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Nanoliposomes Mediate Coenzyme Q_{10} Transport and Accumulation across Human Intestinal Caco-2 Cell Monolayer

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The feasibility of using nanoliposomes as an oral delivery system to improve the intestinal absorption of coenzyme Q_{10} (CoQ_{10}, ubiquinone-10) was examined using Caco-2 cell monolayers as the model of human intestinal epithelium. The apparent permeability coefficient of CoQ_{10} with nanoliposomes as vehicles was increased to be $4.19 \pm 0.76 \times 10^{-6}$ cm/s, which was higher than the favorable intestinal absorption value (1×10^{-6} cm/s). The kinetic data demonstrated that nanoliposomal CoQ_{10} transport occurred via passive diffusion and carrier mediation routes. Nanoliposomes might suppress the modulation activity of the peptide transporter, organic anion transporter, and P-glycoprotein through fluidizing cell membrane. The transported CoQ_{10} was still in the original oxidative form as ubiquinone-10. However, 70−80% of coenzyme Q_{10} accumulated by the cells was in the reduced form (ubiquinol-10, $CoQ_{10}H_2$), suggesting that the conversion of CoQ_{10} to $CoQ_{10}H_2$ takes place in the enterocytes during its absorption. These results indicated that the absorption behavior of CoQ_{10} was modified through nanoliposomes. The bioavailability of CoQ_{10} following its oral administration might be improved with nanoliposomes as the delivery system.

KEYWORDS: Coenzyme Q_{10}; nanoliposomes; transport; accumulation; Caco-2 cells

INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}, ubiquinone-10) is the only endogenously synthesized antioxidant existing in all cell membranes of our body (*1*, *2*). It plays a crucial role in the production of adenosine triphosphate (ATP), which is the energy source for all living cells. Interest in CoQ_{10} has grown recently because of the deficiency of CoQ_{10} associated with several disorders such as diabetes, cardiovascular and neurodegenerative disease. Thus, CoQ_{10} has become one of the most popular nutritional supplements (*2*). However, the bioavailability of CoQ_{10} in human gastrointestinal tract is very low and variable, due to its slow absorption through the gastrointestinal epithelia as well as poor aqueous solubility (*3*).

In order to enhance the absorbability of CoQ_{10}, attempts to increase its solubility have been made by encapsulating CoQ_{10} in liposomes (*4*), which are hydrophilic vesicles consisting of one or more concentric bilayers enclosing aqueous compartments. Previous investigations reported in literature, mostly use small vesicles (nanoliposomes) 50−150 nm in diameters. The size range is a compromise of loading efficiency, stability and distribution in the organisms (*5*, *6*). The stability of liposomes in the gastrointestinal tract was also investigated (*7*, *8*). Despite the interest in the roles of such an oral delivery system, the efficiency of nanoliposomes has not yet been elucidated clearly (*9*) and little is known about the transfer of CoQ_{10} in the form of nanoliposomes across the intestinal epithelium.

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Cell culture models of current interest for the prediction of oral nutrient absorption include human intestinal Caco-2 cells, which has been used to evaluate the penetration of salmon calcitonin (*10*) and insulin (*11*) in liposome formulations. However, for poorly water-soluble nutrient such as CoQ_{10}, it is more difficult to use conventional analytical methods to monitor nutrient transport flux across Caco-2 cell monolayer. For this reason, the ^{3}H-radiolabeled CoQ_{10} ([^{3}H]-CoQ_{10}) at very low concentration (1 nM) with ethyl acetate as the cosolvent has been used to examine mechanism of permeation by Caco-2 cell monolayer (*2*). Considering that the recommended daily dosage of CoQ_{10} ranges from 30 to 100 mg, and a higher dosage such as 1200 mg/d may be required for certain individuals (*3*), it is necessary to investigate the absorptive behavior of CoQ_{10} in a relatively high concentration range. Results of our previous studies showed that the maximum loading capacity of encapsulated CoQ_{10} in nanoliposomes was 40%, and the corresponding CoQ_{10} concentration in nanoliposomal suspension was about 10 mg/mL. Therefore, nanoliposomes might be used as solubilizers to deliver CoQ_{10} at a physiologically relevant concentration level to cultured cells.

The intestinal permeabilities measured after an oral administration normally reflect the absorption in the small intestine (*12*). So far no results have been reported on the influence of nanoliposomes on CoQ_{10} across biological membranes. In this present study, Caco-2 cell monolayers were used to assess the trsnsepithelial transport and intracellular accumulation behavior of CoQ_{10} in the nanoliposome formulation. The effects of nanoliposomes on cell viability and membrane characteristics such as P-glycoprotein activity and fluidity were also investigated.

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MATERIALS AND METHODS

Materials. CoQ_{10} material (98.0%−101.0% purity) was purchased from Nisshin Pharma Inc. (Tokyo, Japan). Heat-inactivated fetal bovine serum (FBS), Hank's balanced salts solution (HBSS), trypsin-EDTA, Dulbecco's Modified Eagle's medium (DMEM), nonessential amino acid solution, L-glutamine, and penicillin−streptomycin were purchased from Gibco Laboratory (Invitrogen Co, Grand Island, NY, USA). Standard CoQ_{10} (98% purity), ubiquinone-9 (CoQ_9), [^{3}H]-mannitol (1 μCi/μL), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), 1,6-diphenyl-1,3,5-hexatriene (DPH, 98% purity), and rhodamine 123 (RH-123) were from Sigma Chemical Co. (St. Louis MO, USA). Egg yolk phospholipid (EPL) was purchased from Chemical Reagent Plant of East China Normal University (Shanghai, China). Cholesterol (Chol), polyoxyethylene sorbitan monooleate (Tween 80), sodium dodecyl sulfate (SDS), and nicotinic acid were obtained from China Medicine (Group) Shanghai Chemical Reagent Co. (Shanghai, China). Cyclosporin A was obtained from Guangdong Medical Drug Co. (Guangzhou, China). Captopril was purchased from Sino-American Shanghai Squibb Pharmaceuticals Ltd. (Shanghai, China). Currently marketed Liquid CoQ_{10} from LifeTime Vitamins (Nutritional Specialties, Inc., Orange, CA) was selected as the reference product for the study.

