Regular Article

Packaging of the Coenzyme Q_{10} into a Liposome for Mitochondrial Delivery and the Intracellular Observation in Patient Derived Mitochondrial Disease Cells

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While Coenzyme Q_{10} (CoQ_{10}) is thought to be effective for the treatment of a variety of diseases, it limits its cellular uptake. Because of the hydrophobic nature of CoQ_{10}, it is reasonable to assume that it could be encapsulated within a liposomal carrier. Several reports regarding the packaging of CoQ_{10} in liposomes have appeared, but detailed investigations of the preparation of CoQ_{10} encapsulated liposomes have not been reported. As a result, information regarding the optimal method of packaging CoQ_{10} in liposomes is not available. In this study, several types of liposomes were prepared using different methods and their characteristics were compared. Since CoQ_{10} is mainly located in the inner mitochondrial membrane, a liposome that targets mitochondria, a MITO-Porter, was used as a model liposome. It was possible to incorporate high levels of CoQ_{10} into the carrier. Transmission electron microscopy analyses showed that an empty MITO-Porter and the CoQ_{10}-MITO-Porter were structurally different from one another. Even though significant structural differences were observed, mitochondrial delivery was not affected in mitochondrial disease fibroblast cells, as evidenced by confocal laser scanning microscopy observations. The results reported herein suggest that the CoQ_{10}-MITO-Porter might be a suitable candidate for the potential medical therapy of mitochondria-related diseases.

Key words mitochondrial drug delivery; liposome; physicochemical property; nanotechnology; mitochondrial disease

Coenzyme Q_{10} (CoQ_{10}), 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 CoQ_{10} 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)] Since then, substantial amounts of laboratory and animal data on CoQ_{10} have been published. These findings indicate that, in addition to its function as an electron carrier, CoQ_{10} acts as an antioxidant and can prevent damage to cellular components caused by free radicals and can also stabilize cell membranes.[4,5)] The effect of CoQ_{10} for the treatment of a variety of medical conditions, including mitochondrial diseases,[6)] cancer,[7)] cardiovascular diseases[8)] and neurodegenerative diseases[9)] has attracted the interest of researchers. Even though the therapeutic effect of CoQ_{10} 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 CoQ_{10} is taken up in the blood stream, making the treatment of CoQ_{10} deficiencies difficult. Even though the absorption levels of CoQ_{10} 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 CoQ_{10}, the use of a liposomal carrier for the delivery might be possible. Since CoQ_{10} 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 CoQ_{10}, 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. CoQ_{10} can then exert its pharmaceutical effect on the mitochondrial membrane, where endogenous CoQ_{10} functions as an important functional molecule.

Earlier research showed that CoQ_{10} 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 CoQ_{10} encapsulation efficiency, attempts to optimize CoQ_{10} encapsulation by varying the initial CoQ_{10} concentration have not been reported. In addition, a comparison of the characteristics of CoQ_{10} encapsulated liposomes among different packaging methods have not been reported, which is important information, in terms of establishing the optimal method for packaging CoQ_{10} 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 CoQ_{10} encapsulation efficiency and the drug/lipid ratio of the carrier. As CoQ_{10} 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, physiochemical properties

