AAPS PharmSciTech **2003; 4 (3) Article 32 (http://www.pharmscitech.org).**

Preparation and Characterization of Novel Coenzyme Q_{10} Nanoparticles Engineered from Microemulsion Precursors

Submitted: April 7, 2003; Accepted: May 27, 2003

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ABSTRACT

The purpose of these studies was to prepare and characterize nanoparticles into which Coenzyme Q_{10} (CoQ_{10}) had been incorporated (CoQ_{10}-NPs) using a simple and potentially scalable method. CoQ_{10}-NPs were prepared by cooling warm microemulsion precursors composed of emulsifying wax, CoQ_{10}, Brij 78, and/or Tween 20. The nanoparticles were lyophilized, and the stability of CoQ_{10}-NPs in both lyophilized form and aqueous suspension was monitored over 7 days. The release of CoQ_{10} from the nanoparticles was investigated at 37°C. Finally, an in vitro study of the uptake of CoQ_{10}-NPs by mouse macrophage, J774A.1, was completed. The incorporation efficiency of CoQ_{10} was approximately 74% ± 5%. Differential Scanning Calorimetry (DSC) showed that the nanoparticle was not a physical mixture of its individual components. The size of the nanoparticles increased over time if stored in aqueous suspension. However, enhanced stability was observed when the nanoparticles were stored at 4°C. Storage in lyophilized form demonstrated the highest stability. The in vitro release profile of CoQ_{10} from the nanoparticles showed an initial period of rapid release in the first 9 hours followed by a period of slower and extended release. The uptake of CoQ_{10}-NPs by the J774A.1 cells was over 4-fold higher than that of the CoQ_{10}-free nanoparticles ($P < .05$). In conclusion, CoQ_{10}-NPs with potential application for oral CoQ_{10} delivery were engineered readily from microemulsion precursors.

KEYWORDS: Coenzyme Q_{10}, nanoparticles, microemulsion, stability, cell uptake

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INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}), also known as ubiquinone or ubidecarenone, is a fat-soluble, vitamin-like substance found in the cells of many organisms. CoQ_{10} is involved in a variety of essential cellular processes such as acting as a redox component of transmembrane electron transport systems in the respiratory chain of mitochondria and as a stabilizing agent in cellular membranes.[1] CoQ_{10} is an essential component for production of cellular energy in the form of adenosine triphosphate (ATP) and acts as an antioxidant.[1] It is used as a nutritional supplement and also in the treatment of cardiovascular disorders such as angina pectoris, hypertension, and congestive heart failure.[2] Also, many studies have reported the immunostimulating action of CoQ_{10}.[3] However, because of the molecule's long side chain of 10 isoprenoid units, CoQ_{10} is poorly water-soluble.[4] It is thought that the incomplete and slow absorption of CoQ_{10} from the gastrointestinal tract is attributed to its poor water solubility and high molecular weight.[5]

Because of its poor aqueous solubility, CoQ_{10} has presented a challenge in the development of formulations for oral administration. Many different approaches for formulating CoQ_{10} have been reported; some of these have been introduced into the market as oil-based or powder-filled capsule formulations. However, the oral bioavailability of these formulations differs widely.[6] For example, Kishi et al[6] compared the plasma CoQ_{10} level of 6 marketed CoQ_{10} preparations in healthy volunteers. Administration of CoQ_{10} soft capsules increased the plasma level to 2.7 times above the starting level. Weis et al[7] compared the bioavailability of CoQ_{10} powder-filled capsules and 3 preparations containing CoQ_{10} suspensions in soybean oil, with and without emulsifiers. In this study, the oil-based formulation without emulsifiers provided superior results. The differences in bioavailability may be because the bioavailability studies were performed based on labeled doses rather than actual doses, and the quality control for neutraceuticals is frequently not stringent. Other reported formulation strategies include a redis-

persible dry emulsion,[8] the complexation of CoQ_{10} with cyclodextrins,[9] and self-emulsifying drug delivery systems.[10] Compared with the currently marketed CoQ_{10} products, such as soft gel, some of these alternative formulations showed enhanced oral bioavailability in animal and/or human studies. However, there continues to be a need to develop improved formulations for the oral delivery of CoQ_{10}.

Recently, nanoparticles have been actively explored as a delivery system for both small drug molecules and macromolecules. It has been shown that nanoparticles can improve the oral bioavailability of drugs with poor absorption characteristics.[11-13] For example, Maincent et al[11] reported that the oral bioavailability of vicamine-incorporated nanoparticles was enhanced compared with an oral solution of the drug. It was also shown that a formulation of cyclosporine-A into nanoparticles increased its absorption and oral bioavailability.[12] In addition, Mathiowitz et al[13] showed that the bioavailability of dicumarol particles (5 μm) resulted in a 112% increase in bioavailability over a dicumarol suspension. It was shown that the absorption pathways as well as efficiency were affected by particle size. Because of the lipophilic nature of the matrix, lipid-based solid nanoparticles are considered to be particularly useful for the delivery of lipophilic drugs.[14] The apparent water solubility of the lipophilic drugs is enhanced by incorporating them in the lipid-based nanoparticles, and the small nanoparticles create a larger surface area and therefore favor increased absorption. Moreover, the intestine has special mechanism(s) to absorb particles, and there may be a size-exclusion phenomenon in the gastrointestinal absorption of particles, with 100 nm particles showing a significantly higher uptake (10- to 250-fold higher) than larger particles (500 nm to 10 μm).[15]

CoQ_{10} is a poorly water-soluble compound exhibiting low oral bioavailability. It has been hypothesized that the bioavailability of CoQ_{10} can be improved by incorporating the compound into submicron particles. CoQ_{10} has low oral bioavailability. It is given in small doses and has very poor water solubility, which makes it an excellent candidate to be incorporated in lipid-based nanoparticles. Seikmann et al[4] and Bunjes et al[14] reported on the preparation and physicochemical characterization of CoQ_{10}-incorporated nanoparticles (CoQ_{10}-NPs). These authors demonstrated that CoQ_{10} could be incorporated into nanoparticles composed of several different matrixes. Other CoQ_{10}-NPs include poly(ethylene imine) dodecanoate complex prepared by the complexation of poly(ethylene imine) with dodecanoic acid,[16] and CoQ_{10} containing poly(methyl methacrylate) nanoparticles prepared by microfluidization.[17]