Preparation and Characterization of CoQ_{10} Nanoliposomes. CoQ_{10} nanoliposomes were prepared by ethanol injection and sonication method as described previously (*13*). Bench-top batches in the laboratory were prepared on a 20 mL scale. CoQ_{10} (20 mg) was dissolved in 2 mL of warm ethanol (about 55 °C) together with the lipids composed of EPL, Chol, and/or Tween 80. The ethanol solution was rapidly injected using a syringe as a pump into 20 mL of warm hydration media (0.01 M phosphate buffer solution, 150 mM NaCl, and PBS, pH 7.4) at 55 °C with magnetically stirring. After agitation for 30 min, the ethanol was removed by rotary evaporation (55 °C, 0.1 MPa) to form an aqueous dispersion of liposomes. The final phospholipid concentration in the incubations was adjusted to 12.5 mg/mL with deionized water. The prepared liposomal suspension was then submitted to a probing sonication process in an ice bath for 4 min at a power of 350 W and with cycles of 1-s sonication followed by 1-s rest using a vibra cell sonicator (VCX500, Sonics & Materials, INC, 20 kHz) to the desired size. Following sonication, nanoliposomes were annealed at 4 °C for 2 h. The titanium fragments shed from the probe and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at 11,000*g* for 30 min at 4 °C. The entire process was carried out in the dark under nitrogen protection to minimize the oxidation and degradation of the lipid mixtures and CoQ_{10}. Finally, nanoliposomes were filled into vials (the headspace of the vials was blanketed with nitrogen) and kept in the refrigerator (about 4 °C in the dark). CoQ_{10} (60 mg and 130 mg) was also formulated with nanoliposomes using the same procedure.

The CoQ_{10} concentration in nanoliposomal suspensions and the encapsulation efficiency were calculated according to the method as described in our previous report (*13*). The CoQ_{10} concentration in nanoliposome suspensions were 1.13 mM, 3.59 mM, and 7.53 mM. The encapsulation efficiency of CoQ_{10} was found to be higher than 95%. The *z*-average particle size of the CoQ_{10} nanoliposomes (EPL/Tween 80/Chol= 2.5:1.8:0.4, w/w) was about 54 nm as determined with a ZetaSizer Nano S (Malvern Instruments Ltd., Malvern, United Kingdom) at 25 ± 0.1 °C.

Cell Culture. Caco-2 cells (American type Culture Collection, Rockville, MD, USA) were grown routinely on 75 cm^2 plastic culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C in an atmosphere of 5% carbon dioxide (CO_2) and 90% relative humidity. The medium was complete DMEM supplemented with 10% FBS, 1% nonessential amino acid solution, 1% L-glutanmine, and 1% penicillin−streptomycin. For the transport experiments, cells were seeded at a density of 5×10^4 cells/cm^2 onto permeable polycarbonate inserts (4.2 cm^2, 0.45 μm pore size, Millipore, USA) in 6-well tissue culture plates (NUNC, Roskilde, Denmark). The integrity of the cell monolayers grown on the permeable membrane was ensured by determining the transepithelial electrical resistance (TEER) of the monolayers at 37 °C using a Millicell-ERS apparatus (Millipore, Bedford, MA, USA) and [^{3}H]mannitol transport. When the TEER values reached 600 $\Omega \cdot$cm^2, the cells were used for experiments. The cell monolayers were considered to be tight when the permeability of mannitol was < 1% of the dose/h. The formation of the cell monolayer was observed and checked with a CM-10 transmission electron microscope (Philips, Holand).

Determination of Cell Viability. Mitochondrial activity of the Caco-2 cells was determined by using the MTT reagent. This assay is based on the reduction of the yellow MTT reagent to the blue formazan crystals by mitochondrial dehydrogenase released from viable cells. Briefly, the Caco-2 cells were seeded onto a 96-well plate at a density of 2×10^5 cells/well and allowed to grow to 95% confluence. The cells were incubated with 100 μL of PBS containing different concentrations and composition of nanoliposomes or without nanoliposomes (control) at 37 °C for 4 h. The weight ratio of EPL/Chol/Tween 80 was 2.5:0.4:1.8. The original concentration of Tween 80 was 0.9% (w/v). Sodium dedocyl sulfate (SDS) at 0.1% (w/v) was used as the positive control, and DMEM served as the negative control. After 4 h, the supernatant was removed by aspiration, the cells were washed twice with PBS, and then 20 μL of MTT (5 mg/mL) in PBS was added per well followed by further incubation at 37 °C for 2 h. The MTT/medium was removed, and 100 μL of dimethyl sulfoxide was added to each well by shaking to dissolve crystals. The absorbance was determined at 490 nm with a reference at 630 nm by using a microplate reader (Powerwave X, Bio-Tek Instruments, Inc., USA). The cell viability was expressed as a percentage of the absorbance of cells treated with test samples compared to that of the negative control.

Transepithelial Transport and Cellular Accumulation of CoQ_{10}. Transport of CoQ_{10} across the Caco-2 cell monolayers was studied using monolayers after 21−24 days of seeding. Before the experiment, the monolayers were preincubated with PBS at 37 °C for 20 min, and TEER was measured. PBS on both sides of the cell monolayers was removed by aspiration and then rinsed three times with PBS. For the transport of CoQ_{10} from the apical (AP)-to-basolateral (BL) direction, 1.5 mL of PBS containing samples (nanoliposomal CoQ_{10}, pure CoQ_{10} powder dispersed in PBS, or marketed Liquid CoQ_{10}) with the same CoQ_{10} concentration (359 μM) was added to the AP (donor) compartments, and 2 mL of PBS was added to the BL (receiver) compartments. The monolayers were incubated at 37 °C in a shaker (50 rpm). Aliquots of 1 mL were sampled from the receiver compartments at 15, 30, 45, 60, 90, 120, and 180 min followed by an immediate replenishment with the same volume of prewarmed fresh PBS. For the transport of CoQ_{10} from the basolateral (BL)-to-apical (AP) direction, 2 mL of PBS containing nanoliposomal CoQ_{10} was added to the BL (donor) compartments, and 1.5 mL of PBS was added to the AP (receiver) compartments. The other procedures were the same as those described above.

