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Optimization in the Preparation of Coenzyme Q_{10} Nanoliposomes

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The optimal formulation of coenzyme Q_{10} (CoQ_{10}) nanoliposomes and the feasibility of production in a pilot scale were investigated. The nanoliposomes were prepared by ethanol injection and sonication techniques for a desired vesicle size in the laboratory. Optimization of formulation in the preparation of CoQ_{10} nanoliposomes was achieved by an orthogonal array design. The best formulation was found to be phospholipid/CoQ_{10}/cholesterol/Tween 80 (2.5:1.2:0.4:1.8, w/w) with phosphate buffer solution (pH 7.4, 0.01 M) as the hydration media. The *z*-average diameter (D_z) was about 68 nm. The encapsulation efficiency was greater than 95% with a retention ratio higher than 90% and a particle size change lower than 10% after storage at 4 °C in the dark for 90 days. CoQ_{10} incorporation resulted in a dramatic increase of the microviscosity of nanoliposomes and inhibited the peroxidation of phospholipid. The D_z of CoQ_{10} nanoliposomes produced in a pilot scale was about 67 nm. Results suggest that the technology developed by this investigation is practical to produce the CoQ_{10} nanoliposomes with the expected encapsulation quality and stability not only in the laboratory but also in a pilot scale.

KEYWORDS: Coenzyme Q_{10}; nanoliposomes; preparation; orthogonal design

INTRODUCTION

Coenzyme Q_{10} (CoQ_{10}, ubiquinone-10) is a fat-soluble, vitamin-like nutrient for every organ in the body (*1*, *2*). It is essential for cell respiration and electron transfer, helping to control the production of energy in the heart cells. Although CoQ_{10} is available in foods such as beef, eggs, fish, and organ meats, the absorbability of CoQ_{10} becomes more difficult with age (*3*). As a result, use of CoQ_{10} as a dietary supplement or nutraceutical has increased dramatically in the past decade. However, because of its higher molecular mass (836.36 Da) and poor water solubility, the absorption of CoQ_{10} for oral administration is limited and it is best delivered in a fat- or lipid-soluble medium for maximum absorption. For this reason, most suppliers recommend that it should be taken with fatty foods, which is not suitable for general health recommendation (*3*).

Many different approaches have been used to improve in vitro dissolution and absorbability of CoQ_{10}. Some of the approaches include complexation with cyclodextrin, solubilized in a blend of Tween 80 and medium chain triglycerides, preparation of redispersable dry emulsion, and solid dispersion (*4*). The CoQ_{10} incorporation into poly(methyl methacrylate) nanoparticles (*5*), self-emulsifying delivery systems (*1*, *3*, *6*, *7*), self-microemulsifying delivery systems (*2*), or liposomes (*8*−*11*) has been developed recently. Among these carrier systems, liposomes are hydrophilic vesicles consisting of one or more concentric phospholipid (PL) bilayers enclosing aqueous compartments (*12*−*14*). Liposomes enhance the shuttling of nutrients with poor water solubility from the intestinal lumen fluids into the enteric cells via bile salt−polar lipid mixed micelles and vesicles (*14*). Their sizes range from 20 nm to several dozens micrometers (μm). Small liposomes (nanoliposomes) with diameters of the order of 100 nm are frequently used as a carrier due to their better distribution in the organisms (*15*). In addition, nanoliposomes have the advantages of nanoparticles, which improve the adhesion to and absorption into the intestinal epithelial cells. In this case, the nanoliposomal delivery system might be a highly effective way to improve CoQ_{10} absorption and provide a faster and longer lasting dosage of CoQ_{10} into the systemic circulation for maximum effect.

However, their physical and chemical instability during manufacturing and storage is a major obstacle that prevents many promising nanoliposomal candidates moving from the experimental scale to the market (*16*). It has been shown that the stability of nanoliposomes is sensitive to several technical parameters, especially the liposomal formulations. Because various formulation parameters potentially affect the encapsulation quality and stability, the optimization of formulation represents a critical step in the development of CoQ_{10} nanoliposomes. As far as we know, there has been no systematic investigation on the optimal formulation in the preparation of CoQ_{10} nanoliposomes. To date, most of the optimum preparation conditions are achieved using an one-variable-at-a-time method. Even though this method has been successfully employed in

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Table 1. Definition and Trial Levels of Factors in Orthogonal Array Experiment

factor	variable	level 1	level 2	level 3
A	Chol:PL (w/w)	0.3:2.5	0.4:2.5	0.5:2.5
B	Tween 80:PL (w/w)	1.6:2.5	1.8:2.5	2.0:2.5
C	CoQ_{10}:PL (w/w)	1.2:2.5	1.7:2.5	2.1:2.5
D	NaCl concentration (M)	0	0.07	0.15

many previous investigations, it should be noted that the one-variable-at-a-time optimization is a slower process than the methods based on statistics, such as factorial designs and response surface designs. Factorial design is capable of simultaneously determining the influence of different factors on the measured responses. Orthogonal array design (OAD) is a type of fractional factorial design, in which orthogonal here means balanced, separable, or not mixed (*17*).

In this study, experimental designs with $OA_9(3^4)$ (a matrix used for experimental arrangement) were used to optimize the formulation in the preparation of CoQ_{10} nanoliposomes with responses, such as encapsulation efficiency (EE), core material retention ratio (RR), *z*-average diameter (D_z), and *z*-average diameter change (ΔD_z) of CoQ_{10} nanoliposomes. The effect of the CoQ_{10} incorporation on the nanoliposomes stability was investigated by measurement of microviscosity and lipid peroxidation. Pilot scale production of CoQ_{10} nanoliposomes prepared with optimum formulations developed in the laboratory was further investigated using an ethanol injection method for technical feasibility and reproducibility.

MATERIALS AND METHODS

Materials. CoQ_{10} material (98.0−101.0% purity) was purchased from Nisshin Pharma Inc. (Tokyo, Japan). Standard CoQ_{10} (98% purity) was from Sigma Chemical Co. (St. Louis, MO). Egg yolk PL was purchased from Chemical Reagent Plant of East China Normal University (Shanghai, China). Analytical grade cholesterol (Chol), Tween 80, and ethanol were obtained from China Medicine (Group) Shanghai Chemical Reagent Corp. (Shanghai, China). The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH, 98% purity) was purchased from Sigma Chemical Co. All other chemicals used were of reagent grade.