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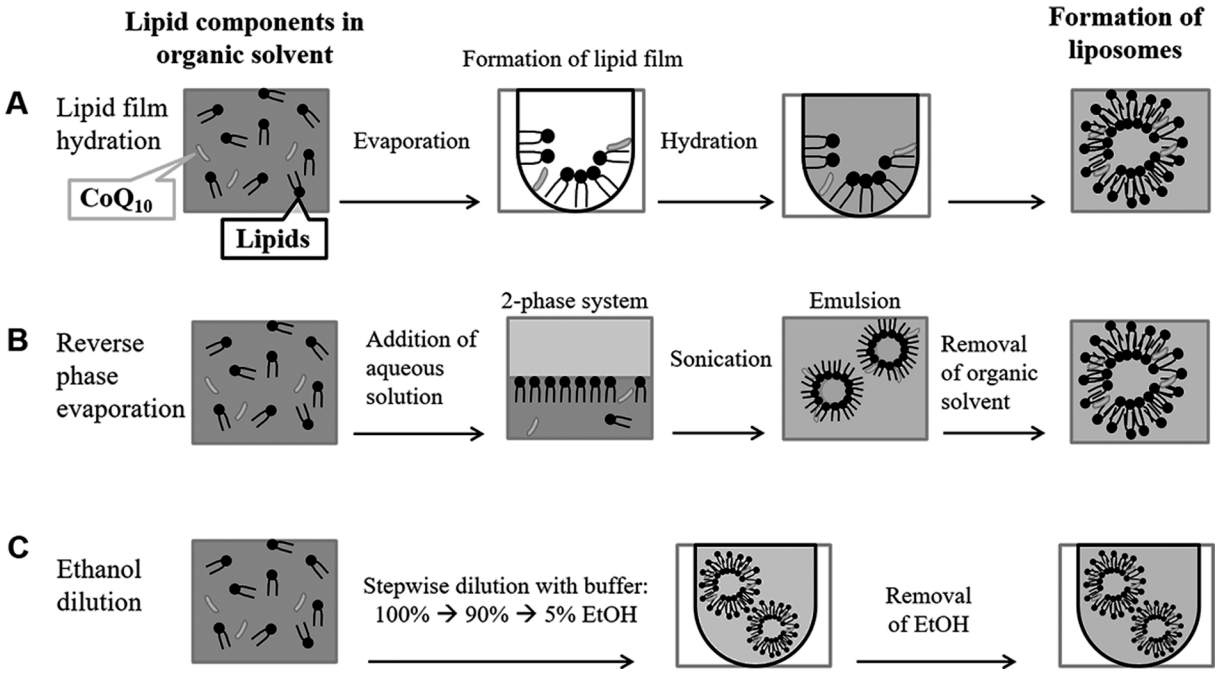

Fig. 1. Schematic Image of Liposome Preparation

Schematic images of liposomes prepared by the lipid film hydration method (A), the REV method (B) and the ethanol dilution method (C).

such as size and its distribution—the polydispersity index (PDI)—, the surface charge (ζ-potential) and the concentrations of encapsulated CoQ_{10} were determined. The structure of CoQ_{10}-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-D-Arg-FK-Dmt-D-Arg-FK-NH_2, Dmt=2, 6-dimethyltyrosine)[20] was obtained from Toray Research Center (Tokyo, Japan). CoQ_{10} was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were commercially available reagent-grade products.

Construction of CoQ_{10}-MITO-Porter CoQ_{10} was added to the lipid components in concentrations of 5, 10, 15, 20 and 25 mol% of the total lipid. A CoQ_{10} 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 physiochemical properties such as size and the concentration of encapsulated CoQ_{10} and TEM analysis, the liposomes were used before being modified with the peptides.

Preparation of CoQ_{10}-Liposomes by Lipid Film Hydration Method

Liposomes were prepared by previously described methodology.[12] Lipid stock prepared in chloroform, and CoQ_{10} 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 mM 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×$\boldsymbol{g}$, 10 min).

Preparation of CoQ_{10}-Liposomes by REV Method

Liposomes were prepared by a previously described method.[21] Stock lipid solutions in chloroform, and CoQ_{10} 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 N_2 gas, the liposome solution was sonicated for 30 s in a bath-type sonicator. To remove excess drug, the solution was centrifuged at (4°C, 20000×$\boldsymbol{g}$, 10 min) twice.

Preparation of CoQ_{10}-Liposomes by Ethanol Dilution Method

These liposomes were prepared by a previously described method.[14] Lipid, CoQ_{10} 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 CoQ_{10} Amount Encapsulated in MITO-Porter Fluids associated with the liposome suspension were removed with a stream of N_2 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 CoQ_{10} recovery rate was calculated as follows: (CoQ_{10} amount of sample)/(initial CoQ_{10} 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% (of total lipid) NBD-DOPE and the fluorescence intensities of the carriers (excitation at 460 nm and emission at 534 nm) were measured using an EnSpire™2300 Multilabel Reader (PerkinElmer, Inc.). The NBD-DOPE recovery rate was calculated as follows: (NBD-DOPE amount of sample)/(initial NBD-DOPE amount). Drug/lipid ratio was calculated as follows: Final amount of CoQ_{10} (mol)/final amount of total lipid (mol).