Cyclosporin A (an inhibitor of P-glycoprotein), captopril (an inhibitor of peptide transporter, PepT-1), and nicotinic acid (a substrate of monocarboxylic acid transporter, MCT1) were dissolved in PBS at an initial concentration of 10 μM. Before the experiments, the monolayers were preincubated with these substrates/inhibitors at 37 °C for 20 min. Solutions on both sides of the cell monolayers were then removed by aspiration and washed three times with fresh PBS. CoQ_{10} nanoliposomes were diluted with PBS containing these substrates/inhibitors (10 μM) and added to the AP (donor) compartments. The other procedures were the same as those described above.

The TEER value was measured to ensure the integrity of the membrane. Donor samples were collected to calculate the mass balance and ensure the sink conditions.

At the end of the incubation, the cells were rinsed four times with ice-cold PBS. The resultant monolayers were harvested in 0.5-mL ice-cold PBS containing 10% (v/v) ethanol and stored at −20 °C for subsequent analysis of the accumulation of CoQ_{10}. Upon analysis, frozen samples were thawed, and the cell homogenates were sonicated in an ice bath for 30 s at a power of 100 W and with cycles of 1-s sonication followed by 3-s rests, using a vibra cell sonicator (model JY92-II, Ningbo Scientz Biotechnology co., LTD, Zhejiang, China). The protein content of the sonicated material was determined using the Bradford assay (*14*). The data of cellular accumulation are expressed as nmol CoQ_{10} per mg cell protein.

Cellular Uptake of RH-123. To evaluate the effect of nanoliposomes on P-glycoprotein activity, the cellular uptake of RH-123 in the presence of nanoliposomes were conducted. Experiments were performed using Caco-2 cells after 15 days of seeding. Cell monolayers were washed twice with 1 mL of PBS before adding 1 mL of nanoliposomes to wells of the 12-well culture dish. Cultures were incubated at 37 °C for 30 min. RH-123 (5.0 μM) was added into each well and then incubated at 37 °C for 30 min.

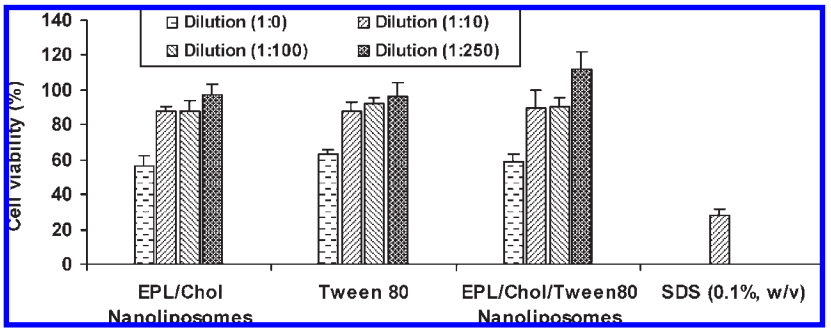

Figure 1. Cytotoxicity of nanoliposomes and surfactants with different dilution factors after 4 h of incubation with cells using the MTT method (compared to the negative control). Each data represents the mean of three measurements.

Cyclosporin A (10 μM), an inhibitor of P-glycoprotein, was used as a positive control. Cells treated with PBS were used as a negative control (blank). The uptake was terminated by removing the medium and washing monolayers four times with ice-cold PBS. Then, the monolayers were harvested in 1-mL 1% (w/v) Triton X-100. RH-123 was quantified using a spectrofluorimeter (model 650−60, Hitachi, Ltd., Tokyo, Japan) operating at an excitation wavelength of 504 nm and an emission wavelength of 550 nm.

Fluorescence Polarization. To evaluate the variation of cell membrane fluidity, fluorescence polarization studies were carried out at 37 °C using a spectrofluorimeter (model 650−60, Hitachi, Ltd., Tokyo, Japan) fitted with a polarizer attachment. Caco-2 cell suspensions (2.5 mL, 2×10^5 cells/mL in PBS) were mixed with appropriate concentrations of Tween 80 and nanoliposomes followed by the addition of 2.5 μL of fluorescent probe DPH solution (1 mM stock solution in tetrahydrofuran), and then incubated at 37 °C for 30 min in darkness (*15*). Cells were also incubated with cholesterol (final concentration 0.25 mM) and benzyl alcohol (final concentration 30 mM), in order to study the influence of the known cell membrane rigidizer (cholesterol) and fluidizer (benzyl alcohol) as positive control groups (*15*). Steady state anisotropy was measured. The wavelengths of excitation and emission were 360 and 430 nm, respectively. For reflecting the effect of samples on cell membrane fluidity, the steady state anisotropy in the presence of samples was compared to that of the negative control group (PBS), on a percentage basis.

Extraction and Analysis of Ubiquinone-10 (CoQ_{10}) and Ubiquinol-10 ($CoQ_{10}H_2$). One milliter aliquot of samples obtained from the transport study in Caco-2 cells was taken into centrifuge tubes. Then 25 μL of internal standard CoQ_9 (4 μg/mL) was added followed by vortex-mixing. This solution was deproteinized by precipitating out the proteins with 1 mL of ethanol, and the first extraction was performed using 3 mL of *n*-hexane. The mixture was vortex-mixed for 3 min and then centrifuged at 2000 rpm for 15 min at ambient temperature to separate the hexane and aqueous layers. The top layer (first extraction) was transferred into a fresh centrifuge tube. The second extraction was carried out with the same sample by adding 2 mL of *n*-hexane. The combined supernatants from the two extraction steps were then dried under a stream of nitrogen, reconstituted in 100 μL of ethanol, and analyzed by the reverse-phase HPLC method. For samples obtained from accumulation in Caco-2 cells, a 0.4 mL aliquot was taken into centrifuge tubes. The other procedures for analysis were the same as those described above.