Preparation of CoQ_{10} Nanoliposomes. The ethanol injection and sonication method (*12*) was modified slightly in this experiment. Benchtop batches in laboratory were prepared on a 20 mL scale. CoQ_{10} was dissolved in 2 mL of warm ethanol (about 55 °C) together with the lipids composed of different ratios of PL, Chol, and Tween 80 (**Table 1**). 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, PBS, pH 7.4) at 55 °C with magnetic 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 PL 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 350 W with a sequence of 1 s of sonication and 1 s rest using a vibra cell sonicator (VCX500, Sonics & Materials, Inc., 20 kHz) to the desired size. In all cases, the initial turbid liposomal suspension was well translucent after sonication. 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 11000*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).

Preparation of CoQ_{10} nanoliposomes scaling up to 1 L was carried out with the ethanol injection technique developed by Xian Libang Pharmaceutical Technology Corporation (Xian, China). The preparation system consisted of vessels for the PBS and ethanol/lipid solution, the injection module for mixture, homogenizer, ultrafilter, and extruder (**Figure 1**). The lipid mixture was dissolved in warm ethanol with stirring at 55 °C. The buffer solution was also tempered to 55 °C. As shown in **Figure 1**, the buffer solution was pumped from vessel 2 to the mixing chamber 5, where the CoQ_{10}/lipid/ethanol solution was injected and immediately diluted into the PBS. To prevent oxidation of wall and core materials used during the procedure, the solutions were continuously purged with nitrogen. The final ethanol concentration in the resulting liposome suspension was about 10% (v/v). The liposome suspensions were processed for three cycles with a high-pressure homogenizer (model NS3075; max pressure, 150 MPa; rated flow, 10 dm^3/h; Niro Soavi S.p.A, Italy) at room temperature. Ethanol was removed by cross-flow ultrafiltration (FlexStand Benchtop Pilot System with hollow fiber cartridges; nominal molecular mass cut off, 10 kDa; A/G Technology Corp., United States). Ultrafiltration was carried out until the removed filtrate volume reached 10 times the sample volume. The samples were then transferred into the sterilized container by extrusion through three layer polycarbonate filters (Nuclepore, 100 nm, Whatman, United States) using Lipex extruder (Northern Lipids Inc., Canada).

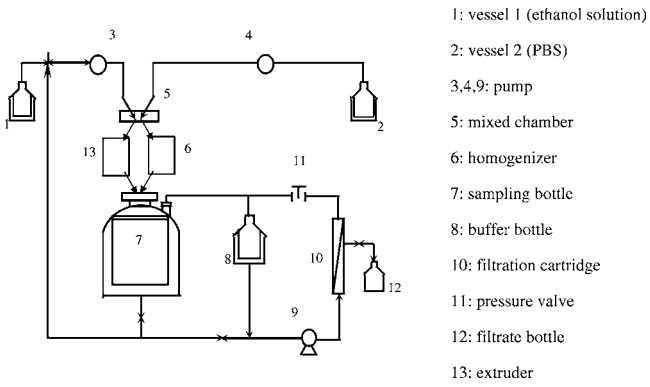

Figure 1. Schematic sketch of the pilot plant for the production of liposomes. The buffer solution was pumped from vessel 2 to mixed chamber 5, where the ethanol solution was pumped from vessel 1 and injected into chamber 5 and immediately diluted. The liposome suspensions were processed with a high-pressure homogenizer 6. Ethanol was removed by cross-flow ultrafiltrater 7. The samples were then transferred into the sterilized container by extruder 13.

Table 2. Assignment of the Factors and Their Levels Using an OA_9 (3^4) Matrix along with the Results of the Effects of Selected Variables on the Response Indices

no.	A	B	C	D	EE (%)	RR (%)[a]	D_z (nm)	ΔD_z (%)[a]
1	0.3:2.5	1.6:2.5	1.2:2.5	0	98.29	96.34	59.08	5.48
2	0.3:2.5	1.8:2.5	1.7:2.5	0.07	96.89	97.72	65.12	10.64
3	0.3:2.5	2.0:2.5	2.1:2.5	0.15	97.13	98.18	72.45	12.60
4	0.4:2.5	1.6:2.5	1.7:2.5	0.15	96.10	101.38	75.90	7.79
5	0.4:2.5	1.8:2.5	2.1:2.5	0	97.90	99.71	96.53	1.45
6	0.4:2.5	2.0:2.5	1.2:2.5	0.07	98.09	97.80	63.22	6.74
7	0.5:2.5	1.6:2.5	2.1:2.5	0.07	93.01	99.74	127.8	2.19
8	0.5:2.5	1.8:2.5	1.2:2.5	0.15	98.75	98.44	71.92	3.77
9	0.5:2.5	2.0:2.5	1.7:2.5	0	97.82	98.16	73.79	3.42

[a] Values are obtained after 1 month of storage at 4 °C in the dark place. (A) Chol/PL weight ratio, (B) Tween 80/PL weight ratio, (C) CoQ_{10}/PL weight ratio, and (D) NaCl concentration.

Optimization Strategy. On the basis of preliminary experiments, some experimental parameters were not varied. Throughout our experiments, the PL concentration was confirmed at 1.25% (w/v). The ratio of ethanol phase and PBS phase was also fixed at 1:10 (v/v).

Table 1 displays the four main factors selected in the optimization study designated as A (Chol/PL ratio), B (Tween 80/PL ratio), C (CoQ_{10}/PL ratio), and D (NaCl concentration in PBS). A standard three-

level OAD with an OA_9 (3^4) matrix was used to examine the four-factor system. In this study, the interactions among the different variables were not incorporated in the matrix, and focus was placed on the main effects of the four most important factors. Subscript 9 denotes the number of the experimental runs. A run involved the corresponding combination of levels to which the factors in the experiment were set. All factors had three levels, which were coded as 1, 2, and 3. The assignment of the factors and levels is listed in **Table 2**. All experiments were performed in triplicate.

The EE, RR, D_z, and ΔD_z of CoQ_{10} nanoliposomes were considered to be the response indices as quality evaluation to encapsulate CoQ_{10}. The experimental results were then analyzed by the Yates technique to extract independently the main effects of these factors, followed by the analysis of variance (ANOVA) to determine which factors were statistically significant.

