. It was observed that liposomes prepared by the lipid film hydration methods contained the lowest CoQ₁₀ concentrations. This finding was surprising, since CoQ₁₀ is a highly hydrophobic molecule and the lipid film hydration method is considered to elevate lactate levels.22) Dulbecco’s modified Eagle’s medium (DMEM) with high glucose and fetal bovine serum (FBS) were purchased from the Invitrogen Corporation (Carlsbad, California). The cells were maintained in DMEM with high glucose and 10% FBS, supplemented with penicillin and streptomycin. Cells were grown in 10 cm dishes at 37°C under 5% CO₂ until reaching approximately an 80% confluence. Cell passage was performed every 2–4 d.
be very suitable for packaging of hydrophobic molecules into liposomes. Due to the multi-lamellar structures when the lipid film hydration method is used, high amounts of hydrophobic molecules can be incorporated in the membranes. Unexpectedly, in this case, liposomes prepared by the REV method and the ethanol dilution method contained considerably more CoQ10. The loss of CoQ10 in liposomes prepared by the lipid film hydration method might be caused by the presence of excessive CoQ10, which would influence liposome formation. We observed that during the hydration, it was not possible to completely remove the CoQ10 from the glass tube which suggests that a part of CoQ10 was lost in this step (Fig. S1). It is noteworthy that no precipitation of CoQ10 was observed during its preparation via the REV method and the ethanol dilution method.

On the other hand, when the REV method and ethanol dilution methods were used to prepare liposomes, CoQ10 appeared to be successfully encapsulated. Surprisingly, the highest drug concentration was observed in liposomes prepared by the REV method, a method that usually produces liposome with an increased aqueous phase. To compare all of the prepared liposomes regarding their drug encapsulation efficiency (drug/lipid ratio), the recovery of lipids was investigated in the next step.

**Lipid Recovery Ratio and Calculation of Drug/Lipid Ratio of CoQ10 Encapsulated Liposomes**

All liposomes were prepared containing 0.1 mol% NBD-DOPE (a fluorescent labelled lipid). The fluorescence was measured and the lipid concentrations and lipid recovery rates were calculated (Table S3). As shown in Fig. 4, the highest lipid concentrations were observed for liposomes that were prepared by the REV method. Liposomes prepared by the ethanol dilution method, that contained a high drug concentration, had a low lipid concentration. It is possible that a part of the lipids had adsorbed to the filter used in the ultrafiltration procedure (the amicon filter) during one of the ethanol removal steps. In addition, during the lipid film hydration method, some of the lipids appeared to have been lost in the preparation process. In this case, it is likely that high concentrations of CoQ10 interfere with the formation of liposomes during the hydration step, leading to a visible film, consisting of CoQ10 and lipids, at the bottom of the glass tube (Fig. S1).

The molar drug/lipid ratios are shown in Table 1. Preparation by the ethanol dilution method and the lipid film hydration method results in liposomes with very low total lipid concentrations, therefore a high drug/lipid ratio. Values of the drug/lipid ratio >1 suggest that the carriers consist of an unusual liposomal structure. To further investigate the structure of the liposomes, TEM analyses were carried out.

**TEM Analysis**

In order to obtain additional information regarding the structure of liposomes, TEM analyses were carried out. Samples were prepared using the lipid film hydration method, the REV method and the ethanol dilution method with an initial loading of CoQ10 of 20 mol%. To compare to the structure of empty liposomes, additional carriers containing 0 mol% CoQ10 were prepared by the REV method.

As expected, the empty liposomes prepared by REV method consisted of a single-lamellar structure with an aqueous phase (Fig. 5A). However, the CoQ10 containing liposomes prepared by the REV method had a quite different structure (Fig. 5C): a multi-lamellar liposome was formed with CoQ10 not only encapsulated in the outer bilayer but also in the inner phase, resulting in an “onion”-like structure. Since such a multi-lamellar structure could not be observed in empty liposomes prepared by the same method, it can be assumed that CoQ10 is the key factor for the formation of this onion-like structure. As previously reported, CoQ10 alters the properties of lipid bilayers and can lead to the condensation of membranes by forming dense oil-like structures that are composed of a large proportion of CoQ10 mixed with the lipids. It is noteworthy that no precipitation of CoQ10 was observed during its preparation via the REV method and the ethanol dilution method.