HPLC was carried out using an HPLC system equipped with a 1525 binary pump and a 2996 photodiode array detector (Waters, Milford, MA, USA). The detection wavelength was set at 275 and 290 nm. HPLC analysis of samples was performed using a SunFire C_{18} ODS analytical column (150 × 4.6 mm, 5 μm) (Waters, Milford, MA, USA). The column temperature was maintained at 35 °C. The mobile phase was a mixture of ethanol/methanol (70:30, v/v). The flow rate was maintained at 1 mL/min. The standard curve for CoQ_{10} in transport samples was linear within the range of 0.0512−20.48 μg/mL ($A_s/A_i = 8.2012C + 0.704$, $R^2 = 0.9993$). The standard curve for CoQ_{10} in accumulation samples was linear within the range of 0.02−10.24 μg/mL ($A_s/A_i = 4.0618C + 0.0313$, $R^2 = 0.9995$). The standard curve for $CoQ_{10}H_2$ in accumulation samples was linear within the range of 0.256−8.192 μg/mL ($A_s/A_i = 1.2249C$-0.0884, $R^2 = 0.9906$). Where, A_s/A_i is the peak area ratio of CoQ_{10} (or $CoQ_{10}H_2$) to CoQ_9, and C is the concentration of CoQ_{10} (or $CoQ_{10}H_2$).

HPLC-PDA-MS analysis was performed on a Waters 2690 HPLC system (Waters, Milford, MA, USA) equipped with a 996 photodiode array detector (PDA). The PDA was set to a range between 200 to 400 nm. HPLC analysis of samples was performed using a SunFire C_{18} ODS analytical column (150 × 2.1 mm, 5 μm) (Waters, Milford, MA, USA). The column temperature was maintained at 35 °C, and injection volume was set to 5 μL. The mobile phase was a mixture of ethanol/methanol (50:50, v/v). The flow rate was set at 0.3 mL/min. The mass spectrometry (MS) analysis as performed in positive electrospray ionization (ESI+) mode on a Waters Platform ZMD 4000 (Waters, Milford, MA, USA). Typical running parameters were as follows: capillary voltage, 3.87 kV; cone voltage, 25 V; source temperature, 120 °C; analyzer vacuum, 2.6×10^{-3} Pa; gas flow, 4.2 L/h. Spectra were scanned over a mass range of 200−1200 m/z. CoQ_{10} and $CoQ_{10}H_2$ were identified by comparing the retention time of the peak of samples with standards in HPLC. The MS spectra were also used to identify CoQ_{10} and $CoQ_{10}H_2$ transporting across and accumulating in the Caco-2 cell monolayers.

Data Analysis. In each transport experiment, the slope of the linear portion of the plot of total amount of CoQ_{10} transported versus time (180 min) was obtained. Apparent permeability coefficients (P_{app}) of CoQ_{10} were calculated in both apical to basolateral and basolateral to apical directions according to the following equation:

$$P_{app}(\text{cm/s}) = \frac{dQ/dt}{60 \times A \times C_0}$$

where, the dQ/dt (μg/min) is the CoQ_{10} permeation rate, A is the cross-sectional area (4.2 cm^2), and C_0 (μg/mL) is the initial CoQ_{10} concentration in the donor compartment at $t = 0$ min.

Statistical Analysis of Data. Results are expressed as the mean ± SD of three or more determinations. Statistical significance was tested by two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) and were considered significant at $P < 0.05$.

RESULTS

Cytotoxicity of Nanoliposomes. The mitochondrial activity of the Caco-2 cells was relative to the concentration of nanoliposomes (**Figure 1**). When nanoliposomes were diluted (1:10, 1:100, and 1:250) with PBS to give a final EPL concentration lower than 0.125% (w/v), the cell viability was higher than 90%. Meanwhile, Tween 80 with a concentration lower than 0.1% (w/v) showed no significant effect on the cell viability. The results were similar to that of the negative control treated cells and were significantly higher as compared to that of positive control treated cells (SDS). The results also suggested that nanoliposomes had no visible influence on the mitochondria of the cells. Thus, CoQ_{10} nanoliposomes were used in a 10-fold dilution throughout the transport and accumulation experiments. Moreover, the TEER values for the Caco-2 cell monolayers after treatment with nanoliposomes showed excellent recovery (data not shown), implying the monolayer integrity during the transport experiments.

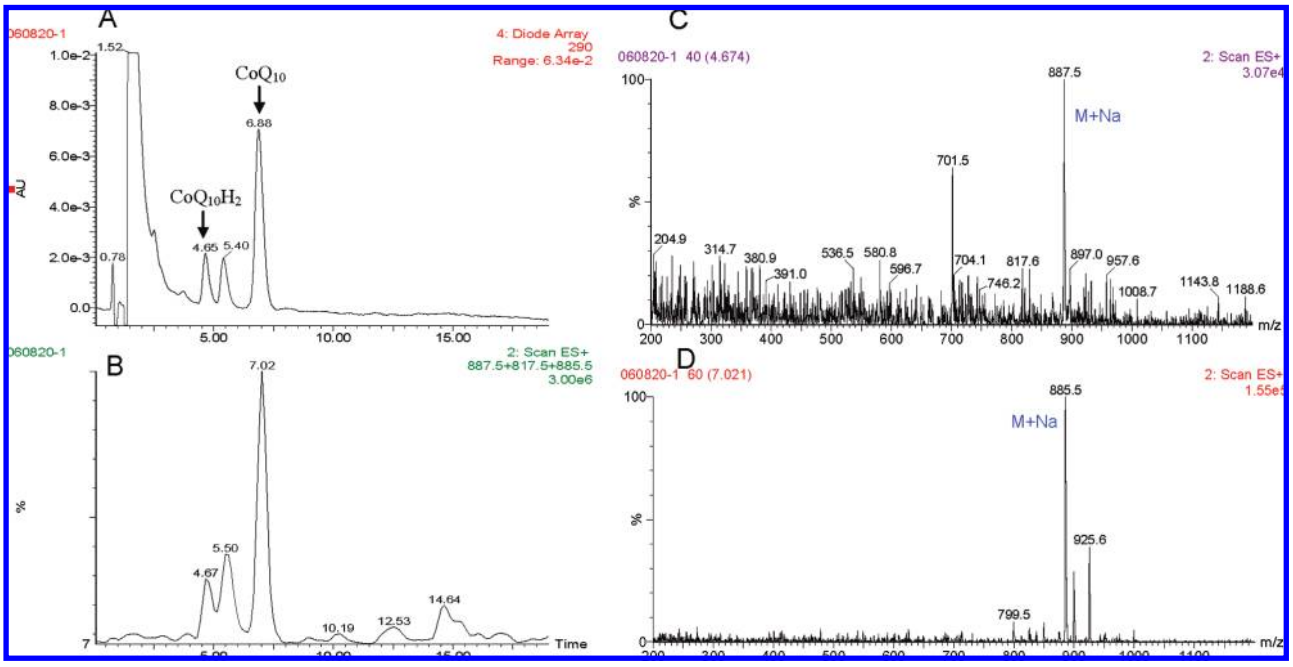

Figure 2. Chromatogram of extracted sample accumulating in Caco-2 cell. (**A**) HPLC chromatogram; (**B**) ESI-MS total ion current (TIC) chromatogram; (**C** and **D**) ESI-MS spectra. The peak at 5.4 min is the internal standard CoQ_9.