TEM Analysis The TEM analysis of the MITO-Porters was carried out at the Hanaichi UltraStructure Research Institute (Okazaki, Japan). The sample solution was dropped on a carbon film grid, and washed with distilled water. After dropping a 2% solution of uranyl acetate on the grid, the samples were observed by HITACHI H-7600 at 100 kV TEM (Hitachi High-Tech Science Systems Corporation; Ibaraki, Japan) with negative stain.

Cell Culture G625A fibroblasts were obtained from a mitochondrial disease patient at the Sapporo City General Hospital. The clinical experiments were approved by the Faculty of Pharmaceutical Sciences, Hokkaido University (No. 2014-003 from 2014/10/17), Hokkaido University Hospital (No. 14-061 from 2015/1/1) and Sapporo City General Hospital (No. H26-050-224 from 2015/1/14). The G625A fibroblasts carry a heteroplasmic mutation in the tRNA for phenylalanine in the mitochondrial DNA, leading to a decreased complex III activity. The phenotype includes epilepsy, hearing loss and elevated 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% CO_2 until reaching approximately an 80% confluence. Cell passage was performed every 2–4 d.

CLSM Observation Liposomes containing 0.5 mol% NBD-DOPE and an initial amount of 0 and 15 mol% CoQ_{10} 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, 8×10^4 cells/mL, incubation at 37°C, 5% CO_2). 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 CoQ_{10} 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.[14] 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 CoQ_{10} (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 CoQ_{10} 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 CoQ_{10}, whereas the largest increase was observed when the REV method was used. This suggests that CoQ_{10} 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 CoQ_{10} concentration.

Evaluation of the Recovery Ratio of CoQ_{10} among the Different Preparation Methods The final CoQ_{10} (μM) concentration in relation to the initial CoQ_{10} 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 CoQ_{10} concentrations. This finding was surprising, since CoQ_{10} is a highly hydrophobic molecule and the lipid film hydration method is considered to

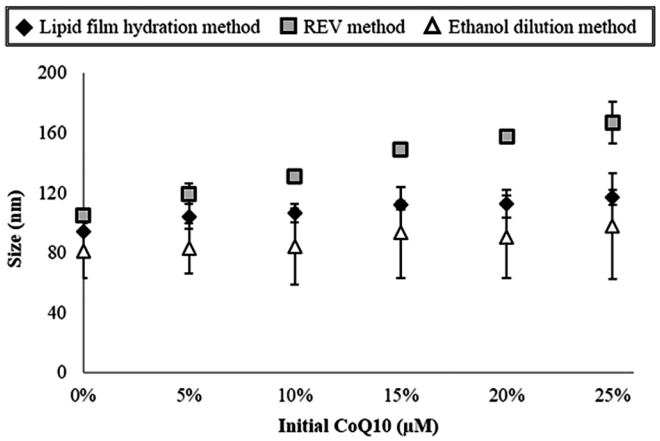

Fig. 2. Effect of the Initial Amount of CoQ_{10} to Prepare Liposomes on Liposome Size

Diameters of liposomes prepared by the lipid film hydration method (diamonds), the REV method (suquares) or the ethanol dilution method (triangles) in relation to initial amount of CoQ_{10}. Data represent mean±S.D. (n=3).

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 CoQ_{10}. The loss of CoQ_{10} in liposomes prepared by the lipid film hydration method might be caused by the presence of excessive CoQ_{10}, which would influence liposome formation. We observed that during the hydration, it was not possible to completely remove the CoQ_{10} from the glass tube which suggests that a part of CoQ_{10} was lost in this step (Fig. S1). It is noteworthy that no precipitation of CoQ_{10} 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, CoQ_{10} 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 CoQ_{10} 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 absorbed 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 CoQ_{10} interfere with the formation of liposomes during the hydration step, leading to a visible film, consisting of CoQ_{10} 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 CoQ_{10} of 20 mol%. To compare to the structure of empty liposomes, additional carriers containing 0 mol% CoQ_{10} 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 CoQ_{10} containing liposomes prepared by the REV method had a quite different structure (Fig. 5C): a multi-lamellar liposome was formed with CoQ_{10} 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 CoQ_{10} is the key factor for the formation of this onion-like structure. As previously reported, CoQ_{10} 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 CoQ_{10} mixed with the lipids.[16] We suspect that this is achieved by CoQ_{10} inducing a change in the curvature of the lipid layer. By positioning the CoQ_{10} molecules among the lipids, a tight multi-lamellar packaging between the layers occurs (Fig. 6).