Cui and Mumper[18-20] have reported on a novel nanoparticle system engineered from oil-in-water (O/W) microemulsion precursors. The microemulsions, formed at increased temperature (50°C-55°C), were composed of emulsifying wax as the oil phase and nonionic, cationic, or anionic surfactants to form nanoparticles with the corresponding surface charges upon simple cooling to room temperature. This microemulsion precursor strategy has some advantages[18,21]: (1) the engineering process can be easily adapted to include many different excipients, (2) well-defined as well as uniform solid nanoparticles (<100 nm) may be reproducibly engineered without the use of expensive and/or damaging high-torque mechanical mixing, microfluidization, or homogenization, and (3) no organic solvents are used during the manufacturing process. This nanoparticle system has been used as a carrier for DNA and proteins as well as for other drugs and sensors.[18-20,22] In the present study, this novel nanoparticle engineering procedure was used to incorporate CoQ_{10}. The overall objective of this study was to develop and characterize CoQ_{10}-NPs from the microemulsion precursors. In addition, an in vitro cell uptake study was carried out to investigate whether incorporation of CoQ_{10} can enhance the uptake of nanoparticles by mouse macrophages. Makabi-Panzu et al[23] reported that incorporation of CoQ_{10} in liposomes enhanced the uptake of the liposomes by J774A.1 macrophage cells.

MATERIALS AND METHODS

Materials

Coenzyme Q_{10}, emulsifying wax, polysorbate 20 (Tween 20), polysorbate 80 (Tween 80), sucrose, and lactose were purchased from Spectrum Chemical Products, Inc (New Brunswick, NJ). Brij 78 was obtained from Aldrich Chemical Co (Milwaukee, WI). Sephadex G-75 and trehalose were obtained from Sigma Chemical Co (St Louis, MO). Fluorescein-labeled DOPE (1, 2-Dioleoyl-sn-Glycero-3-Phosphati dylethanolamine-N-[Carboxyfluorescein]) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL). High-performance liquid chromatography (HPLC)–grade methanol and n-hexane were purchased from Fisher Scientific (Fair Lawn, NJ). Roswell Park Memorial Institute (RPMI) 1640 culture medium, fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were obtained from Gibco BRL (Grand Island, NY).

Engineering of CoQ_{10} Nanoparticles from Microemulsion Precursors

The engineering of nanoparticles was completed as previously described, with modifications.[19] Briefly, varying amounts of CoQ_{10} (0, 0.5, 1, and 2 mg) and emulsifying wax (2 mg) were melted at 55°C. A specific volume of water was added to the melted wax and stirred until a homogenous milky suspension was obtained. Then, a calculated volume of Brij 78 and Tween 20 stock solutions were added to the homogenate while stirring to obtain a clear microemulsion (final volume = 1 mL). Nanoparticles were engineered by simple and direct cooling of this warm microemulsion to room temperature in the same container. The droplet size of the warm microemulsion was measured at 55°C using a Coulter N4 Plus submicron particle sizer (Coulter Corporation, Miami, FL) at 90° light scattering for 90 seconds. For particle sizing of the CoQ_{10}-NPs, the nanoparticle suspension was diluted 20-fold with deionized and filtered (0.22 μm) water, and the particle size was measured at 90° light scattering for 90 seconds at 25°C.

To study the effect of the concentration of each surfactant, nanoparticles with increasing concentration of 1 surfactant (Brij 78 or Tween 20, 0-6 mM) were engineered, while the concentration of the other surfactant (Tween 20 or Brij 78) was fixed at 6 mM.

Purification of Coenzyme Q_{10} Nanoparticles

To separate free Brij 78, Tween 20, and unentrapped CoQ_{10} from the CoQ_{10}-NPs, gel permeation chromatography (GPC) using Sephadex G-75 was performed. Sephadex G-75 was presoaked with deionized and filtered (0.22 μm) water overnight and then packed in a 14 × 230 mm plastic column. Two hundred microliters of the cured CoQ_{10}-NPs suspension were applied to the column, and the eluent was collected in 1-mL fractions in plastic vials. The collected fractions were then analyzed by laser light scattering using the Particle Sizer to identify the fraction containing CoQ_{10}-NPs. In all cases, the fourth fraction contained the greatest number of CoQ_{10}-NPs, as evidenced by the intensity from laser light scattering. Free Brij 78, Tween 20, and CoQ_{10} were removed by GPC using deionized and filtered (0.22 μm) water as the mobile phase.

Transmission Electron Microscopy

The size and morphology of the CoQ_{10}-NPs were observed using transmission electron microscopy (TEM) (Philips Tecnai 12 Transmission Electron Microscope, FEI Company, Hillsboro, OR) in the Electron Microscopy & Imaging Facility at the University of Kentucky Medical Center, Lexington, KY. A carbon-coated 200-mesh copper specimen grid (Td Pella, Inc, Redding, CA) was glow-discharged for 1.5 minutes. CoQ_{10}-NPs suspension deposition on the grid and uranyl acetate staining were completed as previously described.[18]

HPLC Method

For quantitative determination of CoQ_{10}, a reverse-phase HPLC method was used.[24] The mobile phase was methanol and n-hexane (90:10, vol/vol). The HPLC system consisted of a model P2000 pump with a model UV1000 UV detector (both from Thermo Separation Products [TSP], San Jose, CA), a model AS1000 autosampler (TSP), SCM1000 vacuum membrane degasser (TSP), and a Dell Optiplex computer. The injection volume was 20 μL. The analysis was performed at a flow rate of 1.5 mL/min with the UV detector at 275 nm. A Supelcosil LC-18-S reverse phase column (250 × 4.6 mm, 5μm) and a C-18 precolumn (20 × 4.0 mm, 5 μm) from Supelcol (Bellefonte, PA) were used.