Table 1. Apparent Permeability Coefficients (P_{app}) and Cellular Accumulation of CoQ_{10} as Well as $CoQ_{10}H_2$ in Various Formulations for 180 min in the Absorptive (AP-BL) Direction[a]

formulation	$P_{app} \times 10^{-6}$ (cm/s)	accumulation of CoQ_{10} (nmol/mg protein)	accumulation of $CoQ_{10}H_2$ (nmol/mg protein)
CI	0.033 ± 0.030	4.89 ± 1.14	0.92 ± 0.05
CII	0.34 ± 0.13	3.49 ± 0.52	4.35 ± 1.02
FI	0.65 ± 0.50	1.22 ± 0.25	2.79 ± 0.42
FII	4.19 ± 0.76[b]	0.52 ± 0.05[c]	1.53 ± 0.16[c]

[a] CI, CoQ_{10} powder dispersion; CII, marketed liquid CoQ_{10}; FI, CoQ_{10} nanoliposomes (EPL/Tween 80/Chol = 2.5:0.0:0.4, w/w); FII, CoQ_{10} nanoliposomes (EPL/Tween 80/Chol = 2.5:1.8:0.4, w/w). Each data point represents the mean of three monolayers. The CoQ_{10} concentration in the donor compartment was about 359 μM. [b] $P < 0.01$ (significant increase versus the controls). [c] $P < 0.05$ (significant decrease versus the controls).

Identification of CoQ_{10} and $CoQ_{10}H_2$. In order to identify the CoQ_{10} form transporting across and accumulating in the Caco-2 cell monolayers, HPLC-PDA-MS analysis was carried out. As the formation of fragment ion for the transport sample was $[M + Na]^+$ and its m/z was 885.5, the transported CoQ_{10} was still present as ubiquinone-10, i.e., the oxidized state (data not shown). However, it was interesting to find that the CoQ_{10} accumulated by the Caco-2 cell monolayers existed in two forms: ubiquinone-10 and ubiquinol-10 ($CoQ_{10}H_2$) (**Figure 2**). The formation of fragment ion for the accumulated sample was $[M + Na]^+$ at m/z 887.5 (**Figure 2C**) and 885.5 (**Figure 2D**). The results indicated that the conversion from ubiquinone-10 to ubiquinol-10 took place in the enterocytes during its absorption.

Effect of Nanoliposomes on Transepithelial Transport and Cellular Accumulation of CoQ_{10}. The calculated apparent permeability coefficients of CoQ_{10} are presented in **Table 1**. The P_{app} of CoQ_{10} in the apical to basolateral direction was estimated to be $0.33 \pm 0.30 \times 10^{-7}$ cm/s when measured for the CoQ_{10} powder dispersion, consistent with poor absorbability of the lipophilic nutrient. The apparent permeability of CoQ_{10} across Caco-2 cell monolayers was increased by 10-fold for marketed liquid product and 20-fold for nanoliposomes primarily composed of EPL. However, the P_{app} values of these two formulations were still low as compared to the value that is generally known to be necessary for favorable (100%) intestinal absorption (i.e., 1×10^{-6} cm/s) (*16*). The increase of P_{app} was much more pronounced when CoQ_{10} was applied in the formulation of nanoliposomes composed of EPL, Tween 80, and Chol, which have been proven to be the optimal formulation during preparation and storage (*13*). The results showed that the influence of nanoliposomal composition was significant for the enhancement of CoQ_{10} transport.

It was evident that the encapsulation of CoQ_{10} in nanoliposomes reduced the intracellular accumulation. The accumulation percentage of $CoQ_{10}H_2$ accounting for total Q_{10} in the cell monolayers treated with CoQ_{10} powder dispersion was just about 16%, which is lower than that of marketed liquid product (55%) and EPL/Tween 80/Chol nanoliposomes (75%).

Effect of CoQ_{10} Concentration on Transport and Accumulation Behavior. According to the results in **Figure 3A**, the transport rate of CoQ_{10} in the formulation of nanoliposomes was concentration dependent and almost showed linearity. The P_{app} values in the apical to basolateral direction at 37 °C for three levels of donor concentrations (113.4, 359.4, and 753.3 μM) were estimated to be $6.31 \pm 1.42 \times 10^{-6}$, $4.19 \pm 0.76 \times 10^{-6}$, and $5.42 \pm 0.45 \times 10^{-6}$ cm/s. The corresponding transport percentages were 4.54 ± 1.02%, 3.02 ± 0.54%, and 3.90 ± 0.32%, which showed no significant concentration dependence. Cellular accumulation of CoQ_{10}, $CoQ_{10}H_2$, and total Q_{10} were all proportional to the concentration of CoQ_{10} in the donor compartment (**Figure 3B**), whereas the percentage of $CoQ_{10}H_2$ accounted for 70−80%, which was independent of the donor concentration.