EE, RR, and Loading Capacity (LC) of CoQ_{10} Nanoliposomes. The EE, RR, and LC, were calculated according to the following formulas:

$$\%\ \mathrm{EE} = \frac{[\text{total } CoQ_{10}] - [\text{free } CoQ_{10}]}{[\text{total } CoQ_{10}]} \times 100 \quad (1)$$

$$\%\ \mathrm{RR} = \frac{[CoQ_{10} \text{ encapsulated after storage}]}{[CoQ_{10} \text{ encapsulated initially prepared}]} \times 100 \quad (2)$$

$$\%\ \mathrm{LC} = \frac{[\text{total } CoQ_{10}] - [\text{free } CoQ_{10}]}{[\text{lipid content}]} \times 100 \quad (3)$$

The total CoQ_{10} content in CoQ_{10} nanoliposomes was determined by Tween 80 solubilization and UV spectrophotometry. One milliliter of Tween 80−ethanol solution (10% w/v) was added to a certain volume of CoQ_{10} nanoliposomes and mixed by vortex to disrupt the liposomal bilayer structure. The total volume for each flask was made up to 10 mL with deionized water to give a final surfactant concentration of about 1% (w/v), followed by incubation at 40 °C for 30 min. One milliliter of deionized water was added to one set (still existing as oxidative form ubiquinone), while 1 mL of freshly prepared $NaBH_4$ (7 mg/mL) was added to the other set to reduce ubiquinone to ubiquinonol. The CoQ_{10} concentration was detected spectrophotometrically by the change in absorbance (ΔA) at 275 nm prior to and after reduction of CoQ_{10}. A standard calibration curve was prepared with 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 mL of CoQ_{10} standard solutions according to the above procedure, in which the concentration of CoQ_{10} was 119.4 μg/mL and 1% w/v Tween 80 aqueous solution was used as the solubilizing media. With the CoQ_{10} concentration (C) as the abscissa and ΔA as the ordinate, a regression curve was plotted and the regression equation obtained was $\Delta A = 0.014C + 0.0102$ ($r^2 = 0.9995$), where C is the concentration of CoQ_{10} (μg/mL).

The free CoQ_{10}, which was not incorporated into nanoliposomes, was removed by *n*-pentane washing as described by Degli et al. (*18*). Five milliliters of *n*-pentane was added to the sample and mixed by vortex for 3 min. The aqueous phase (nanoliposomes) was separated from the pentane phase by centrifugation at 2000 rpm for 20 min. The pentane phase was then removed using a disposable pipet, followed by bubbling with nitrogen gas to evaporate *n*-pentane. The residual substance was further dissolved in 10 mL of absolute ethanol. All of the samples were read against a blank, which was obtained by using *n*-pentane to wash CoQ_{10}-free nanoliposomes. The concentration of CoQ_{10} was detected spectrophotometrically by the change in absorbance (ΔA) at 275 nm prior to and after reduction of CoQ_{10} using freshly prepared 30 μL of aqueous solution of $NaBH_4$ (7 mg/mL). A standard calibration curve was performed using ethanol as the solvent with 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 mL of CoQ_{10} standard ethanol solutions (121.1 μg/mL) following the above procedure. With the CoQ_{10} concentration (C) as the abscissa and ΔA as the ordinate, a regression curve was plotted and the regression equation obtained was $\Delta A = 0.0144C + 0.0094$ ($r^2 = 0.9995$), where C is the concentration of CoQ_{10} (μg/mL).

Particle Size Analysis. The average particle size of the nanoliposomes was determined with a ZetaSizer Nano S (Malvern Instruments, Malvern, United Kingdom) at a temperature of 25 ± 0.1 °C. The ZetaSizer was modified with a He/Ne laser (λ = 633 nm, Spectra Physics, Mt. View, United States). The intensity of the laser light scattered by the samples was detected at an angle of 90° with a photomultiplier. The undiluted nanoliposome samples of 2 mL were put into a polystyrene latex cell and measured with a refractive index of 1.33. For each specimen, 10 autocorrelation functions were analyzed using a cumulant analysis. From this analysis, the D_z was obtained, which is an approximation of the diameter of the liposomes. The particle size distribution was characterized using the polydispersity index (PDI), which is a measure for the width of the size distribution.

The changes of particle size (ΔD_z) reflecting the physical stability of nanoliposomes were calculated using the formula:

$$\%\ \Delta D_z = \frac{[D_z \text{ after storage at 4 °C for 1 month}] - [D_z \text{ initially prepared}]}{[D_z \text{ initially prepared}]} \quad (4)$$

Relative Particle Size Change. Turbidity (T) was used to estimate relative sizes of liposomes via spectrophotometry, using a wavelength that would not be absorbed by the particles in suspension, so that absorbance does not compete with light scattering. A compromise was usually reached with wavelengths in the 400−500 nm range (*19*). Here, the relative particle size change of the nanoliposomes was obtained through the determination of absorbance (A) in a 1 cm thick cuvette at 500 nm (A_{500nm}), in a spectrophotometer, model 722 (Exact Science Apparatus Ltd., Shanghai, China). According to Rayleigh−Gans−Debye (RGD) theory, the absorbance (A) is proportional to turbidity ($T = 2.303A/l$, where l is the length of light path) (*20*), so an increase in absorbance is interpreted as an increase of particle size in the system. The variation in turbidity at 500 nm was expressed using the formula

$$\%\ \Delta A_{500nm} = \frac{[A_{500nm} \text{ after storage at 4 °C for 1 month}] - [A_{500nm} \text{ initially prepared}]}{[A_{500nm} \text{ initially prepared}]} \times 100 \quad (5)$$

Microviscosity of Liposomal Bilayer Membranes. DPH as a fluorescent probe was dissolved in tetrahydrofuran (2×10^{-3} mol/L). The DPH solution was diluted to 2×10^{-5} mol/L with PBS before use. It was added to the dilute liposome and then incubated at 37 °C for 1 h. The weight ratio of PL to probe was 2700:1. The microviscosity (η) of liposomes was determined by fluorescence polarization (P), which can be calculated according to the following equation:

$$\eta = 2P/(0.46 - P) \quad (6)$$

$$P = (I_{0,0} - GI_{0,90})/(I_{0,0} + GI_{0,90}),\ G = I_{90,90}/I_{90,0} \quad (7)$$

where $I_{0,0}$ and $I_{0,90}$ are the fluorescence intensities of the emitted light polarized parallel and vertical to the exciting light, respectively, and G is the grating correction factor (*21*). The fluorescence intensities were measured at room temperature (20 °C) with a fluorescence spectrophtometer (650-60, Hitachi, Ltd., Tokyo, Japan), and excitation and emission wavelengths were 365 and 430 nm, respectively.