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 CoQ_{10} is a requirement for producing a multi-lamellar structure. These findings suggest that CoQ_{10} 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 CoQ_{10} changed the liposomal structure to a significant extent, resulting in multi-lamellar carriers. The encapsulation of CoQ_{10} into liposomes has been reported in previous studies.[15–18] 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 CoQ_{10} liposomes was very different, *i.e.*, single-lamellar liposomes with aqueous phases. These results support the hypothesis, that high concentrations of CoQ_{10} changes the structure of liposomes.

Intracellular Observation of the CoQ_{10}-MITO-Porter in Patient-Derived Mitochondrial Disease Cells Due to the fact that high concentrations of CoQ_{10} can be incorporated and that the CoQ_{10}-MITO-Porter has mitochondria-targeting properties, it would be predicted to be a suitable candidate for the treatment of mitochondrial diseases. It appears the CoQ_{10}

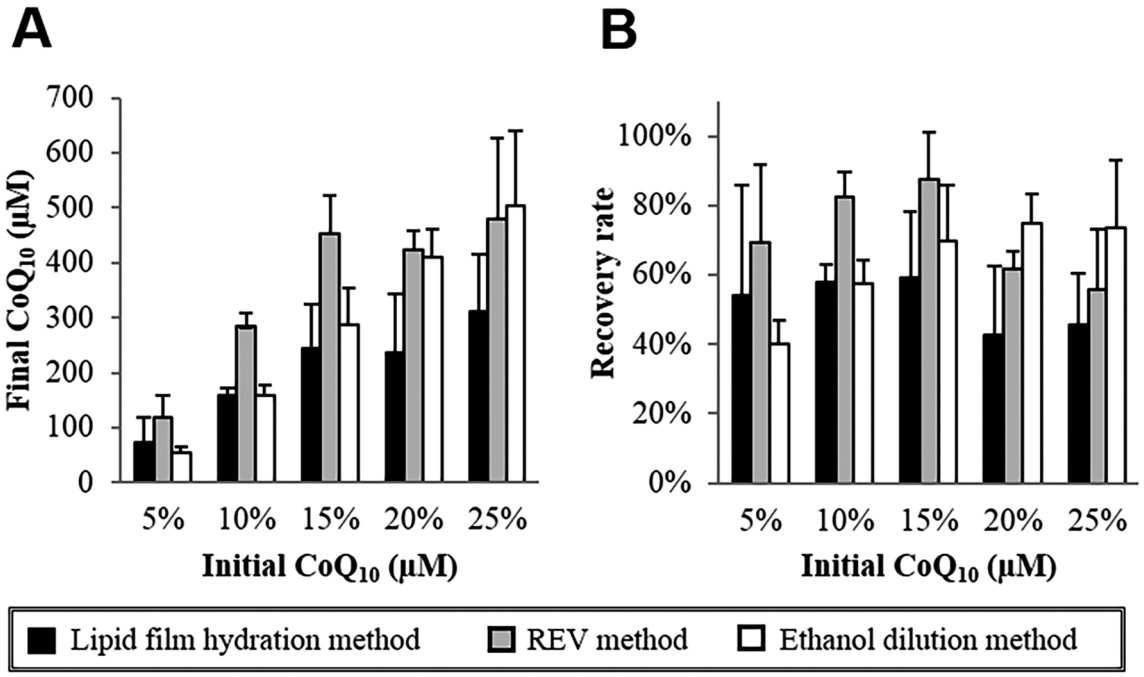

Fig. 3. Evaluation of the Recovery Ratio of CoQ_{10} of CoQ_{10}-Liposomes for the Different Preparation Methods

Final CoQ10 concentrations in relation to initial CoQ_{10} (A). Recovery rate of CoQ_{10} (B). Data represent mean±S.D. (n=3).