Directional Permeability Differences. The transepithelial fluxes of CoQ_{10} in the formulation of nanoliposomes across Caco-2 cell monolayers in the absorptive (apical to basolateral; AP-BL) and

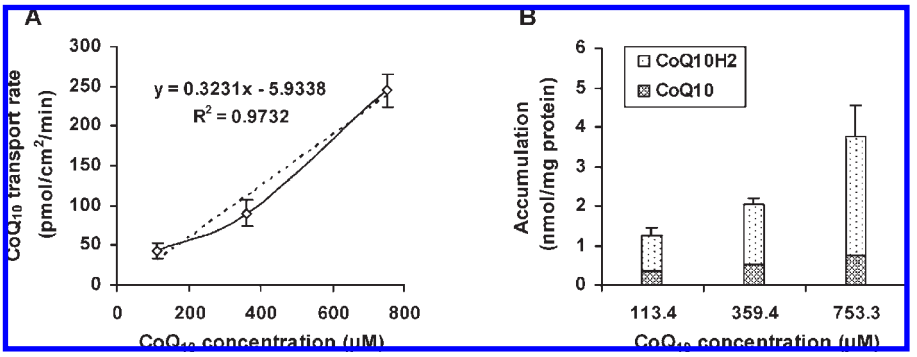

Figure 3. Concentration dependence of transport rate (**A**) and cellular accumulation (**B**) of CoQ_{10} in the nanoliposomal formulation for 180 min in the absorptive (AP-BL) direction. Each data point represents the mean of three monolayers.

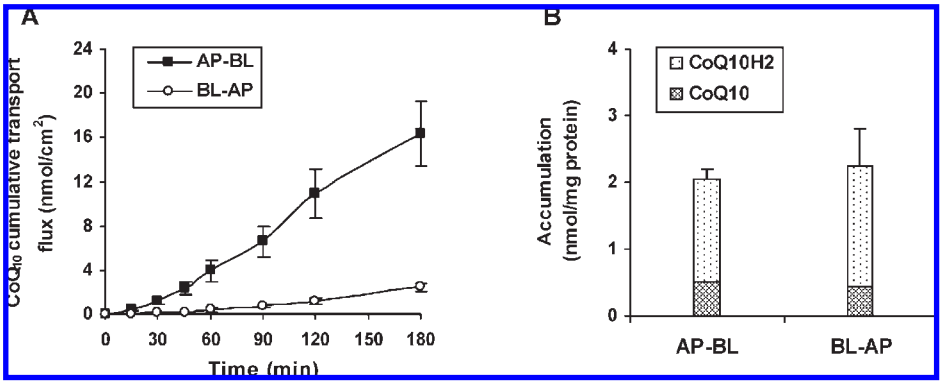

Figure 4. Transepithelial fluxes (**A**) and cellular accumulation (**B**) of CoQ_{10} in the nanoliposomal formulation in the absorptive (AP-BL) and secretory (BL-AP) directions. Each data represents the mean of three monolayers. The CoQ_{10} concentration in the donor compartment was about 359 μM.

Table 2. Apparent Permeability Coefficients and Cellular Accumulation of CoQ_{10} in the Formulation of Nanoliposomes (NL) in the Presence of Some Transporter Inhibitors/Substrates[a]

samples	$P_{app} \times 10^{-6}$ (cm/s)	accumulation of CoQ_{10} (nmol/mg protein)	accumulation of $CoQ_{10}H_2$ (nmol/mg protein)
control	4.19 ± 0.76	0.52 ± 0.05	1.53 ± 0.16
NL + nicotinic acid	4.39 ± 0.27	0.46 ± 0.07	1.31 ± 0.36
NL + captopril	4.73 ± 0.68	0.53 ± 0.06	1.25 ± 0.13
NL + cyclosporin A	7.23 ± 0.80^b	0.40 ± 0.03	1.09 ± 0.16

[a] Each data point represents the mean of three monolayers. Control refers to cells treated with CoQ_{10} nanoliposomes in the absence of the transporter inhibitor/substrate. The CoQ_{10} concentration in the donor compartment was 359 μM. [b] $P < 0.05$ (significant increase versus the other treatments).

secretory (basolateral to apical; BL-AP) directions are shown in **Figure 4A**. The cumulative amount of CoQ_{10} across Caco-2 cell monolayers was time dependent and showed linearity over 180 min. The cumulative transport amount of CoQ_{10} for the AP-BL was found to be greater as compared to that obtained from BL-AP transport. The secretory P_{app} was calculated to be $0.63 \pm 0.09 \times 10^{-6}$ cm/s, which was significantly lower than that of the absorptive direction, and the ratio of $P_{app\ (AP\text{-}BL)}$ to $P_{app\ (BL\text{-}AP)}$ was 6.65. However, the direction had no obvious effect on the cellular accumulation of CoQ_{10} and $CoQ_{10}H_2$ (**Figure 4B**).

Competitive Inhibition Studies with Substrate/Inhibitor of Known Transporters. The directional dependence of transport of CoQ_{10} in the nanoliposomal formualtion suggests that it is likely to be mediated by some active transporters located in the apical side. Furthermore, possible involvement of multiple transporters such as organic cation/carnitine transporters, peptide transporter, and organic anion transporter have also been indicated at a concentration (1 nM) of [^{3}H]-CoQ_{10} (*2*). Therefore, the AP-BL transport of CoQ_{10} (359 μM) in the formulation of nanoliposomes was investigated in the presence of captopril (an inhibitor of peptide transporter, PepT-1) and nicotinic acid (a substrate of monocarboxylic acid transporter, MCT1). As CoQ_{10} is lipophilic, the effect of active efflux pump P-glycoprotein (P-gp) was also considered. Wherein, cyclosporin A was used as an inhibitor of P-gp. The P_{app} values in the presence of captopril and nicotinic acid were not significantly different from that obtained from the control in the absence of substrate/inhibitor (**Table 2**). The presence of cyclosporin A enhanced the apical to basolateral absorption of CoQ_{10}. The P_{app} value was estimated to be $7.23 \pm 0.80 \times 10^{-6}$ cm/s, which was significantly increased as compared to the values of the other three groups. In addition, cellular accumulation of CoQ_{10}, $CoQ_{10}H_2$, and total Q_{10} were not obviously altered in the presence of substrate/inhibitor (**Table 2**).

Effect of Nanoliposomes on Inhibition of P-gp. As the cyclosporin A affected the transport of CoQ_{10} in the formulation of nanoliposomes, thus RH-123 was used as an inhibitor of P-gp to investigate the influence of nanoliposomes on the active efflux pump. An RH-123 sequestering might be the cause for the decline in the P-gp inhibitory activity (*17*). A previous study has revealed that phospholipids might be recognized by P-gp (*18*), whereas the presence of nanoliposomes primarily composed of EPL had no significant effect on the cellular uptake of RH-123 (**Figure 5**).