Determination of Incorporation Efficiency

The incorporation efficiency of CoQ_{10} in the nanoparticles was determined using ultrafiltration as previously described[25] with slight modification. The percentage of incorporated CoQ_{10} in the nanoparticles was determined by filtering the CoQ_{10}-NPs dispersion using centrifugal filter tubes (Eppendorf Scientific, Inc, Westburg, NY) with a molecular weight cut-off of 50 kDa. The concentrations of CoQ_{10} in the CoQ_{10}-NPs (total CoQ_{10}) and the ultrafiltrate (free CoQ_{10}) were determined using HPLC. The experiment was performed in triplicate. Incorporation Efficiency (IE) was calculated using the following equation:

$$IE = 100\% \times (\text{total } CoQ_{10} - \text{free } CoQ_{10})/\text{total } CoQ_{10} \quad (1)$$

Freeze–Thaw Experiment

GPC-purified CoQ_{10}-NPs were diluted with either deionized water or aqueous solution of sucrose, lactose, and trehalose so that the final disaccharide concentrations varied from 0% to 10% (wt/vol). The diluted nanoparticle suspensions (1 mL) were then placed in glass vials (7 mL), sealed, and frozen at –20°C overnight. The frozen samples were thawed at room temperature, and the particle sizes were measured.

Lyophilization

GPC-purified CoQ_{10}-NPs were diluted with a sucrose solution to make a final sucrose concentration of 1% (wt/vol). The diluted nanoparticle suspensions (1 mL) were then added to glass vials (7 mL) and frozen at –20°C overnight. The samples were then lyophilized for 24 hours using a Labconco freeze-dryer model 4.5 (Labconco Corporation, Kansas, MO) at a temperature of –45°C and a vacuum of 130 mbar for 24 hours. After lyophilization, the vials were sealed, wrapped in aluminum foil, and stored at room temperature for 7 days.

Short-Term Stability of CoQ_{10} Nanoparticles

A short-term stability study of the CoQ_{10}-NPs was carried out at room temperature and at 4°C for 7 days. The vials containing CoQ_{10}-NP suspensions and lyophilized cakes of CoQ_{10}-NPs were sealed and wrapped in aluminum foil. Prior to the measurement of particle size, the lyophilized samples were resuspended with deionized and filtered (0.22 μm) water to the original volume. The CoQ_{10}-NP dispersions were diluted 20-fold with deionized and filtered (0.22 μm) water prior to particle-size measurement.

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed on a differential scanning calorimeter (TA 2920 DSC, TA Instruments, New Castle, DE). Approximately 2 mg of samples were accurately weighed into DSC aluminum pans. An empty pan was used as a reference. The samples were cooled to 10°C at the scan rate of 5°C/min and equilibrated at 10°C for 10 minutes. Then, the samples were heated at a rate of 5°C/min from 10°C to 90°C. All experiments were carried out in triplicate.

In Vitro Release of CoQ_{10} from Nanoparticles

The CoQ_{10}-NPs were purified by GPC and concentrated by ultrafiltration (molecular weight cut-off of 50 kDa). One milliliter of the concentrated CoQ_{10}-NPs in suspension was placed in a dialysis bag with a molecular cut-off of 50 kDa (Spectrum Chemical Products, Inc). The dialysis bag was immersed in 10 mL of 5% (vol/vol) Tween 80 maintained at 37°C in a shaking water-bath (120 rpm). At predetermined time points, 0.5 mL of the sample was withdrawn and immediately another 0.5 mL of fresh medium was added. After 22 hours, the total medium was replaced with fresh medium at every time interval. CoQ_{10} concentration was determined using HPLC. The experiment was carried out in triplicate.

Incorporation of Fluorescein-Labeled DOPE in the Nanoparticles With and Without CoQ_{10}

Fluorescein-labeled DOPE-incorporated nanoparticles were prepared for cell uptake study. Fluorescein-labeled nanoparticles with and without CoQ_{10} (2 mg/mL) were prepared by mixing 200 μg of fluorescein-labeled DOPE (final 10%, wt/wt of emulsifying wax) with emulsifying wax (2 mg/mL) prior to microemulsion preparation. Deionized and filtered water (910 μL) was added to the melted wax and stirred until a homogeneous milky suspension was obtained. Brij 78 and Tween 20 solutions were subsequently added to make final concentrations of 6 mM of each into the homogenate while stirring to obtain a clear microemulsion. Nanoparticles were formed by simple and direct cooling of this warm microemulsion to room temperature in the same container. Nanoparticles were purified and reconcentrated as described above.

In Vitro Cell Uptake by Macrophages

Uptake studies were performed as previously described using a mouse monocyte-macrophage cell line (J774A.1), obtained from the American Type Culture Collection (ATCC, Rockville, Maryland).[23] The culture was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin as monolayers under an atmosphere containing 5% CO_2 at 37°C. Cells were plated in 48-well plates at a density of 5×10^5 cells/well and incubated overnight. The cells were then co-incubated with GPC-purified fluorescein-labeled CoQ_{10} nanoparticles and fluorescein-labeled CoQ_{10}-free nanoparticles at 37°C and 4°C, respectively, for 1 hour. Cells were harvested by removing the media and washing with phosphate-buffered saline (PBS) buffer (10 mM, pH 7.4) 3 times. The cells were then lysed by adding 500 μL of lysis buffer (Promega, Madison, WI), incubating for 5 to 10 minutes, and freeze-thawing 3 times. The cell lysates were diluted with water to 1 mL, and the fluorescence intensity was determined using a F-2000 fluorescence spectrophotometer (excitation 497 nm, emission 521 nm; Hitachi Instruments, Danbury, CT). Unlabeled nanoparticles were also incubated at 4°C and 37°C as controls. Incubation at 4°C was included to study the binding of the nanoparticles on the macrophage cell surface. Nanoparticle uptake was measured as the ratio

of the fluorescence determined in the cell lysates to the total amount of fluorescence added. The experiments were carried out in triplicate. Data were reported as the percentage of uptake increase at 37°C over the uptake increase at 4°C.

$$\% \text{ uptake increase} = 100\% \times (\text{uptake at } 37^{\circ}\text{C} - \text{uptake at } 4^{\circ}\text{C})/\text{uptake at } 4^{\circ}\text{C} \quad (2)$$

Statistical Analyses

Statistical analyses were completed using a 1-way analysis of variance (ANOVA) followed by pair-wise comparisons with Fisher's protected least significant difference procedure (PLSD). It was assumed that the observations were normally distributed. A P-value of ≤ .05 was considered to be statistically significant.