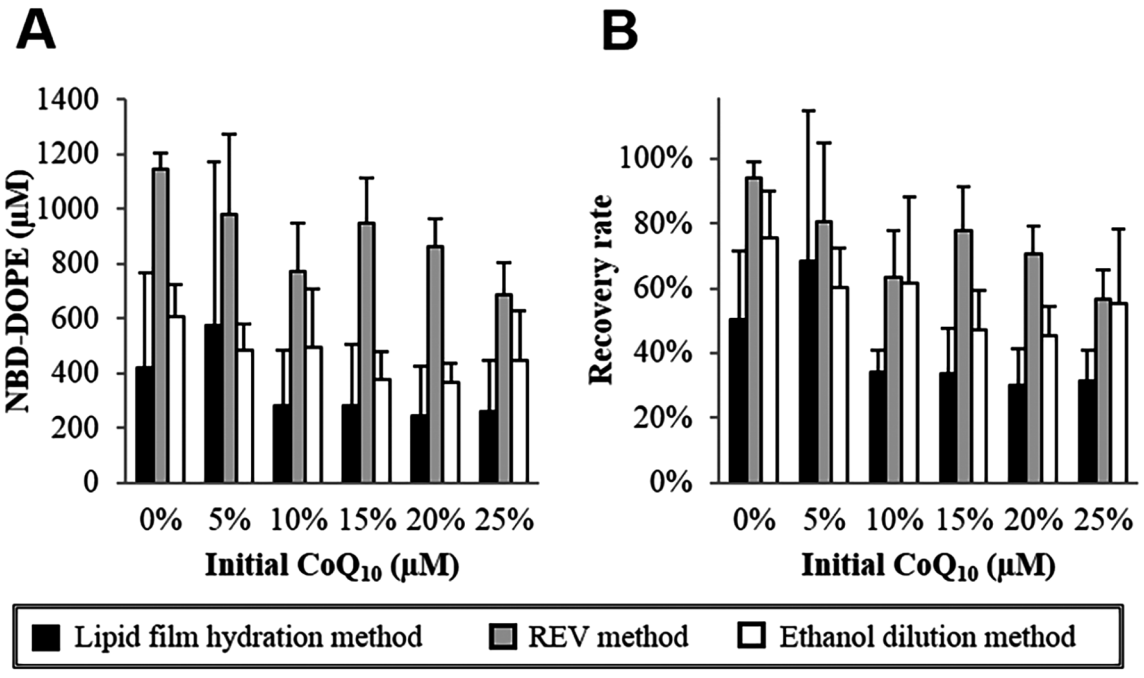

Fig. 4. Evaluation of Lipid Recovery Rate of CoQ_{10} Encapsulated Liposomes for the Different Preparation Methods

Final NBD-DOPE concentrations in relation to initial CoQ_{10} (A). Recovery rate of NBD-DOPE recovery rate (B). Data represent mean±S.D. (n=3).

Table 1. Drug/Lipid Ratio of CoQ_{10}-Liposomes Prepared by the Different Methods

Method to prepare liposomes	Initial CoQ_{10} (mol%)					
	0	5	10	15	20	25
Lipid film hydration method	0	0.06±0.06	0.36±0.04	0.57±0.20	0.99±0.25	0.94±0.05
REV method	0	0.13±0.06	0.38±0.06	0.48±0.06	0.49±0.04	0.69±0.11
Ethanol dilution method	0	0.12±0.04	0.35±0.12	0.78±0.18	1.15±0.19	1.23±0.47

Data represent mean±S.D. (n=3).

might not be incorporated into a liposome as a drug cargo but is instead is 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