Further studies showed that the presence of nanoliposomes composed of EPL, Tween 80, and/or Chol significantly enhanced the intracellular accumulation of RH-123 in Caco-2 cells as compared to that in the control (blank). It was also found that the P-gp activity was concerned with the percentage of Tween 80 in wall materials of nanoliposomes.

Effect of Nanoliposomes on Cell Membrane Fluidity. As most of the transport proteins are membrane bound, the physical state of the cell membrane is probably important in the regulation of transporter function (*15*). Fluidization of the cell membrane usually caused the decline of P-gp activity. DPH is a fluorescent probe to evaluate the fluidity of the hydrophobic core of the cell membrane lipid bilayer. As expected, cholesterol led to an obvious decrease in membrane fluidity (i.e., increase in steady state anisotropy of DPH). Benzyl alcohol did the opposite. The surfactant Tween 80 and nanoliposomes resulted in a significant decrease in the anisotropy of DPH (**Table 3**), suggesting fluidization of the lipid bilayers of the Caco-2 cell membrane. Furthermore, the lower the concentration, the smaller the decreased percentage of fluidity. In this regard, the permeability increase of CoQ_{10} in the formulation of nanoliposomes would appear to be related to the modulation of cell membrane fluidity.

DISCUSSION

The absorption of an orally administered nutrient depends on the transport characteristics, which in turn are determined by the biopharmaceutical (solubility and permeability) and physiological factors (*2*). CoQ_{10} has a very low bioavailability when administered orally because of low solubility and permeability. Liposomes can provide a protective layer for CoQ_{10} molecules,

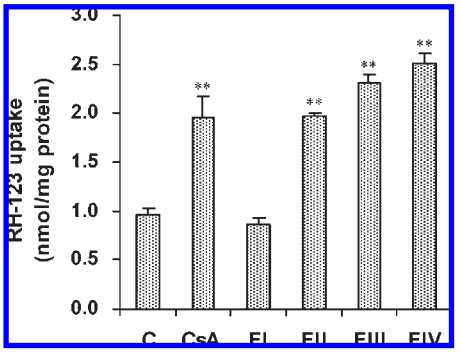

Figure 5. RH-123 uptake after the treatment of nanoliposomes with different compositions. C, control cells treated with PBS; CsA, cyclosporin A, an inhibitor of P-gp; FI, EPL/Tween 80/Chol = 2.5:0.0:0.0 (w/w); FII, EPL/Tween 80/Chol = 2.5:1.8:0.0 (w/w); FIII, EPL/Tween 80/Chol = 2.5:1.8:0.4 (w/w); FIV, EPL/Tween 80/Chol = 1.75:3.25:0.0 (w/w). The CoQ_{10} concentration in the donor compartment was about 359 μM. $^{**}P < 0.01$ (significant increase versus the control).

and there are a number of studies indicating that nutrient or drug molecules in liposome form can pernetrate through the cell membrane more effectively (*11*). The development of nanoliposomes for oral delivery of water-insoluble nutrient CoQ_{10} aimed at improving the solubility and absorption.

Cytotoxicity evaluation carried out using nanoliposomes as transport vehicles showed that it would be used safely up to a 1:10 dilution. The corresponding maximum CoQ_{10} concentration might reach about 1 mg/mL in PBS, which is significantly higher than that of previous cell experiments. As for the difficulty of determining CoQ_{10} concentrations across the Caco-2 cell monolayers encountered by some researchers (*2*), it was found that the reverse-phase HPLC method was sensitive enough for our studies and that the method avoided the unsafe factors caused by radiolabeled materials. Moreover, the forms of CoQ_{10} accumulated in cell monolayers might be directly detected by HPLC, which helps one know whether the metabolism takes place during absorption.

The intracellular accumulation reflects the membrane affinity of CoQ_{10} that may be likely due to covalent binding to cellular proteins in Caco-2 cells. Moutardier et al. (*12*) found that the increase of the transepithelial transport of the liposomal form of 5-fluorouracil was not linked to an increase in the tissue accumulation of the drug. They also suggested that the optimization of the transepithelial passage of the drugs by the liposomal formulations depends on the nature of the drug and the concentration but does not appear to be linked to the tissue accumulation. Our study supported their conclusion by providing evidence that encapsulation of CoQ_{10} in nanoliposomes reduced cell accumulation.

The transepithelial transport of CoQ_{10} across Caco-2 cell monolayers was enhanced with nanoliposomes as vehicles. Tween 80 was found to exhibit an enhancing effect in the absorptive transport of CoQ_{10}. Inclusion of phospholipid with surfactant Tween 80 resulted in transport increase across the cell monolayers, which has been reported in the past (*19*). Surfactants in the presence of a lipid bilayer make the liposome structure highly sensitive to deformation, which is helpful to enhance the encapsulated nutrient delivery across barriers. Formulation of CoQ_{10} with EPL and cholesterol leads to the formation of rigid liposomes; however, the addition of a surfactant makes the liposomes more flexible. The flexibility or deformability could make nanoliposomes transport across the cell more easily as compared to liposomes without the surfactant. We also found that the nanoliposomal formulations were able to significantly enhance CoQ_{10} transport without influencing TEER. This indicated that the increased permeability from the formulations was not a result of cell membrane damage.

The concentration dependence of nanoliposomal CoQ_{10} transport was not obvious in the studied range, suggesting the involvement of passive diffusion. However, the secretive (BL-AP)

Table 3. Influence of Nanoliposomes (NL), Surfactant, and Known Fluidity Modulators on the Anisotropy of DPH[a]

fluidity modulators/nanoliposomes/ surfactant	DPH anisotropy (% of control)[b]
NL (EPL/Tween 80/Chol = 2.5:1.8:0.0, w/w) (EPL = 1.25%, w/v)	51.0 ± 1.6^c
NL (EPL/Tween 80/Chol = 2.5:1.8:0.4, w/w) (EPL = 1.25%, w/v)	68.2 ± 0.5^c
NL (EPL/Tween 80/Chol = 2.5:1.8:0.4, w/w) (EPL = 0.125%, w/v)	79.3 ± 2.0^c
NL (EPL/Tween 80/Chol = 2.5:1.8:0.4, w/w) (EPL = 0.0125%, w/v)	88.6 ± 3.5^c
Tween 80 (0.9%, w/v)	42.7 ± 6.8^c
Tween 80 (0.09%, w/v)	55.4 ± 4.2^c
Tween 80 (0.009%, w/v)	69.3 ± 3.6^c
cholesterol (0.2 mM)	139.4 ± 3.6
benzyl alcohol (30 mM)	44.8 ± 5.2