RESULTS AND DISCUSSION

CoQ_{10} is poorly soluble in water and is representative of many Class IV compounds that exhibit low solubility and permeability. The oral bioavailability of currently available CoQ_{10} preparations is very low, although reports have differed widely. There continues to be a need to develop improved formulations for the oral delivery of CoQ_{10} and compounds with similar physical and chemical properties. Incorporation of CoQ_{10} into submicron particles is an approach that may overcome these challenges by increasing the surface area available for dissolution, or by facilitating transcytotic absorption of the intact nanoparticles. Previously, Cui and Mumper have reported a novel procedure to engineer nanoparticles from O/W microemulsion precursors.[18] The microemulsion precursors were composed of emulsifying wax as the oil phase and surfactant(s) with appropriate charges. A nonionic emulsifying wax, composed of cetyl alcohol and polysorbate 60 in a molar ratio of about 20:1, was the core material of the nanoparticles. The wax (melting point, 50°C -55°C) is typically used in cosmetics and topical pharmaceutical formulations and is generally regarded as a nontoxic and nonirritating material. For example, cetyl alcohol is present in Exosurf Neonatal (GlaxoSmithKline, Triangle Park, NC) for lung administration. Polysorbate 60 is used in many pharmaceutical products including parenterals.

For the preparation of neutral nanoparticles, one of the surfactants being used was Brij 78. Cui and Mumper further showed that the nanoparticles prepared with emulsifying wax (2 mg/mL) and Brij 78 (4-6mM) were most stable, whereas increasing concentrations of Brij 78 resulted in less stable nanoparticle suspensions.[20] In the present studies, a CoQ_{10} preparation based on the emulsifying wax/Brij 78 nanoparticles was developed and characterized.

Preparation of CoQ_{10} Nanoparticles

An initial preparation of CoQ_{10}-NPs was carried out by mixing increasing concentrations of CoQ_{10} (0-2 mg/mL) together with emulsifying wax (2 mg/mL), while keeping the final Brij 78 surfactant concentration at 6 mM. As expected, when no CoQ_{10} was added (CoQ_{10} = 0 mg/mL), 6 mM of Brij 78 was sufficient to form a microemulsion with 2 mg/mL of emulsifying wax, resulting in nanoparticles after cooling to room temperature (**Figure 1A**). However, when CoQ_{10} as low as 0.5 mg/mL was added, 6 mM of Brij 78 was not sufficient to form a microemulsion; the systems did not turn clear even after prolonged stirring at increased temperature. The particle size reported in **Figure 1A** was most likely due to the precipitation of wax and CoQ_{10}. Tween 20, with an Hydrophilic-Lipophilic Balance (HLB) of 16.7, favors the formation of O/W microemulsions.[26] Therefore, increasing concentrations of Tween 20 (0, 2, 4, and 6 mM), as a cosurfactant, were used together with Brij 78 (6 mM) to form a microemulsion. As shown in **Figure 1A**, when 2 mg/mL of CoQ_{10} was used, 4 or 6 mM of Tween 20 with 6 mM Brij 78 was enough to form a microemulsion and lead to nanoparticles with a size of 50 to 100 nm. The size of the nanoparticles was dependent on the concentration of CoQ_{10} and Brij 78, whereas emulsifying wax (2 mg/mL) and Tween 20 (6 mM) concentrations were fixed (**Figure 1B**). Again, if CoQ_{10} was included, using Tween 20 alone at 6 mM was not sufficient to form a microemulsion; this was addressed by using both Tween 20 and Brij 78 as surfactants. The final formulation chosen for further studies was the one with the following composition: emulsifying wax (2 mg/mL), CoQ_{10} (2 mg/mL), Tween 20 (7.5 mg/mL) and Brij 78 (7 mg/mL); the Tween 20 and Brij 78 concentrations are each equivalent to 6 mM. This formulation resulted in nanoparticles containing a greater amount of CoQ_{10} and having the smallest size. Smaller nanoparticles were selected since the study by Desai et al[15] suggested that a size exclusion phenomenon exists in the gastrointestinal absorption of particles, with 100 nm particles showing a significantly higher uptake (10- to 250-fold higher) than larger particles (500 nm to 10 μm). **Figure 1C** shows that a microemulsion based on CoQ_{10} and emulsifying wax could be engineered using Brij 78 and Tween 20 as surfactants. Similar to what was reported