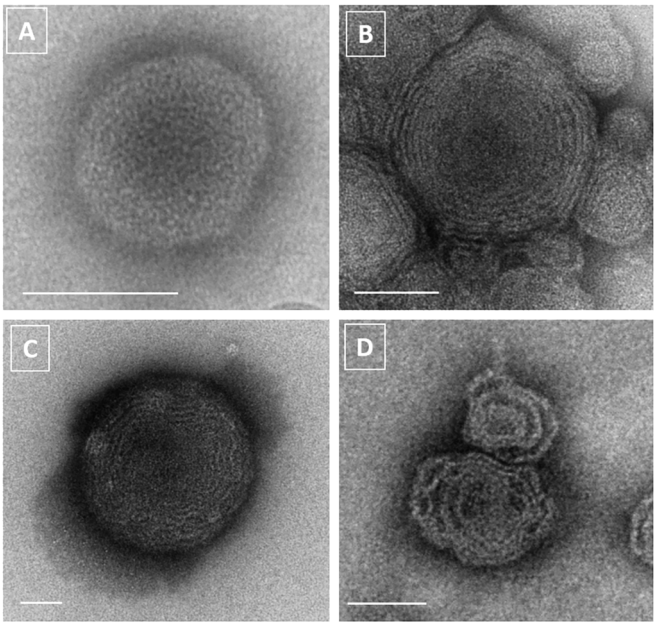

Fig. 5. TEM Images of CoQ_{10}-Liposomes

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.

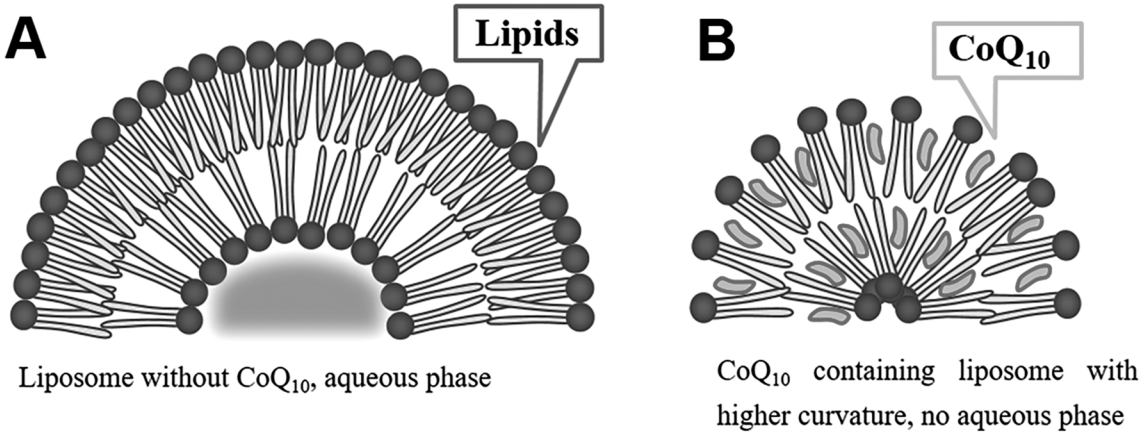

Fig. 6. Schematic Images of Change of Liposomes Structure Induced by CoQ_{10}

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).