[a] The results are expressed as the mean value ± standard deviation ($n = 3$). [b] Control anisotropy value for DPH was 0.192 ± 0.023. [c] $P < 0.05$ (significant decrease versus the rigidizer control (cholesterol).

permeability was significantly lower as compared to the absorptive direction, which indicates that transporter proteins are related to permeability and that the transporter system locates on the AP side. This is in agreement with the finding with [^{3}H]-CoQ_{10} at a lower concentration of 1 nM. The reported studies have also given preliminary evidence for CoQ_{10} transport by carrier-mediated transport mechanism (*2*). To further confirm whether the specific transporters would still be involved in the permeability of CoQ_{10} in the formulation of nanoliposomes, preliminary studies for competitive inhibition were performed in the presence of a specific substrate/inhibitor of known transporters. Caco-2 cells have been shown to possess the peptide transporters and organic anion transporters as well as active efflux pump P-gp in the apical, which are similar to small intestinal epithelium. If AP-BL transport in the presence of captopril (an inhibitor of peptide transporter) or nicotinic acid (a specific substrate of organic anion transporters) is inhibited, it might indicate that the peptide transporter and organic anion transporters would still be involved in the permeability of CoQ_{10} with nanoliposomes as vehicles. In contrast to the hypothesis, the permeability of CoQ_{10} was not altered significantly in the presence of these two competitive compounds. One likely reason is that the variable transporter concentrations on the cell membrane have been deduced to be related with the observed low and variable bioavailability of CoQ_{10} in humans after oral administration (*2*). Moreover, CoQ_{10} is often used for adjuvant treatment of many cardiovascular diseases coadministered with statins and antihypertensive drugs such as captopril. The use of statins decreases the plasma and cellular concentrations of CoQ_{10} (*20*). It was also found that statin drugs are transported by MCT1; therefore, some researchers speculated that CoQ_{10} and statins might share the same transporter(s), thus causing competition and subsequent reduction of CoQ_{10} levels (*2*). Herein, nicotinic acid, a specific substrate for MCT1, did not inhibit the permeability of CoQ_{10} in the formulation of nanoliposomes in our studies. These results suggest that the effect of two transporters on the permeability of CoQ_{10} might be suppressed when using nanoliposomes as transport vehicles. The CoQ_{10} permeability was enhanced by cyclosporin A (an inhibitor of P-gp), suggesting the involvement of P-gp in the transport of CoQ_{10}. Also, this indicates that nanoliposomes enhancing the CoQ_{10} transport might have the capability of regulating the activity of P-gp. Further confirmation of this hypothesis was obtained by investigating the effect of nanoliposomes on the cellular uptake of RH-123 (a substrate of P-gp) and membrane fluidity. The inhibitory effects of nanoliposomes containing Tween 80 on the P-gp activity were similar to or even higher than that of cyclosporin A. The fluidity of the cell membrane was enhanced by nanoliposomes and Tween 80, which was relative to the concentration. The structural characteristics of the Tweens impart both lipophilic and hydrophilic properties to the surfactants, allowing them to insert between the lipophilic tails of the bilayers, resulting in disruption of the lipid-packing arrangement and fluidization of the lipid membrane, thus promoting the diffusion of the drug across Caco-2 cells (*21*). P-gp is located at the hydrophobic region in the apical side of Caco-2 cell monolayers. DPH is a fluorescent probe targeting the hydrophobic core of the cell membrane lipid bilayer. Taking these into account, we might deduce that the increase in cell membrane fluidity induced by nanoliposomes is a cause of the impairment of P-gp function. Likewise, most of the transport systems are cell membrane bound, and the fluidity change of the cell membrane with nanoliposomes as transport vehicles is also probably important in the modulation of other transporters involved in the CoQ_{10} permeability.

The permeability increase of the nutrient CoQ_{10} would also depend on the mode of interaction of the nanoliposomes with the cells. This could be due to the fact that the liposomes may preferentially interact with cells via several mechanisms, including intermembrane transfer, adsorption, fusion, and endocytosis allowing CoQ_{10} and phospholipids to internalize into cells and to localize into the plasma membrane and subcellular organelles, and to affect the cell association, the transmembrane flux, or the binding of CoQ_{10} to intracellular targeting sites (*18*, *22*). Further work should be carried out in later studies to clearly explain the mechanism of the alteration.

A large amount of enzymes such as bioreductive enzymes (i.e., microsomal cytochrome P450 reductase) are expressed within Caco-2 cells (*23*), which are similar to the human small intestinal epithelium, so that the accumulation form in cells might reflect whether the form transformation takes place during transport. Exogenous CoQ_{10} after being converted to its reduced form may protect cells from oxidative stress by ubiquinone reductase such as microsomal NADH-cytochrome b_5 and NADPH-cytochrome P-450 reductases, DT-diaphorase, and the NADH dehydrogenase associated with the mitochondrial outer membrane (*24*, *25*). We observed that there exists the redox turnover of CoQ_{10} in Caco-2 cells regardless of the administered formulation in our study. Furthermore, the $CoQ_{10}H_2/CoQ_{10}$ molar ratio accumulated in cells almost kept constant when using nanoliposomes as transport vehicles of CoQ_{10}, which was greater than the controls.

In conclusion, nanoliposomes can be used as an effective solubilizer of CoQ_{10} so as to increase the concentration of CoQ_{10} in an aqueous system at several hundred fold without causing significant cell toxicity. The bioreduction of CoQ_{10} occurred during its absorption. The apparent permeability coefficient of CoQ_{10} in the formulation of EPL/Tween 80/Chol nanoliposomes was increased, suggesting an improved bioavailability following its oral administration. The findings of this study support the significance of investigating the permeation of formulated water-insoluble nutrients, as opposite to unformulated nutrients, by the Caco-2 human intestinal cell model.

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