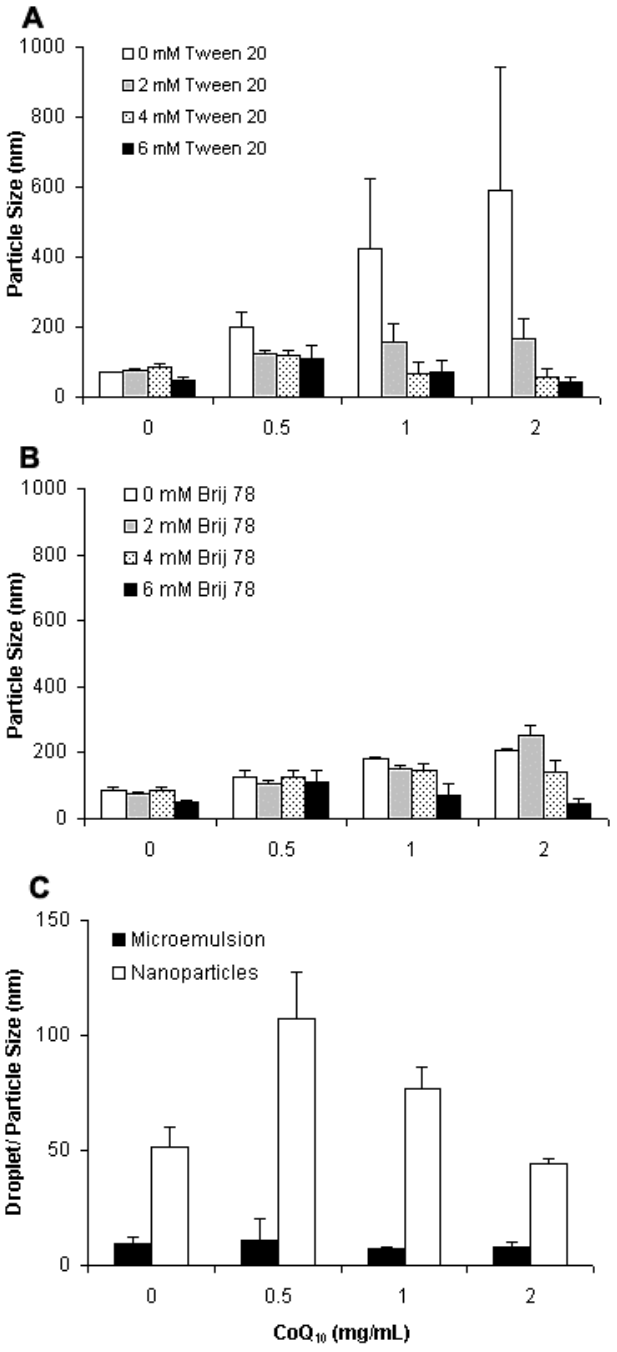

Figure 1. Preparation of CoQ_{10}-NPs. The CoQ_{10}-NPs were prepared using of CoQ_{10} (0, 0.5, 1, and 2 mg/mL), emulsifying wax (2 mg/mL), and Brij 78 (0 mM-6 mM) and Tween 20 (0 mM-6 mM) as surfactants: (A) Brij 78 (6 mM) and Tween 20 at 0 mM, 2 mM, 4 mM, and 6 mM; (B) Tween 20 (6 mM) and different concentrations of Brij 78 (0 mM, 2 mM, 4 mM, and 6 mM); (C) Preparation of CoQ_{10}-NPs (2 mg/mL emulsifying wax) from microemulsion precursors using Brij 78 (6 mM) and Tween 20 (6 mM) as surfactants. The data reported for the microemulsion droplet size and nanoparticle size are the mean ± SD (n = 3).

by others, the size of the cured nanoparticles was larger than the corresponding microemulsion droplet size.[18]

Incorporation Efficiency of CoQ_{10} in the Nanoparticles

Ultracentrifugation was initially attempted without success to determine the incorporation efficiency. This may be due to the fact that the density of the CoQ_{10}-NPs was lower than the aqueous medium in which they were dispersed. However, using ultrafiltration, the percentage of CoQ_{10} incorporated into the nanoparticles was estimated to be 74% ± 5%. The same value was obtained even after the nanoparticles were diluted 1000-fold before being transferred to the ultrafiltration tubes. A dilution of 1000-fold should theoretically eliminate the CoQ_{10} solubilized by micelles. The critical micelle concentration (CMC) values for Brij 78 and Tween 20 are approximately 1 mM and 0.06 mM, respectively. Sufficient drug loading capacity is one of the prerequisites for the nanoparticles as carriers for drug delivery.[27] Chen et al[28] reported that the characteristics of the surfactant might affect the incorporation efficiency significantly. Brij 78 may increase the solubility of CoQ_{10} in aqueous solution. Tween 20 can also be a solubilizing agent for poorly water-soluble compounds. An incorporation efficiency of 74% indicated that most of the CoQ_{10} was entrapped in the nanoparticles.

Transmission Electron Microscopy

A transmission electron microscopy (TEM) image of the unpurified CoQ_{10}-NPs engineering using emulsifying wax (2 mg/mL), CoQ_{10} (2 mg/mL), Brij 78 (6 mM), and Tween 20 (6 mM) is shown in **Figure 2**. The particles were spherical. The diameters of most of the particles were 50 to 100 nm. The size determined by TEM agreed well with that measured by laser light scattering photon correlation spectroscopy.

Lyophilization and Short-Term Stability of CoQ_{10} Nanoparticles

A preliminary short-term stability study showed that the size of CoQ_{10}-NPs increased over time if stored as an aqueous suspension at room temperature. This was also observed with other nanoparticle preparations.[18] Although the mechanisms of particle size increase are unknown, this observation indicated the need for alternative methods to store the nanoparticles. Among the many ways to improve the stability of nanoparticles,

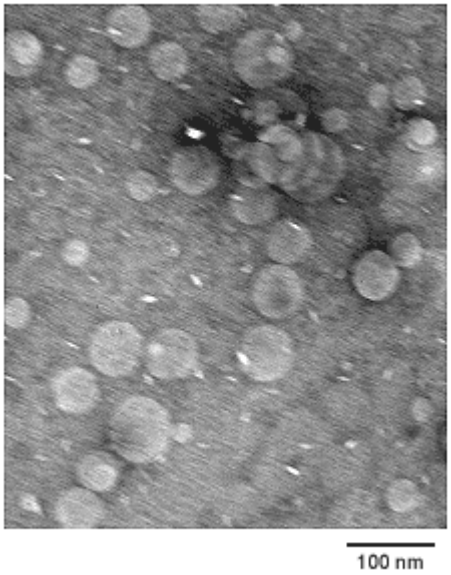

Figure 2. TEM of CoQ_{10}-NPs. The nanoparticles were engineered from microemulsion precursors composed of emulsifying wax (2 mg/mL), CoQ_{10} (2 mg/mL), Brij 78 (6 mM) and Tween 20 (6 mM). The microemulsions were prepared at 50°C to 55°C and were then cooled at room temperature to form nanoparticles.

Cui et al[21] reported that lyophilization might be a successful approach. Therefore, the feasibility of lyophilizing CoQ_{10}-NPs was investigated, and the stability of the nanoparticles in lyophilized form was compared with that in aqueous suspension.

Because freezing is a critical stage of lyophilization, a freeze–thaw study was used as a pretest to select appropriate excipient(s) with the greatest potential for serving as a cryoprotectant. If an excipient failed to protect the nanoparticles during the first step of lyophilization, it would not be considered for further study.[21,27] Numerous studies have shown the protective effect of disaccharides in preventing particle aggregation during the freeze-drying process.[29] A simple freeze–thaw experiment was carried out to determine whether or not a cryoprotectant was required for the lyophilization of these CoQ_{10}-NPs, and if so, to select the appropriate cryoprotectant and concentration for further lyophilization studies. In this study, the cryoprotective abilities of 3 disaccharides (lactose, sucrose, and trehalose) were evaluated. As shown in **Figure 3**, cryoprotectants at appropriate concentrations were required to maintain the size of nanoparticles upon freeze–thawing. For example, as little as 0.5% to 1% (wt/vol) disaccharides in the suspension was enough to prevent the nanoparticles from subsequent aggregation upon thawing. This is in contrast to the suspensions in which no cryoprotectant was added, where the size of the CoQ_{10}-NPs increased to over 250 nm on thawing. Of the 3 disaccharides studied, sucrose and trehalose yielded better results than lactose. Interestingly, as Cui et al[21] reported, an excessive concentration of the disaccharides led to larger particles and more aggregation.