Malondialdehyde (MDA) Value. The MDA value was determined as an index of the PL peroxidation. The MDA value was detected spectrophotometrtically by the thiobarbituric acid (TBA) reaction following the method of Weng and Chen (*22*) with a slight modification. MDA, a final product of fatty acid peroxidation, reacted with TBA to form a colored complex that had a maximum absorbance at 535 nm. A solution containing TBA (15% w/v), trichloroacetic acid (0.37% w/v), and hydrochloric acid (1.8% v/v) was added to 1 mL of liposomal sample. The system was properly mixed and heated for 30 min at 100 °C in a water bath. The sample was cooled, and the total volume was added to 10 mL followed by centrifugation at 2500 rpm for 5 min. Clear solutions were obtained with this procedure suitable for direct spectrophotometric measurement. MDA (ng/mL) was expressed by multiplying a coefficient of 4.15×10^3 to the absorbance at 535 nm (*23*). MDA per milligram of PL was equal to MDA (ng/mL) dividing

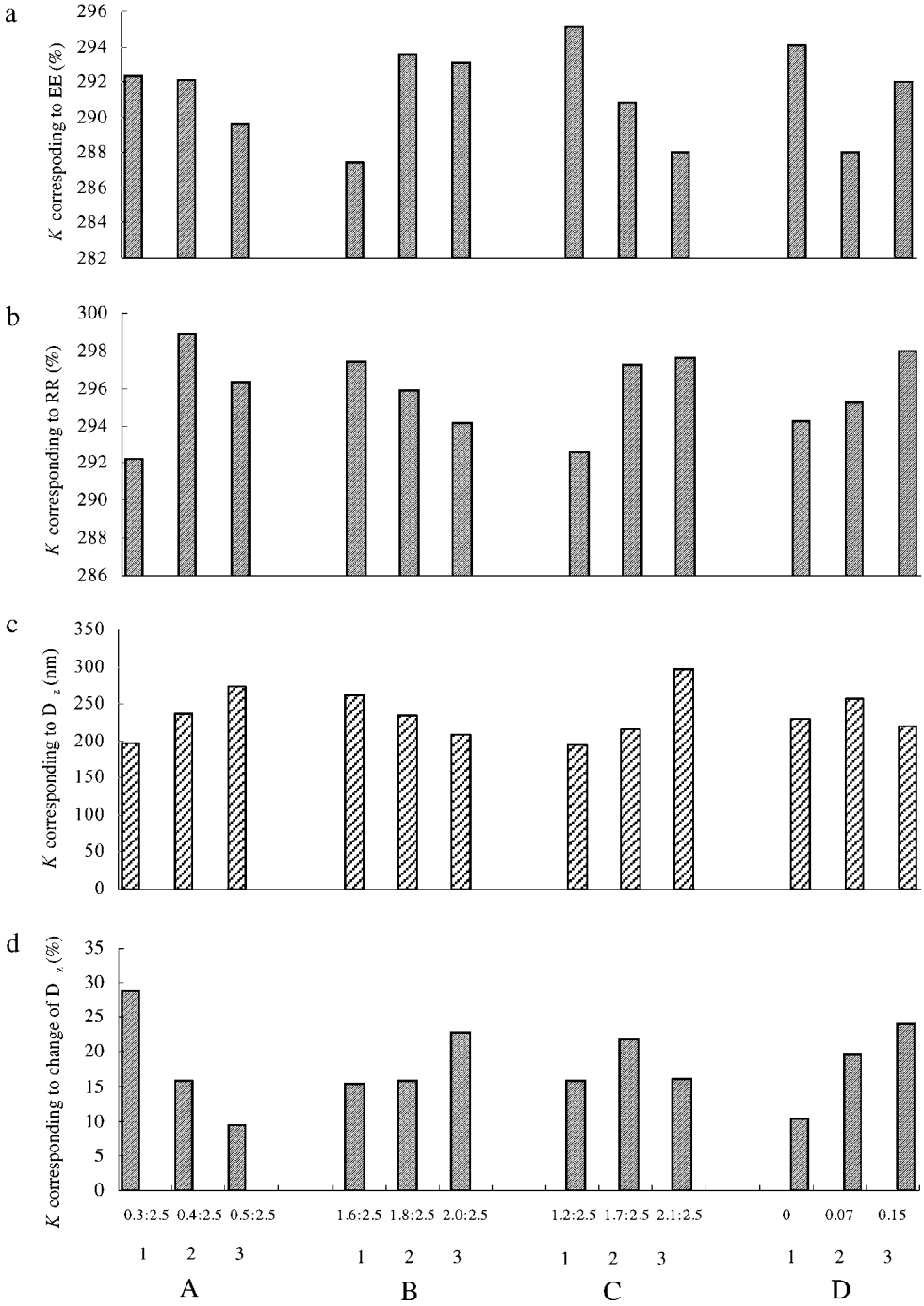

Figure 2. Main effect analysis of indexes. *K* denotes the sum of responses at each level. The numbers 1, 2, and 3 denote the levels of each factor (**A**, **B**, **C**, and **D**). (**A**) Chol/PL weight ratio, (**B**) Tween 80/PL weight ratio, (**C**) CoQ_{10}/PL weight ratio, and (**D**) NaCl molar concentration.

PL content per milliliter. The PL concentration was assessed by phosphorus assay according to the colorimetry of molybdenum blue (*24*).

Statistical Analysis. All experiments were conducted at least in triplicate, and values were averaged. The standard deviation was calculated. The statistically significant difference was evaluated with SPSS 10.0 for windows.

RESULTS AND DISCUSSION

Experimental Designed Data Analysis. In this optimization study with OAD, four response indices were considered as follows: high EE, high RR, appropriate D_z, and small ΔD_z. **Table 2** shows the mean values of each set of triplicate runs. The results of OAD experiments were subjected to direct observation analysis (also called range analysis) and ANOVA.

The *K* values (K_1, K_2, and K_3, the subscript denotes the level), the sum effect of four factors at three levels on four response indices, are presented in **Figure 2** for direct observation analysis. Superiority and inferiority levels of the four factors were evaluated by comparing their sum effect at three levels. It was found that lower CoQ_{10} level led to an obvious decrease of D_z (**Figure 2c**) and improved EE (**Figure 2a**). Additionally, higher Chol levels decreased the EE slightly (**Figure 2a**) and increased D_z (**Figure 2c**), whereas the effect of Tween 80 was opposite (**Figure 2a**,**c**). **Figure 2a**,**b** illustrates that the four factors imparted a little different influence on EE and RR within the

Table 3. Variance Analysis of Orthogonal Experiment for Formulation Optimization[a]

index	source of variation	type III sum of squares	DF[b]	mean square	*F* value[c]	*P*
EE	B	7.75	2	3.88	1.99	>0.1
	C	8.51	2	4.26	2.19	>0.1
	pooled error[d]	7.78	4	1.95		
	total	24.05	8			
RR	A	7.50	2	3.76	3.42	>0.1
	C	5.28	2	2.64	2.41	>0.1
	pooled error[d]	4.39	4	1.10		
	total	17.17	8			
D_z	A	984.65	2	492.32	2.78	>0.1
	C	1962.40	2	981.20	5.55	<0.1
	pooled error[d]	706.95	4	176.74		
	total	3654.00	8			
ΔD_z	A	64.43	2	32.22	6.95	<0.05
	D	32.98	2	16.49	3.56	>0.1
	pooled error[d]	18.55	4	4.64		
	total	115.96	8			

