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Purified and surfactant-free coenzyme Q10-loaded biodegradable nanoparticles

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Abstract

The intent of this work was to synthesize and comprehensively characterize ubiquinone-loaded, surfactant-free biodegradable poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles in vitro. Surfactant-free, empty and ubiquinone (CoQ10)-loaded biodegradable nanoparticles were synthesized by nanoprecipitation, and the physicochemical properties of these nanoparticles were analyzed with a variety of techniques. Nanoprecipitation consistently yielded individual, sub-200 nm, surfactant-free empty and CoQ10-loaded nanoparticles, where the physical and drug encapsulation characteristics were controlled by varying the formulation parameters. CoQ10 release was sustained for 2 weeks but then plateaued before 100% CoQ10 release. A novel, nondestructive purification protocol involving transient sodium dodecyl sulfate (SDS) adsorption to nanoparticles followed by centrifugation and dialysis was developed to yield purified, surfactant-free, CoQ10-loaded nanoparticles. This protocol permitted removal of unencapsulated CoQ10, prevented centrifugation-induced nanoparticle aggregation and preserved the surfactant-free and drug encapsulation properties of the nanoparticles. These CoQ10-loaded nanoparticles are promising as sustained drug delivery devices due to their extended CoQ10 release. Importantly, a surfactant-free nanoprecipitation procedure is presented that in combination with a novel purification step enables the synthesis of individual and purified CoQ10-loaded nanoparticles.

Keywords: Nanoprecipitation; PLGA nanoparticles; Coenzyme Q10; Drug delivery; Surfactant-free

1. Introduction

Coenzyme Q10 (CoQ10), also called ubiquinone, is an extremely lipophilic antioxidant found in lipid membranes, especially the inner mitochondrial membrane. It is a cofactor in the electron transport chain, where it transfers free electrons from complexes I and II to complex III during oxidative phosphorylation and ATP synthesis (Ebadi et al., 2000). In addition to its bioenergetic and free radical scavenging functions, CoQ10 regenerates alpha-tocopherol, another critical antioxidant (Lass and Sohal, 1998). Experimental evidence established CoQ10's effectiveness at preventing lipid peroxidation (Forsmark-Andree et al., 1997) and decreasing apoptosis (Papucci et al., 2003). Additionally, early clinical trials showed minor improvements in neurological disorders due to CoQ10 therapy (Shults et al., 2002; Shults and Haas, 2005). These promising characteristics have spurred great interest in CoQ10 as a supplemental therapy for cardiovascular and mitochondrial disorders (Mortensen, 1993). Unlike symptomatic treatment, CoQ10 antioxidant therapy may slow the progression and exacerbation of diseases. However, the long isoprenoid side chain imparts extreme hydrophobicity to the CoQ10 molecule and thus renders it poorly bioavailable as a therapeutic. Therefore, there has been much interest in designing new technologies for efficient delivery of this promising lipophilic antioxidant.

Nanoparticles formulated as drug delivery devices are an innovative approach for CoQ10 therapeutics. Biodegradable poly(lactide-*co*-glycolide) (PLGA) nanoparticles are partic-

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ularly interesting due to PLGA's clinical biocompatibility, tunable biodegradation and resorbable by-products (Hans and Lowman, 2002). PLGA nanoparticles loaded with drugs are preferable to traditional oral, intramuscular or intravenous therapies because they flow through capillaries, protect molecules from enzymatic degradation, enable delivery of non-traditional molecules, are modifiable for receptor targeting and can be internalized by cells for cytoplasmic drug delivery (Soppimath et al., 2001; Sahoo et al., 2002). Furthermore, they solubilize hydrophobic drug molecules, such as CoQ10, by encapsulating and separating drugs from aqueous physiological environments. Motivated by these advantages, several groups have employed emulsion-based techniques to encapsulate CoQ10 within nondegradable micro- and nanoparticles for improved oral bioavailability (Kommuru et al., 2001; Hsu et al., 2003). As an alternative to the aforementioned techniques, we investigated the feasibility of CoQ10 encapsulation within biodegradable nanoparticles using nanoprecipitation, a much simpler synthesis protocol. Since its introduction (Fessi et al., 1989), nanoprecipitation has been applied to formulate polymeric nanoparticles loaded with diverse drug molecules. In this simple and fast technique, a water-miscible organic solvent containing the dissolved polymer (with or without drug molecules) is injected into an aqueous nonsolvent; nanoparticles spontaneously precipitate. A major advantage of nanoprecipitation is that the stabilizing surfactants commonly added during nanoparticle synthesis are not required to prevent nanoparticle aggregation. Therefore, putative disadvantages of surfactants, like questionable biocompatibility, irreversible incorporation in the nanoparticles and alteration of nanoparticle surface properties (Sahoo et al., 2002), are eliminated. Furthermore, the nanoparticles are free for surface chemistry modifications and targeting applications. Here, it was demonstrated that CoQ10-loaded nanoparticles can be synthesized rapidly and simply via nanoprecipitation and that surfactants are not required to prevent nanoparticle aggregation in solution.