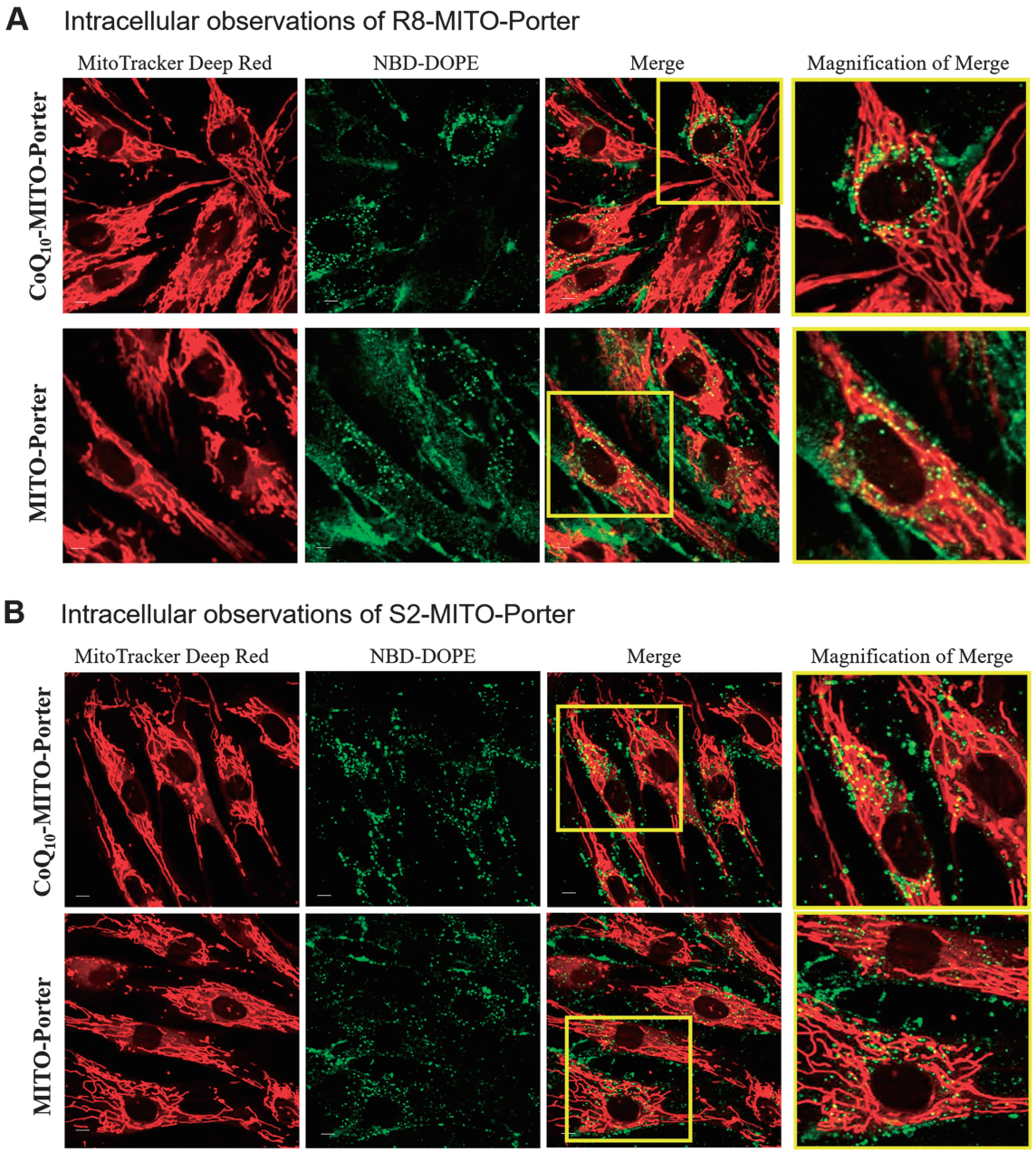

Fig. 7. Intracellular Observations of the CoQ_{10}-MITO-Porter and Empty MITO-Porter

G625A fibroblast cells were incubated with NBD (a green fluorescence molecule) -labeled CoQ_{10}-MITO-Porter and the empty MITO-Porter, modified with 5 mol% R8-peptide (A) and 5 mol% S2-peptide (B). After staining the mitochondria red, the cells were observed by CLSM. NBD-labeled MITO-Porter appeared as yellow clusters when it was localized in mitochondria. Right panels indicate the images of modified merged images. Scale bars; 10 μm.

G625A fibroblast cells used in this study were obtained from a patient with a mitochondria-disorder.

CONCLUSION

The findings reported herein show that CoQ_{10} 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 CoQ_{10}-MITO-Porter by TEM, structural differences between the empty MITO-Porter and the CoQ_{10}-MITO-Porter could be clearly observed. Even though there was a big structural difference between the empty MITO-Porter and the CoQ_{10}-MITO-Porter, mitochondrial delivery was not affected. The CoQ_{10}-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 CoQ_{10}-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.