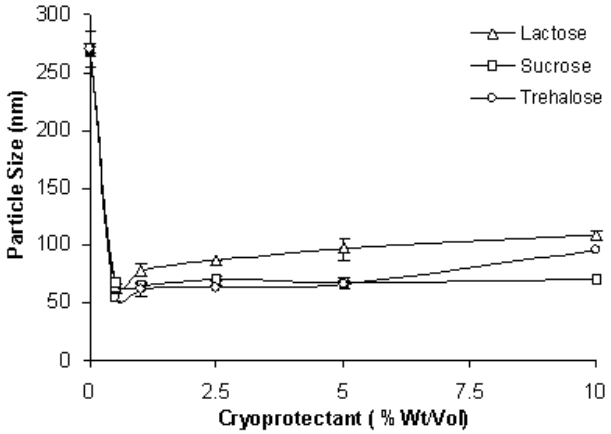

Figure 3. The particle size of the CoQ_{10}-NPs after freeze–thawing with different concentrations of lactose, sucrose, and trehalose as cryoprotectants. Data reported are the mean ± SD (n = 3).

CoQ_{10}-NPs in 1% sucrose (wt/vol) subjected to freezing at –20°C were then lyophilized for 24 hours. Sucrose was selected as the cryoprotectant for this study because it can potentially act as a good excipient for future incorporation of the nanoparticles into solid dosage forms, such as tablets. Following lyophilization, a fluffy, stable, and intact cake occupying the same volume and size of the original frozen mass was obtained. After adding deionized, filtered water, 1 minute of manual shaking was sufficient to ensure complete reconstitution of the lyophilized nanoparticles. The protective effect of sucrose may be attributed to the ability of the sugars to form a glassy amorphous "shell" around the particles, preventing the particles from adhering to one another during the removal of water.[30] As shown in **Figure 4**, after 7 days of storing the CoQ_{10}-NPs as a lyophilized cake at room temperature, no significant increase in particle size was observed. In contrast, the size of CoQ_{10}-NPs stored as an aqueous suspension at room temperature was 4.6-fold greater than their original size. This observation suggested that the storage of CoQ_{10}-NPs in a lyophilized form was suitable to maintain their small size. **Figure 4** also shows

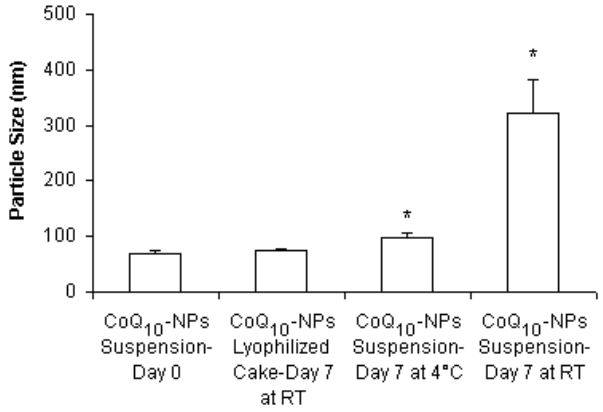

Figure 4. Short-term stability of the GPC-purified CoQ_{10}-NPs stored in the dark at 4°C or room temperature (RT) in an aqueous suspension, or at room temperature as a lyophilized cake for 7 days. Data reported were the mean ± SD (n = 3). * indicates that the size of the nanoparticles stored for 7 days in aqueous suspension was significantly different from the original nanoparticle size (P = .005 and P = .0009 for 4°C and RT, respectively), while that stored as a lyophilized cake was not different from the original particle size (P = .177).

that storage temperature had a great effect on the stability of the aqueous nanoparticle suspensions. When stored at 4°C for 7 days, the size of the CoQ_{10}-NPs increased by only 1.4-fold, in contrast to the 4.6-fold increase in particle size observed when the suspensions were stored at room temperature.

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed to further investigate the physical states of CoQ_{10}-NPs. DSC is a thermal analytical technique, which measures heat flow associated with transitions in materials as a function of temperature. DSC results provide useful information about the physical and chemical changes that involve endothermic or exothermic process or changes in heat capacity. In the present studies, DSC experiments were useful in understanding the potential interactions between emulsifying wax and CoQ_{10} in the nanoparticles as well as in the behavior of the mixture. As shown in **Figure 5**, the DSC profiles of CoQ_{10} (1) and emulsifying wax (4) alone showed apparent endothermic peaks at about 50°C and 55°C, respectively, corresponding to their individual melting points. The DSC profile of a physical mixture of CoQ_{10} and emulsifying wax (3) showed 1endothermic peak at 53°C, which may be attributed to the fusion process of emulsifying wax and CoQ_{10} as well as some interactions between emulsifying wax and CoQ_{10} in the mixture. In contrast, the profile of the lyophilized powder of CoQ_{10}-NPs (2) showed a single broad peak around 40°C, suggesting the existence of unique molecular interactions in these CoQ_{10}-NPs. Taken together, these DSC profiles demonstrated that the CoQ_{10}-NPs, which were engineered from warm microemulsion precursors composed of emulsifying wax and CoQ_{10}, were not a simple physical mixture of their individual components.

In Vitro Release of CoQ_{10} from Nanoparticles

An initial release study to determine the amount of CoQ_{10} loosely associated on the surface of the nanoparticles showed no detectable release over 7 days when the nanoparticles were suspended in water, presumably due to the low aqueous solubility of CoQ_{10}. Therefore, a 5% Tween 80 solution was used as the medium for further study.[28] **Figure 6** shows the release of CoQ_{10} from nanoparticles as a function of time. A rapid release is observed over the first 9 hours during which over 7% of CoQ_{10} was released from the CoQ_{10}-NPs. However, after 9 hours, the release of CoQ_{10} from the nanoparticles apparently slowed down. After 72 hours, a total of 12% CoQ_{10} was released. The subsequent slower release of CoQ_{10} from the nanoparticles suggests that the CoQ_{10} might be dispersed in the emulsifying wax matrix, and its release might be due to diffusion and/or the disintegration of the nanoparticles. The release profile agreed well with the profiles observed for other nanoparticle preparations. For example, Chen et al[28] showed an initial rapid release of paclitaxel from solid lipid nanoparticles prepared by Brij 78 was followed by a slow release. After 24 hours, the amount of paclitaxel that had been released was about 7%. The release profiles of drug-loaded nanoparticles can be affected by the nature of the lipid matrix, surfactant concentration, and the preparation temperature.[31] As will be discussed later, a slow release of free CoQ_{10} is thought to be beneficial since, once being administered orally, the slowly released CoQ_{10} may be passively partitioned into the gastrointestinal tract tissues resulting in improved bioavailability. The released CoQ_{10} will be complementary to the proposed absorption mechanism of CoQ_{10} by the absorption of the CoQ_{10}-NPs via the M cell in the Peyer's patches of the intestine.