In this study, nanoprecipitation was used to produce novel CoQ10-loaded, surfactant-free, biodegradable PLGA nanoparticles, where CoQ10, a promising antioxidant, was controllably incorporated within the nanoparticles. These CoQ10-loaded drug delivery devices may significantly enhance CoQ10's therapeutic efficacy. The physical properties and drug encapsulation characteristics of the nanoparticles were controlled and optimized by varying the nanoprecipitation parameters. Drug release studies demonstrated steady release of CoQ10 for 2 weeks, an exciting result relevant for sustained drug delivery applications. A novel purification protocol is presented which did not alter the initial physicochemical properties of the surfactant-free nanoparticles. Specifically, transient SDS adsorption to synthesized nanoparticles combined with centrifugation and dialysis allowed efficient separation of unencapsulated CoQ10 from CoQ10-loaded nanoparticles without aggregation, which is normally induced by centrifugation in the absence of SDS. These purified CoQ10-loaded biodegradable nanoparticles are promising and novel drug delivery devices.

2. Materials and methods

2.1. Materials

Poly(DL-lactide-*co*-glycolide)–COOH (referred to as PLGA) with 50:50 lactide:glycolide copolymer ratio was purchased from Lactel Absorbable Polymers (Pelham, AL, USA). Coenzyme Q10 was purchased from Spectrum Chemicals and Laboratory Products (Gardena, CA, USA). Omnipur phosphate buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4 at pH 7.4 was referred to as 1× PBS (Fisher Scientific, Pittsburgh, PA, USA). Sodium dodecyl sulfate (SDS) was from Shelton Scientific-IBI (Peosta, IA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA) and used as received, unless noted otherwise. For dialysis and filtration, cellulose membranes with 50 kDa molecular weight cutoff (Membrane Filtration Products, Inc., Seguin, TX, USA) and qualitative Whatman 1 membrane filters (Millipore, Billerica, MA, USA) were used, respectively.

2.2. Nanoparticle preparation

Nanoparticles were synthesized using the nanoprecipitation method (Fessi et al., 1989). Briefly, the specified mass of PLGA was dissolved in 5 mL of a water-miscible organic solvent, typically acetone or acetonitrile. This polymer solution was injected into a specific volume (usually 50 mL) of deionized water (DIH_2O) with magnetic stirring at room temperature. After at least 12 h of organic solvent evaporation into air by magnetic stirring, the nanoparticle suspension was qualitatively filtered to remove dust or large aggregates. For CoQ10-loaded nanoparticles, CoQ10 was dissolved along with PLGA in the solvent before injection into DIH_2O.

2.3. Nanoparticle size and surface charge analysis

The particle size and polydispersity index (PDI) were measured by dynamic light scattering (DLS). DLS was performed at room temperature in DIH_2O with a scattering angle of 90° on the 90Plus Particle Size Analyzer (Brookhaven Instruments Corp., Holtsville, NY, USA). Five 1 min sub-runs per sample were collected and averaged. Zeta potential measurements for surface charge were performed at 25 °C in a folded capillary cell at 150 V and M3-PALS detection using non-invasive backscatter at 173° with an Avalanche photodiode, Q.E. > 50% at 633 nm (Malvern Zetasizer Nano-ZN, Worcestershire, UK). Samples were diluted 1:50 in DIH_2O and measured in triplicate with 20 sub-runs. Zeta potentials (ZP) were calculated using the Smoluchowski limit for the Henry equation with a setting calculated for practical use ($f(ka) = 1.5$).

2.4. Nanoparticle morphology analysis

Surfactant-free nanoparticle morphologies were visualized by scanning electron microscopy (SEM; Supra 40VP FESEM, Zeiss) and atomic force microscopy (AFM; Quesant Q-250, Ambios Technology, Santa Cruz, CA, USA). For SEM, images

were collected at 1 kV and either 30,000× or 6000× magnification. For AFM, standard silicon cantilevers with force constants ∼40 N/m and resonant frequencies ∼137 kHz were used. Topography and phase images were simultaneously collected in tapping mode at a scan rate of 3 Hz under ambient laboratory conditions. To prepare samples, silicon chips were first cleaned in 3:1 97% sulfuric acid:30% hydrogen peroxide Piranha solution for 30 min, followed by vigorous rinsing in DIH_2O. The chips were then silanized in 2% (v/v) 3-aminopropyltriethoxysilane in acetone for 30 min following by acetone rinsing. Then, 5 μL of nanoparticle suspension was adsorbed on silanized silicon chips for 20 min before delicate rinsing in DIH_2O and drying under a nitrogen gas stream.