[a] (A) Chol/PL weight ratio, (B) Tween 80/PL weight ratio, (C) CoQ_{10}/PL weight ratio, and (D) NaCl concentration. [b] DF, degrees of freedom. [c] Critical *F* value: $F_{0.1}(2,4) = 4.32$, $F_{0.05}(2,4) = 6.94$. [d] The sum of squares of the errors of other insignificant factors was combined and treated as a pooled error.

selected range. D_z increased with increasing Chol level (A factor) and CoQ_{10} level (C factor) or decreasing Tween 80 amount (B factor) (**Figure 2c**). On the other hand, ΔD_z decreased with increasing Chol level (A factor) or decreasing NaCl concentration in hydration media (D factor) (**Figure 2d**). The difference between the highest and the lowest among K_1, K_2, and K_3 is defined by the symbol "*R*". The higher the *R* is, the greater the effect on the responses is. **Figure 2** also indicates that the order of the four-factor effect on D_z was C > A > B > D and the order on ΔD_z was A > D > B > C.

To investigate whether the factor effect on indexes was significant or could be ignored, ANOVA is performed. During calculation, the sum of squares of the error along with those of insignificant factors was combined and treated as the estimation of pooled error results, so that the ANOVA could be conducted for data analysis. The ANOVA results are shown in **Table 3**. It was found that within the selected range, only the CoQ_{10} level (factor C) was statistically significant related to D_z ($P < 0.1$). Furthermore, the Chol level (factor A) was statistically significant related to ΔD_z ($P < 0.05$). **Table 3** also shows that these four factors (A, B, C, and D) had no significant influence on the EE and RR within the selected range. The effect order of these four factors on indexes was the same as the *R* value mentioned above.

Because the variables within the selected range had no obvious effect on EE and RR and also the physical stability (with D_z and ΔD_z as criteria) of CoQ_{10} nanoliposomes was a crucial index, the best formulation was obtained as $C_1A_2B_2D_3$ (The subscript after each variable is the optimal level). The resulting optimum formulation composition was PL/CoQ_{10}/Chol/Tween 80 (2.5:1.2:4.0:1.8, w/w) with 0.01 M PBS (pH 7.4) as the hydration media. The results from validating experiments under these optimum conditions were also desirable as compared to each run of the OA_9 experiments. The CoQ_{10} nanoliposomes had a relatively homogeneous size distribution ranged from 20 to 500 nm. The D_z was about 69 nm with the PDI of 0.305. The EE of CoQ_{10} nanoliposomes was greater than 95%. After storage at 4 °C for 3 months, the D_z of the vesicles slightly increased from 69 to 75 nm, but none of these showed visible inhomogeneities. Also, encapsulation quality was not affected obviously during storage with a RR of higher than 90%.

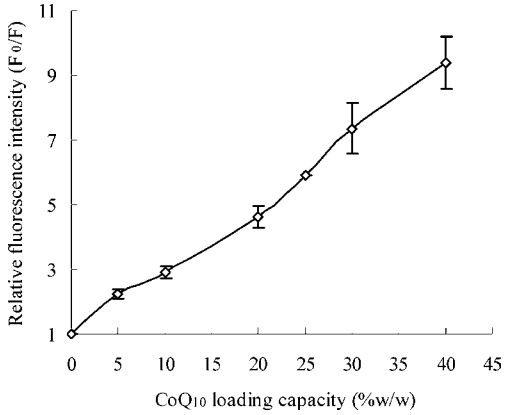

Figure 3. Relative DPH fluorescence intensity as a function of CoQ_{10} LC in PL/Chol/Tween 80 (2.5:0.4:1.8, w/w) nanoliposomes at room temperature. F_0 refers to the fluorescence intensity of CoQ_{10}-free nanoliposomes; *F* refers to the fluorescence intensity of nanoliposomes containing various amounts of CoQ_{10}. Each point represents the mean ± standard deviation ($n = 3$).

Effect of Formulation Composition. The core material CoQ_{10} is an important factor affecting the encapsulation quality and stability of CoQ_{10} nanoliposomes. This may be due to the fact that CoQ_{10} molecules unencapsulated in nanoliposomes would separate out from the liposomes and form crystals. As for the location and physical state (monomer or aggregate) of CoQ_{10} in the liposome membrane, there is a discrepancy, which probably results from different experimental conditions such as the lipids used, method of liposome preparation, and CoQ_{10} concentrations (*25*). The fluorescence probe DPH produces an intense fluorescence emission when incorporated in the hydrophobic membrane region (*26*). The influence of CoQ_{10} on the fluorescence intensity of DPH was investigated to obtain information about the orientation of CoQ_{10} in liposomal bilayers. The relative DPH fluorescence intensity as a function of CoQ_{10} LC in nanoliposomse at room temperature is presented in **Figure 3**. It was found that the DPH fluorescence intensity decreased significantly with the increase of CoQ_{10} LC. The slope of the curve was the largest when the LC varied from 20 to 30% (w/w). The DPH moiety is strongly hydrophobic. The measurement of the transversal location of DPH in lipid membranes by resonance excitation energy transfer suggests that the probe in egg yolk lecithin is predominantly located in the close vicinity of the bilayer center (*25*). The fluorescence quenching of DPH induced by increase of CoQ_{10} LC suggests that the CoQ_{10} molecules come into contact with the membrane zone occupied by DPH and led to increased polarity in the environment of the probe in the hydrophobic interior of bilayer membrane (*27*).