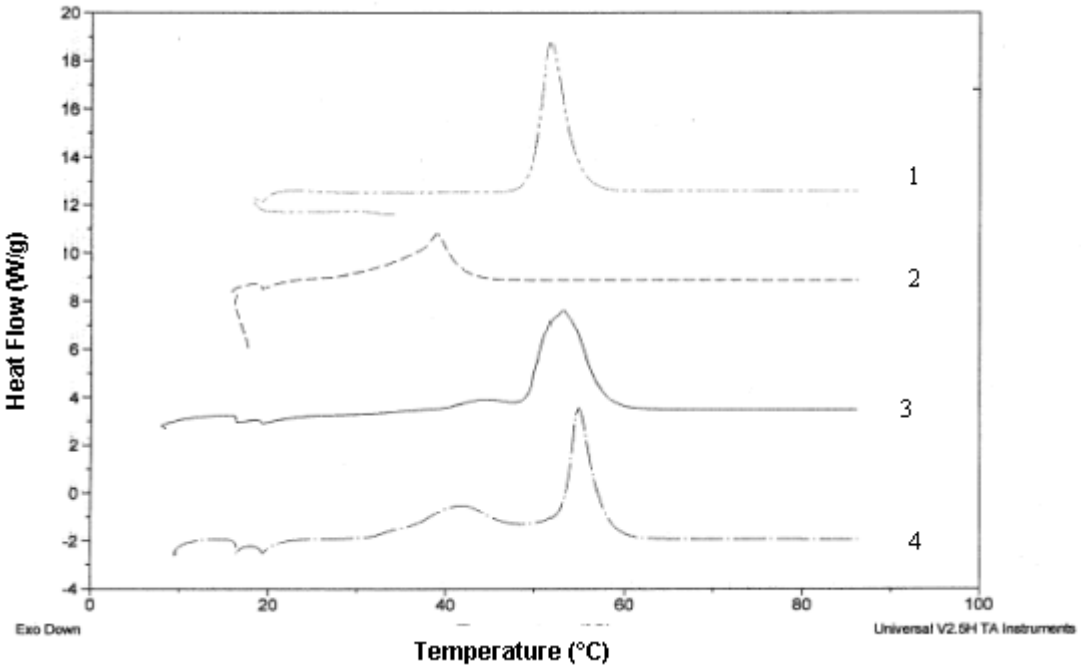

Figure 5. DSC profiles of (1) CoQ_{10}, (2) CoQ_{10}-NPs, (3) physical mixture of CoQ_{10} and emulsifying wax (ratio 2:2), and (4) emulsifying wax. For (2) and (3), the ratios (wt/wt) of the emulsifying wax and CoQ_{10} were the same.

In Vitro Cell Uptake by Macrophages

CoQ_{10} has been shown to enhance the phagocytic activity of macrophages and to increase granulocyte proliferation.[32] For example, Makabi-Panzu et al[23] reported that incorporation of CoQ_{10} in liposomes enhanced the uptake of the liposomes by J774A.1 macrophage cells. Liposomes containing CoQ_{10} resulted in a 10-fold enhancement in cell uptake compared with liposomes without CoQ_{10}. To investigate whether the CoQ_{10} is still active after being incorporated into nanoparticles, an in vitro uptake of nanoparticles with and without CoQ_{10} was carried out using J774A.1 macrophage cells. The nanoparticles (with or without CoQ_{10} incorporated) were labeled with fluorescein. Labeling of nanoparticles with fluorescein was made possible by taking advantage of the lipophilic DOPE group covalently linked to the fluorescein. It was hypothesized that DOPE acted as an anchor by inserting itself in the hydrophobic matrix of the nanoparticles while leaving the hydrophilic fluorescein molecules on the surface of the nanoparticles. As shown in **Figure 7**, labeling did not significantly affect the size of the nanoparticles. Fluorescein-labeled CoQ_{10}-NPs and fluorescein-labeled CoQ_{10}-free nanoparticles were incubated with a fixed number of J774A.1 macrophages (5×10^5) at 4°C and at 37°C for 1 hour. At 4°C, phagocytosis processes are no longer active. Therefore, any association of the

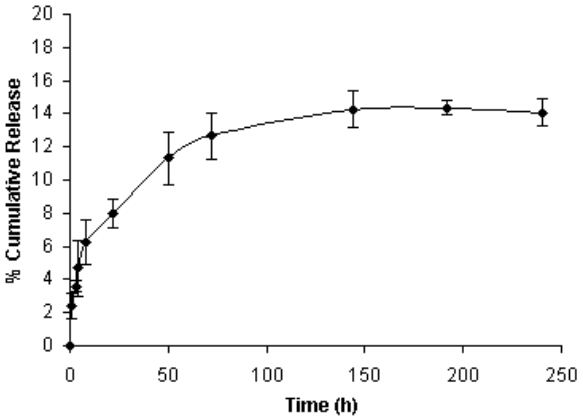

Figure 6. In vitro release profile of CoQ_{10} from nanoparticles. The experiment was performed in 5% Tween 80 solution at 37°C. Data reported are the mean ± SD (n = 3).

nanoparticles with the macrophages under these conditions would be attributed to nonspecific binding. At 37°C, binding and uptake of the nanoparticles was observed. As shown in **Figure 8**, the relative increase in the uptake of CoQ_{10}-free nanoparticles was about 20%. However, the relative increase in the uptake of nanoparticles containing CoQ_{10} was close to 80%. The enhancement in nanoparticle uptake clearly demon-