As shown in Fig. 5B, liposomes prepared by the lipid film hydration method also had a multi-lamellar structure. When the ethanol dilution method was used, the formation of multi-lamellar liposomes was also observed (Fig. 5D). It thus appears that the method used to prepare liposomes has a negligible effect on their structure, whereas the presence of CoQ10 is a requirement for producing a multi-lamellar structure. These findings suggest that CoQ10 is not incorporated into the liposome as a drug cargo but, instead, is an integral component of the liposomal bilayer itself.

Unexpectedly, the presence of high amounts of CoQ10 changed the liposomal structure to a significant extent, resulting in multi-lamellar carriers. The encapsulation of CoQ10 into liposomes has been reported in previous studies. The drug concentrations used in these studies were typically much lower compared to our experiments. Accordingly, in those cases where the structures were examined by TEM, the structure of CoQ10 liposomes was very different, i.e., single-lamellar liposomes with aqueous phases. These results support the hypothesis, that high concentrations of CoQ10 changes the structure of liposomes.

**Intracellular Observation of the CoQ10-MITO-Porter in Patient-Derived Mitochondrial Disease Cells**

Due to the fact that high concentrations of CoQ10 can be incorporated and that the CoQ10-MITO-Porter has mitochondria-targeting properties, it would be predicted to be a suitable candidate for the treatment of mitochondrial diseases. It appears the CoQ10...
**Fig. 3. Evaluation of the Recovery Ratio of CoQ10 of CoQ10-Liposomes for the Different Preparation Methods**

Final CoQ10 concentrations in relation to initial CoQ10 (A). Recovery rate of CoQ10 (B). Data represent mean±S.D. (n=3).

**Fig. 4. Evaluation of Lipid Recovery Rate of CoQ10 Encapsulated Liposomes for the Different Preparation Methods**

Final NBD-DOPE concentrations in relation to initial CoQ10 (A). Recovery rate of NBD-DOPE recovery rate (B). Data represent mean±S.D. (n=3).

**Table 1. Drug/Lipid Ratio of CoQ10-Liposomes Prepared by the Different Methods**

<table>
<thead>
<tr>
<th>Method to prepare liposomes</th>
<th>Initial CoQ10 (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Lipid film hydration method</td>
<td>0.06±0.06</td>
</tr>
<tr>
<td>REV method</td>
<td>0.13±0.06</td>
</tr>
<tr>
<td>Ethanol dilution method</td>
<td>0.12±0.04</td>
</tr>
</tbody>
</table>

Data represent mean±S.D. (n=3).
might not be incorporated into a liposome as a drug cargo but is instead a component of the liposomal bilayer itself. Because of this, a need arose to investigate the effect of the differences in structure between the conventional MITO-Porter and the CoQ_{10}-MITO-Porter on mitochondrial targeting efficiency. To observe the intracellular trafficking of the CoQ_{10}-MITO-Porter in vitro, a G625A fibroblast cell line was used as a model. G625A fibroblasts carry a heteroplasmic mutation in the tRNA for phenylalanine in the mitochondrial DNA, leading to a decreased complex III activity. 22)

Liposomes containing an initial amount of 0 and 15 mol% CoQ_{10} were prepared by the REV method. We used the REV method to package CoQ_{10} in the MITO-Porter, because carriers prepared by this method showed a high recovery rate of CoQ_{10} and NBD-DOPE, as shown in Figs. 3 and 4. These recovery rates indicated maximum values of 15% of the initial CoQ_{10} concentration. The particle size of the MITO-Porter prepared by the REV method increased with increasing initial concentration of CoQ_{10}, as shown in Fig. 2. In the case where the initial concentration of CoQ_{10} was 15%, which is the optimal concentration for CoQ_{10} packaging, the particle diameter was around 160 nm. We conclude that this size is an appropriate size of a nanoparticle for pharmaceutical formulation.