Liposomes are thermodynamically unstable, so the vesicles will aggregate, fuse, flocculate, and precipitate during storage (*14*). The D_z of CoQ_{10}-free nanoliposomes was increased from 117.5 to 260.9 nm after storage at 4 °C for 90 days; however, the D_z of CoQ_{10}-loaded nanoliposomes manufactured in the same way changed just a little (**Figure 5**). The stability of nanoliposomes is concerned with the fluidity of the lipid membrane. Microviscosity of the lipid membrane is a physical parameter used for estimating the fluid of membrane. The fluorescence probe DPH exists in the hydrophobic region and is able to evaluate the microviscosity around DPH in the liposomal bilayer membranes (*21*). The majority of the DPH molecules are oriented with their long axis aligned parallel to lipid acyl chains but with some population oriented parallel to the membrane surface (*25*). Fluorescence polarization is cor-

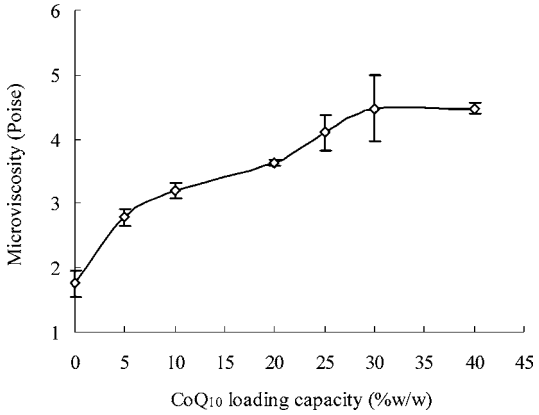

Figure 4. Effect of CoQ_{10} LC on the microviscosity of PL/Chol/Tween 80 (2.5:0.4:1.8, w/w) nanoliposomes. Each point represents the mean ± standard deviation ($n = 3$).

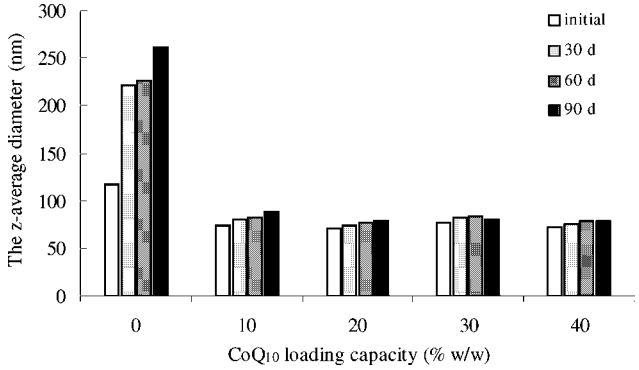

Figure 5. Variation of the D_z of CoQ_{10}-free nanoliposomes and CoQ_{10}-loaded nanoliposomes with different LC during storage at 4 °C. The weight ratio of PL/Chol/Tween 80 was 2.5:0.4:1.8.

Table 4. RR (%) of CoQ_{10} Nanoliposomes with Different LC during Storage at 4 °C in the Dark

day	LC = 10%[a]	LC = 20%	LC = 30%	LC = 40%
30	91.08 ± 2.86** [b]	95.07 ± 1.37	96.90 ± 1.45	96.77 ± 1.10
60	87.70 ± 0.17	90.49 ± 2.31	93.76 ± 1.09	94.77 ± 0.61
90	85.71 ± 2.21**	91.18 ± 1.03	92.66 ± 1.86	93.47 ± 1.38

[a] PL/Chol/Tween 80 (2.5:0.4:1.8, w/w) nanoliposomes. The LC is the weight percentage of CoQ_{10} against the lipids. [b] Data represent means ± standard deviation from three liposome preparations. **$P < 0.01$, significantly different from the RR of nanoliposomes containing CoQ_{10} (20, 30, and 40%).

related to microviscosity near the fluorescent probes, and microviscosity increases with increasing fluorescence polarization (*21*). **Figure 4** shows the microviscosity in nanoliposomes with different CoQ_{10} LCs. The microviscosity was enhanced dramatically once the core material CoQ_{10} was incorporated into bilayers and then increased slowly with the CoQ_{10} LC ranging from 5 to 30% followed by keeping constant when further increasing the CoQ_{10} LC to 40%. The higher the microvicosity is, the lower the fluidity of the lipid membrane is. In this way, the ordering of the lipid molecules in nanoliposomes containing CoQ_{10} is more compact than that of CoQ_{10}-free nanoliposomes, so the fusion or aggregation of nanoliposomes was inhibited by incorporation of CoQ_{10}. This can be used to explain why the core material CoQ_{10} encapsulated leaked out slowly and retained a large amount of CoQ_{10} during storage (**Table 4**).

Egg yolk PL was used as the major component of liposomal membrane, containing partially polyunsaturated fatty acid residues sensitive to oxidative free radicles. Ultrasonic irradiation

Table 5. Variation of the MDA Values in CoQ_{10}-Free and CoQ_{10}-Loaded Nanoliposomes during Storage at 4 °C in the Dark

	samples MDA (ng/mgPL)				
time (days)	CoQ_{10}-free nanoliposomes[a]	CoQ_{10}-loaded nanoliposomes[a]			
		LC = 10%	LC = 20%	LC = 30%	LC = 40%
initially	32 ± 6** [b]	15 ± 2	12 ± 2	14 ± 0	14 ± 1
30	52 ± 4**	14 ± 1	11 ± 0	12 ± 0	13 ± 0
60	55 ± 1**	15 ± 1	12 ± 1	11 ± 0	12 ± 0
90	82 ± 7**	14 ± 1	11 ± 1	8 ± 1	9 ± 3

[a] PL/Chol/Tween 80 (2.5:0.4:1.8, w/w) nanoliposomes. The LC is the weight percentage of CoQ_{10} against the lipids. [b] Data represent means ± standard deviation from three liposome preparations. **$P < 0.01$, significantly different from the MDA values of CoQ_{10}-loaded nanoliposomes.

used to reduce the vesicle size in the laboratory scale might produce oxygen free radicals and then lead to lipid peroxidation (*28*). The MDA, as a final product of fatty acid peroxidation, was detected in our study (**Table 5**). It can be seen that the MDA values of CoQ_{10}-free nanoliposomes initially prepared were significantly higher than those of CoQ_{10}-loaded nanoliposomes ($P < 0.01$). The result is in agreement with the reports by Landi et al. (*28*, *29*) who found that incorporation of ubiquinone-3 (CoQ_3) prevents lipid peroxidation induced by ultrasonic irradiation to disperse egg phosphatidylcholine in the aqueous phase. Antioxidant properties from CoQ_3 to ubiquinone-10 (CoQ_{10}) are similar. One CoQ_3 molecule protects about 20−30 polyunsaturated fatty acid residues and suggests that the antioxidant effect of ubiquinone mostly resides in its ability to trap lipid peroxyl radicals, and thus acts as a chain-breaking antioxidant (*28*). During 3 months of storage at 4 °C, the MDA values in CoQ_{10}-free nanoliposomes showed a significant increase. Nevertheless, the CoQ_{10}-loaded nanoliposomes with different LCs varied from 10 to 40% and showed no distinct differences in the MDA values. The data reported in **Table 4** also show the effects of changing the CoQ_{10} LC on CoQ_{10} stability in nanoliposomes. Incorporation of CoQ_{10} in vesicles can prevent peroxidation, with a concomitant decrease of CoQ_{10} encapsulated content. As compared with 10% of the LC, the remaining percentage of CoQ_{10} incorporated into the liposomes was higher when the LC was of 20, 30, and 40%. This reveals that the CoQ_{10} degradation would have increased with a decrease in the weight ratio of CoQ_{10} and PL.