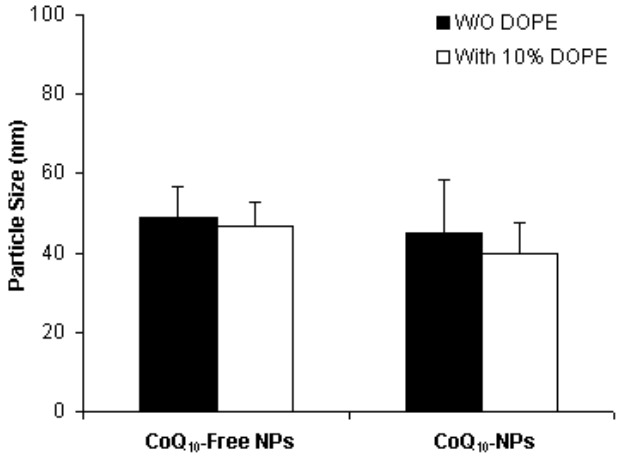

Figure 7. The effect of fluorescein-labeled DOPE incorporation on the particle size of CoQ_{10}-free nanoparticles or CoQ_{10}-NPs. Data reported are the mean ± SD (n = 3). Statistical analyses showed that the particle size of the DOPE-incorporated nanoparticles was not significantly different from the nanoparticles with DOPE (P = .186 and P = .292 for CoQ_{10}-free and CoQ_{10}-NPs, respectively).

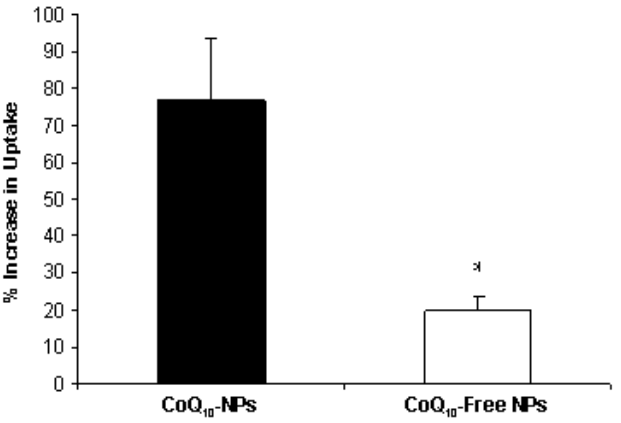

Figure 8. In vitro cell uptake of fluorescein-labeled CoQ_{10}-NPs and fluorescein-labeled CoQ_{10}-free nanoparticles by mouse macrophage J774A.1 cells after incubation of 1 hour at 4°C or 37°C. * indicates that the result for CoQ_{10}-NPs was significantly different from that of the CoQ_{10}-free nanoparticles (P = .0023). Data reported are the mean ± SD (n = 3).

strated that the incorporated CoQ_{10} was still functioning and active in that it can enhance the phagocytotic activity of macrophages. With its amphiphilic property, CoQ_{10} is assumed to mainly insert itself into the emulsifying wax core of the nanoparticles while leaving its hydrophilic head on the surface. Casey et al[3] reported that for the CoQ_{10} to stimulate phagocytosis, the quinoidal moiety with at least 1 partially unsaturated side chain is sufficient. The maintenance of the biological activity of CoQ_{10} precisely pointed out one of the many advantages of the procedure of engineering nanoparticles from microemulsion precursors. Since microemulsions were formed spontaneously, the high-torque and damaging mechanical mixing process and the chemical and photochemical reactions used by others for nanoparticle preparation are thus avoided. Therefore, many therapeutics, including macromolecules such as plasmid DNA, can be incorporated into nanoparticles by this procedure without losing their activities.[19]

This CoQ_{10}-NP formulation described above is intended for oral administration. Although the absorption of particulates in the intestine following oral administration is currently thought to occur by several possible mechanisms, the majority of the available literature evidences suggests that the absorption predominantly takes place at the intestinal lymphatic tissues (ie, the Peyer's patches). The epithelial cell layer overlying the Peyer's patches contains specialized M cells, which are believed to facilitate transcytotic transport across the intestines. Small particles in the intestinal lumen localize to the apical side of M cells and can be internalized by the M cells through transcytosis. The CoQ_{10}-NPs in the present studies are expected to be absorbed via this mechanism. As mentioned earlier, Desai et al[15] reported that there is a particle size–dependent exclusion phenomenon in the gastrointestinal mucosal tissue with 100 nm-size particles showing significantly greater tissue uptake than larger particles. The CoQ_{10}-NPs developed in the present studies were ≤100 nm. Therefore, the size of these nanoparticles will be less likely to be a limiting factor for internalization via the M cells. Part of the future work will be on investigating whether or not the CoQ_{10}-NPs can be effectively transported by the M cells in vivo. Of course, since other parameters of the particles were also reported to affect the particle uptake, further optimization of the nanoparticles, both the composition and the surface properties, might need to be done in future studies.

It should also be pointed out that the long-term goal is to increase the oral bioavailability of CoQ_{10}. Absorption of CoQ_{10} via the uptake of its carrier, the nanoparticles, is of course not the only possible means for CoQ_{10} absorption. If the nanoparticle preparation were to be administered orally, some of the free CoQ_{10} released from the nanoparticles might be passively partitioned into the gastrointestinal tract tissues. Also, due to the small size of the nanoparticles, the resulting lar-

ger total surface area might also favor increased CoQ_{10} absorption.

CONCLUSION

In these present studies, emulsifying wax–based nanoparticles were used as a carrier to incorporate a highly lipophilic drug, CoQ_{10}. CoQ_{10}-NPs less than 100 nm in diameter were engineered from microemulsion precursors. The concentration of final surfactant influenced the particle size. DSC showed that the nanoparticles were not a physical mixture of their individual components. CoQ_{10}-NPs could be lyophilized using low concentrations of disaccharides that served as cryoprotectants during lyophilization. This process was shown to increase the stability of the nanoparticles. The release profile of CoQ_{10} from the nanoparticles demonstrated a rapid release followed by a long period of slow release. An in vitro cell uptake study by macrophage cells demonstrated that the CoQ_{10} was still active after being entrapped in nanoparticles. CoQ_{10}-NPs may be considered as a promising carrier for oral delivery.

ACKNOWLEDGEMENTS

This work was supported in part by a University of Kentucky Graduate Fellowship, Lexington, KY (Cheng-Hsuan Hsu).

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