In this experiment, the carriers included 0.5 mol% NBD-DOPE (green) to permit the trafficking behavior of the liposomes to be observed. After the preparation, the carriers were modified with 5 mol% R8 or S2-peptides. The physicochemical properties are summarized in Table S4. The diameters of the CoQ_{10}-MITO-Porters that were modified with peptides were around 160 nm, suggesting that modifying the CoQ_{10}-MITO-Porter with peptides had negligible effect on particle diameter.

As shown in Fig. 7A, the CoQ_{10}-MITO-Porter modified with R8-peptide was taken up by cells and accumulated in mitochondria (shown in red). We observed many green dots derived from the carriers in the cytosol, and some carriers were localized in red stained mitochondrial, observed as yellow signals. We also observed the same tendency regarding mitochondrial targeting in cells that were treated with an empty R8-MITO-Porter. There appeared to be no significant difference in cellular uptake and mitochondrial targeting activities of the CoQ_{10}-MITO-Porter compared to the empty MITO-Porter.

As a next step, the intracellular trafficking of a CoQ_{10}-MITO-Porter modified with the S2-peptide was investigated. As reported previously, 20) the S2-peptide also efficiently targets mitochondria but has been shown to be less cytotoxic compared to modification with R8-peptide. Figure 7B shows the cellular trafficking of the CoQ_{10}-MITO-Porter and the empty MITO-Porter modified with the S2-peptide. Again, the carriers were taken up by cells and some of them accumulated in mitochondria. It was not possible to distinguish the intracellular trafficking behavior between CoQ_{10}-MITO-Porter and empty MITO-Porter.

The goal of this study was to investigate the cellular uptake of the empty-MITO-Porter and CoQ_{10}-MITO-Porter in G625A fibroblast cells. Even though the structure of CoQ_{10}-MITO-Porter, as seen in TEM images, is altered by the incorporation of CoQ_{10} uptake was not affected. Both targeting ligands (R8 and S2-peptides) ensured cellular uptake with a high affinity for mitochondria. These results are of significance because the

![Fig. 5. TEM Images of CoQ_{10}-Liposomes](image)

TEM images of liposomes without CoQ_{10} prepared by the REV method (A) and CoQ_{10}-liposomes prepared by the lipid film hydration method (B), the REV method (C) and the ethanol dilution method (D). Bars, 50 nm.

![Fig. 6. Schematic Images of Change of Liposomes Structure Induced by CoQ_{10}](image)

Schematic diagrams of liposome without CoQ_{10} which has an aqueous phase (A) and CoQ_{10} containing liposome with higher curvature, no aqueous phase (B).
G625A fibroblast cells used in this study were obtained from a patient with a mitochondria-disorder.

CONCLUSION

The findings reported herein show that CoQ10 can be successfully encapsulated into a liposome for mitochondrial delivery. It was possible to incorporate approximately 50% of the drug into the carrier, a concentration sufficiently high that it changed the structure of the liposome significantly. By analyzing the CoQ10-MITO-Porter by TEM, structural differences between the empty MITO-Porter and the CoQ10-MITO-Porter could be clearly observed. Even though there was a big structural difference between the empty MITO-Porter and the CoQ10-MITO-Porter, mitochondrial delivery was not affected. The CoQ10-MITO-Porter was successfully taken up by fibroblast cells obtained from a patient with a mitochondrial disease and accumulated in mitochondria, as observed by CLSM. The findings suggest that a CoQ10-MITO-Porter represents a potentially suitable candidate for use in medical therapy for mitochondrial related diseases.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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