Chol as an adjuvant membrane component showed a significant influence on the encapsulation quality and physical stability. By varying the amount of Chol in wall material composition, it was found that EE decreased a little, while the stability (with RR and ΔD_z as criteria) was improved during storage when the Chol proportion increased from 0.3:2.5 (Chol/PL, w/w) to 0.5:2.5 (w/w). The reduction of EE is related with the orientation of CoQ_{10} in the liposomal bilayer. CoQ_{10} molecules reside mainly in the hydrophobic region of the membrane. The incorporation of Chol into the membrane resulted in the increase of the microviscosity (**Figure 6**), suggesting the rigidity strength of the membrane (*30*). **Figure 6** also shows the D_z of CoQ_{10}-loaded nanoliposomes with different Chol levels. The increase of the D_z corresponds to the decrease of curvature, which is responsible for the reduction of CoQ_{10} encapsulated in nanoliposomes. Chol interacts with fatty acids of liposomes via hydrogen bonding, increasing the cohesiveness and mechanical strength of the vesicular membrane (*31*); thus, CoQ_{10} encap-

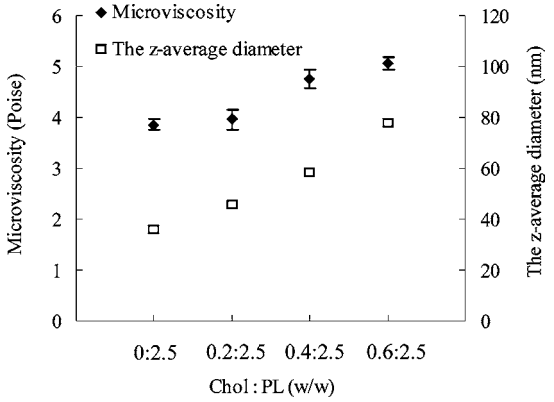

Figure 6. Microviscosity and D_z of CoQ_{10}-loaded nanoliposomes with different Chol levels. The weight ratio of PL/Tween 80 was 2.5:1.8, and the LC of CoQ_{10} was 30%.

sulated in nanoliposomes will not leak out easily, and the fusion or aggregation of nanoliposomes is inhibited during storage.

In addition to the incorporation of Chol into the liposome bilayer, some attempts were made to enhance their steric stability by preparation of polymer-coated liposomes. So far, several substances such as poloxamer, Tween 80, carboxymethyl chitin, carboxymethyl chitosan, and dextran derivatives have been used (*32*). Among them, Tween 80 is a kind of nonionic surfactant that is often used as an emulsifier in food, and its tolerance is only 0.1−1(g/100 g) by FAO/WHO. Tasi et al. (*33*) reported that steric-stabilized liposomes increase the stability of systems that incorporated Tween 80 surfactants into the liposomal bilayer in serum, as compared with the corresponding liposomes without Tween 80. However, there has been no published information on the effect of Tween 80 used in the CoQ_{10} nanoliposomes. Therefore, the effects of CoQ_{10} nanoliposomes incorporated with varying quantities of Tween 80 were examined in this work from the perspective of the encapsulation quality and physical stability. Although the effect of Tween 80 on the four indices such as EE, RR, D_z, and ΔD_z was not statistically significant within the selected range from OAD, the preliminary experiments with a wide range of Tween 80 levels indicated that the encapsulation quality and stability were improved obviously. The physical stability of Tween 80-coated nanoliposomes was evaluated by measuring the change of particle size and the RR of core material after storage at 4 °C for 30 days. Herein, the change of turbidity (A_{500nm}) was used as an index to reflect the relative change of particle size. The interaction between surfactant Tween 80 and lipids was responsible for the change of turbidity in system. The EE and RR improved more concomitantly with increasing the amount of Tween 80 in the formulation from 1.2:2.5 (Tween 80/PL, w/w) to 1.8:2.5 (w/w) as shown in **Figure 7a**. The change of absorbance (ΔA) after storage at 4 °C for 30 days decreased remarkably from 7.87% to zero as Tween 80 increased in the formulation. **Figure 7b** also demonstrates that the liposomal size decreased as the amount of Tween 80 in the formulation increased. This is due to steric repulsion existing among the Tween 80, which is exposed from the outer and inner leaflet of the liposomal bilayer membrane. The Tween 80 exposed from the outer leaflet of the bilayer membrane increases the liposome particle curvature, whereas the Tween 80 exposed to the inner leaflet does the opposite. Therefore, adding Tween 80 reduces the liposomal size since more Tween 80 exists in the outer leaflet than that in the inner leaflet of the liposomal bilayer membrane (*33*). On the basis of these results, Tween 80-coated nanoliposomes had

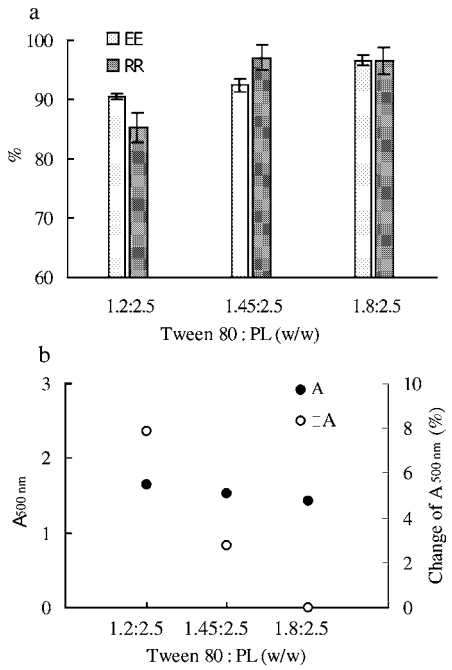

Figure 7. Effect of Tween 80 amount on the encapsulation quality of CoQ_{10} nanoliposomes. The weight ratio of PL/Chol/CoQ_{10} was 2.5:0.4:2.0. The RR and the change of absorbance at 500 nm (ΔA) were obtained after 1 month of storage at 4 °C in the dark place.

resistance to leakage of core material CoQ_{10} and aggregation or fusion of bilayer vesicles.