REFERENCES

1) Turunen M, Olsson J, Dallner G. Metabolism and function of coenzyme Q. *Biochim. Biophys. Acta*, **1660**, 171–199 (2004).
2) Crane FL, Hatefi Y, Lester RL, Widmer C. Isolation of a quinone from beef heart mitochondria. *Biochim. Biophys. Acta*, **25**, 220–221 (1957).
3) Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature*, **191**, 144–148 (1961).
4) Mellors A, Tappel AL. The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. *J. Biol. Chem.*, **241**, 4353–4356 (1966).
5) Crane FL, Navas P. The diversity of coenzyme Q function. *Mol. Aspects Med.*, **18** (Suppl.), S1–S6 (1997).
6) DiMauro S, Hirano M, Schon EA. Approaches to the treatment of mitochondrial diseases. *Muscle Nerve*, **34**, 265–283 (2006).
7) Lockwood K, Moesgaard S, Hanioka T, Folkers K. Apparent partial remission of breast cancer in 'high risk' patients supplemented with nutritional antioxidants, essential fatty acids and coenzyme Q10. *Mol. Aspects Med.*, **15** (Suppl.), s231–s240 (1994).
8) Pepe S, Marasco SF, Haas SJ, Sheeran FL, Krum H, Rosenfeldt FL. Coenzyme Q10 in cardiovascular disease. *Mitochondrion*, **7** (Suppl.), S154–S167 (2007).
9) Beal MF. Mitochondrial dysfunction and oxidative damage in Alzheimer's and Parkinson's diseases and coenzyme Q10 as a potential treatment. *J. Bioenerg. Biomembr.*, **36**, 381–386 (2004).
10) Hidaka T, Fujii K, Funahashi I, Fukutomi N, Hosoe K. Safety assessment of coenzyme Q10 (CoQ10). *Biofactors*, **32**, 199–208 (2008).
11) Zhang Y, Aberg F, Appelkvist EL, Dallner G, Ernster L. Uptake of dietary coenzyme Q supplement is limited in rats. *J. Nutr.*, **125**, 446–453 (1995).
12) Yamada Y, Akita H, Kamiya H, Kogure K, Yamamoto T, Shinohara Y, Yamashita K, Kobayashi H, Kikuchi H, Harashima H. MITO-Porter: A liposome-based carrier system for delivery of macromolecules into mitochondria *via* membrane fusion. *Biochim. Biophys. Acta*, **1778**, 423–432 (2008).
13) Yamada Y, Harashima H. MITO-porter for mitochondrial delivery and mitochondrial functional analysis. *Handb. Exp. Pharmacol.*, (2016).
14) Yamada Y, Nakamura K, Abe J, Hyodo M, Haga S, Ozaki M, Harashima H. Mitochondrial delivery of coenzyme Q *via* systemic administration using a MITO-porter prevents ischemia/reperfusion injury in the mouse liver. *J. Control. Release*, **213**, 86–95 (2015).
15) Verma DD, Hartner WC, Thakkar V, Levchenko TS, Torchilin VP. Protective effect of coenzyme Q10-loaded liposomes on the myocardium in rabbits with an acute experimental myocardial infarction. *Pharm. Res.*, **24**, 2131–2137 (2007).
16) Agmo Hernández V, Eriksson EK, Edwards K. Ubiquinone-10 alters mechanical properties and increases stability of phospholipid membranes. *Biochim. Biophys. Acta*, **1848** (10 Pt A), 2233–2243 (2015).
17) Xia F, Jin H, Zhao Y, Guo X. Preparation of coenzyme Q10 liposomes using supercritical anti-solvent technique. *J. Microencapsul.*, **29**, 21–29 (2012).
18) Lee WC, Tsai TH. Preparation and characterization of liposomal coenzyme Q10 for *in vivo* topical application. *Int. J. Pharm.*, **395**, 78–83 (2010).
19) Futaki S, Ohashi W, Suzuki T, Niwa M, Tanaka S, Ueda K, Harashima H, Sugiura Y. Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjug. Chem.*, **12**, 1005–1011 (2001).
20) Kawamura E, Yamada Y, Harashima H. Mitochondrial targeting functional peptides as potential devices for the mitochondrial delivery of a DF-MITO-Porter. *Mitochondrion*, **13**, 610–614 (2013).
21) Abe J, Yamada Y, Harashima H. Validation of a strategy for cancer therapy: delivering aminoglycoside drugs to mitochondria in HeLa cells. *J. Pharm. Sci.*, **105**, 734–740 (2016).
22) Sudo A, Takeichi N, Hosoki K, Saitoh S. Successful cochlear implantation in a patient with mitochondrial hearing loss and m.625G>A transition. *J. Laryngol. Otol.*, **125**, 1282–1285 (2011).