Dispersion of CoQ_{10} nanoliposomes in NaCl solution promoted a small increase of the average particle size during storage, suggesting that vesicles had aggregated or fused when the NaCl concentration increased from 0 to 0.15 M. This effect can be related to the use of a primary wall material PL with charge. Hydration forces (due to adsorption of hydroxyl groups) are the main repulsive forces in dispersions of liposomes. Close association is a prerequisite of vesicle fusion (*34*). As the salt concentration increases, the thickness of the double layer decreases and the electrostatic repulsion between them diminishes. Consequently, liposomes suspended in NaCl solution with higher concentrations could locate in closer proximity to neighboring vesicles than those dispersed in NaCl solution with lower concentrations, hence promoting the formation of large vesicles.

CoQ_{10} Nanoliposomes Production in a Pilot Scale. The first large-scale production of liposomes was performed in the mid-1980s. The reason for the relatively slow development of successful large-scale production is related to the time-consuming technological problems (e.g., reproducible processes) and quality control issues (e.g., long-term chemical and physical stability) that had to be resolved (*35*). Therefore, in this study, we focused on the establishment of a manufacturing technique with the potential for CoQ_{10} nanoliposome scale-up.

The production of CoQ_{10} nanoliposomes employing the optimal formulation was scaled-up to 1 L per batch by the flow injection procedure, as described in section 2 and shown in **Figure 1**. Ethanol was removed by continuous cross-flow filtration with a 10 kDa cross-flow filtration membrane. The retained liposome suspension was concentrated to 12.5 mg PL/mL. Size and size distribution of liposome suspensions during the process were determined by laser light scattering as shown in **Table 6**. Treatment with the high-pressure homogenizer had the effect of narrowing down the particle size distribution at the same time as the average particle size decreased. Only

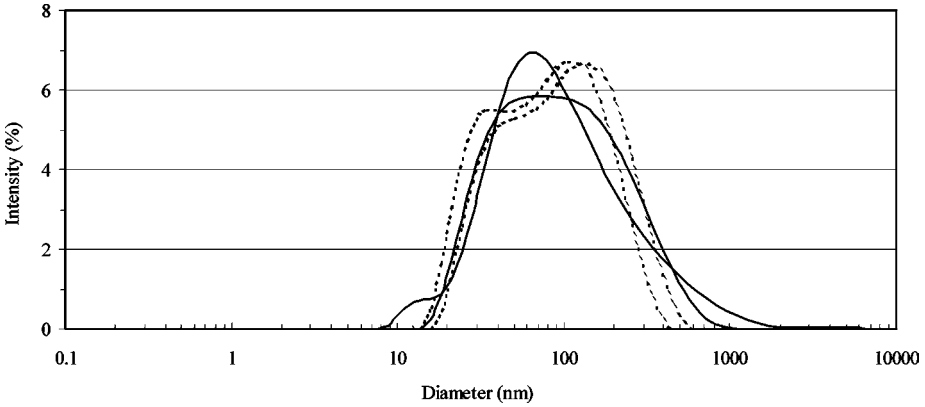

Figure 8. Size distribution by intensity of CoQ_{10} nanoliposome made in the laboratory scale (- - -) and in the pilot scale (—). Nanoliposomes were composed of PL/Chol/Tween 80/CoQ_{10} = 2.5:0.4:1.8:1.2 (w/w).

Table 6. Variation of the D_z and Size Distribution (PDI) of CoQ_{10} Nanoliposomes[a] during Production in the Pilot Scale

sampling point[b]	1	2	3	4
D_z (nm)	147.4	73	69.89	67.04
PDI	0.441	0.345	0.400	0.402
peak 1 (% intensity)	280.5 (98.95)	91.74 (98.22)	130.5 (100)	146.3 (99.8)
peak 2 (% intensity)	18.69 (1.046)	5298 (1.781)		42.95 (0.2013)

[a] PL/CoQ_{10}/Chol/Tween 80 (2.5:1.2:0.4:1.8, w/w) nanoliposomes. [b] Key: 1, after hydration; 2, after homogenization; 3, after filtration; and 4, after extrusion.

minimal alterations with respect to the liposomal size and size distribution were observed during the filtration and extrusion procedure. The D_z of the final CoQ_{10} nanoliposomes was about 67 nm, and the EE was 98.60 ± 3.37%. After storage at 4 °C for 60 days, the D_z just increased to about 69 nm and the remaining percentage of the encapsulated CoQ_{10} in nanoliposomes was 98.46 ± 1.54%. The MDA values of the CoQ_{10}-free nanoliposomes and CoQ_{10}-loaded nanoliposomes initially prepared were 14 ± 4 and 8 ± 3 ng/mg PL, respectively. It was interesting to find that the MDA values of CoQ_{10}-free nanoliposomes obtained from the pilot scale were significantly lower than those of samples prepared with a sonication method in the laboratory scale. Furthermore, the MDA values of CoQ_{10}-free nanoliposomes just showed a slight increase up to 16 ± 4 ng/mg PL after storage at 4 °C for 60 days. As almost no increase of the MDA was detected, results suggest that the preparation process with homogenizer does not promote the radical reaction. The phenomenon agreed with those indicated by Barnadas-Rodriguez et al. (*36*) who found that microfluidization does not increase the soybean phosphatidylcholine liposome oxidation index (conjugated dienes). This is due to the mild preparation procedure using a homogenizer and extruder in the pilot scale to reduce the liposome size as compared with sonication in the laboratory scale. Additionally, the two production batches showed almost the same average particle size, but the size distribution had little difference (**Figure 8**). The maximum size of CoQ_{10} nanoliposomes obtained from the pilot scale seemed to be slightly larger than that of samples obtained from the laboratory scale. So, the extrusion process has to be repeated for several passes to avoid the existence of larger liposomal vesicles in further work. These data also demonstrated the feasibility and reproducibility of the established production technique used in this study for the production of CoQ_{10} nanoliposomes.

In summary, CoQ_{10} nanoliposomes with higher EE (EE > 95%) and RR (RR > 90%) as well as desired D_z (D_z < 100 nm) and change of D_z (ΔD_z < 10%) was obtained. Optimal formulation for the laboratory preparation of CoQ_{10} nanoliposomes was provided by effectively controlling the factors in determining the quality with statistical techniques employed in this study. This optimal formulation was also suitable for large-scale production. Pilot-scale production revealed that the production of CoQ_{10} nanoliposomes was technically feasible with ethanol injection by controlling the PL/CoQ_{10}/Chol/Tween 80 ratio (2.5:1.2:0.4:1.8, w/w) and the hydration media (0.01 M PBS). The scale-up technique also has advantages of mild preparation conditions and the avoidance of hazardous solvents and forces. The information generated by this study is helpful for engineers to flexibly adjust the system performance and variation in the encapsulation quality and stability of CoQ_{10} nanoliposomes used as nutraceuticals.

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