2.5. Determination of CoQ10 encapsulation

To measure maximum yield, encapsulation efficiency (EE) and drug content, unencapsulated CoQ10 was separated from the CoQ10-loaded nanoparticles by centrifugation (RC-5C Plus, Sorvall, Asheville, NC, USA) at 17,200 × *g* for 30 min at 25 °C. The unencapsulated CoQ10 in the supernatant was measured spectrophotometrically at 275 nm and quantified via a standard curve. The nanoparticle pellet was washed three times with 30 mL DIH_2O, resuspended in DIH_2O and frozen at −80 °C. After freezing, the nanoparticles were lyophilized for 2 days (Virtis Benchtop SLC, Gardiner, NY, USA). The lyophilized nanoparticle mass (for yield) was measured before solubilizing the nanoparticles in acetonitrile. The CoQ10 encapsulation properties were calculated for process suitability assessment purposes with yield = 100 × (mass of the lyophilized nanoparticles/('correction factor' × (CoQ10 + PLGA mass dissolved in the solvent))). CoQ10 encapsulation was determined spectrophotometrically at 275 nm. CoQ10 incorporation was measured by EE = 100 × (CoQ10 mass in nanoparticles/('correction factor' × CoQ10 mass in solvent) and drug content = 100 × (CoQ10 mass in nanoparticles/mass of the lyophilized nanoparticles). The 'correction factor' = (volume nanoparticle suspension centrifuged/volume nanoparticle suspension after overnight evaporation). It accounted for the small loss of nonsolvent, CoQ10 and PLGA during overnight evaporation and the actual volume of nanoparticle suspension analyzed. This correction factor was needed to reliably compare nanoparticles of different batches. In CoQ10-loaded nanoparticles, it is important to remember that the lyophilized nanoparticle mass included the encapsulated CoQ10 mass.

2.6. In vitro CoQ10 release

Drug release from the nanoparticles was measured in a cell-free system. Empty and CoQ10-loaded nanoparticles were diluted 1:1 (v:v) in Petri dishes with physiologic buffer (1× PBS) at a typical concentration of 0.33 mg/mL. These nanoparticle suspensions were placed in a humidified incubator so that a biological environment was simulated for CoQ10 release. At specified time points, the nanoparticle samples were collected and analyzed for yield, EE, drug content and unencapsulated CoQ10, as described above. Empty nanoparticle suspensions were used as controls.

2.7. Nanoparticle purification

A novel, transient SDS adsorption protocol was developed to separate unencapsulated CoQ10 from CoQ10-loaded nanoparticles. After nanoprecipitation, SDS was added to the nanoparticle suspension (0.3% (w/v) SDS) and allowed to transiently adsorb to nanoparticle surfaces for 1 h. Nanoparticles were pelleted by centrifugation at 17,200 × *g* for 30 min at 25 °C and unencapsulated CoQ10 was aspirated with the supernatant. These SDS modified, CoQ10-loaded nanoparticles were resuspended in DIH_2O by sonication and dialyzed against 1 L DIH_2O for 48 h, with five changes of DIH_2O. The nanoparticle suspension was then qualitatively filtered, and these CoQ10-loaded nanoparticles were considered "purified" for further experimentation.

2.8. Determination of surface-adsorbed SDS

Purified CoQ10-loaded nanoparticles were comprehensively analyzed for residual surface-adsorbed SDS after 48 h of dialysis. First, the methylene blue active substances assay was used (Hayashi, 1975; Arand et al., 1992). In this assay, purified CoQ10-loaded nanoparticle samples were reacted with equivolume methylene blue reagent (1% (v/v) H_2SO_4, 5% (w/v) Na_2SO_4, 0.025% (w/v) methylene blue), which caused the formation of a water-insoluble methylene blue-SDS salt. Then, chloroform was added to this solution (2:1 v:v) to extract the insoluble salt. The remaining aqueous layer was removed, and the absorbance was measured spectrophotometrically at 561 nm. SDS in DIH_2O was used as a standard, and residual SDS was reported per nanoparticle mass. X-ray photoelectron spectroscopy (XPS) employing a monochromatic 1486.6 eV Al Kα-X-ray small spot source and multichannel detector (SSI S-Probe Monochromatized XPS Spectrometer) was used to determine the chemical composition of different nanoparticle samples adsorbed on silanized silicon chips. A concentric hemispherical analyzer was operated in constant analyzer transmission mode to measure the binding energies of emitted photoelectrons. The binding energy scale was calibrated by the gold Au $4f_{7/2}$ peak at 83.9 eV with linearity verified by the copper Cu $3p_{1/2}$ and copper Cu $2p_{3/2}$ peaks at 76.5 and 932.5 eV, respectively. Survey spectra were collected from 0 to 1100 eV with pass energy of 160 eV. All spectra were referenced by setting the C 1s peak to 285.2 eV to compensate for residual charging effects. Data for percent atomic composition and ratios were calculated using analysis software bundled with the instrument. ZP experiments for surface charge analysis were performed as described above.

3. Results and discussion

3.1. Surfactant-free nanoparticle synthesis and physicochemical characterization

Nanoprecipitation was used to synthesize surfactant-free empty and CoQ10-loaded PLGA nanoparticles. The parameters

(A)

Parameter	Variable	Size (nm)	PDI
Solvent Type	Acetone	165 ± 1.6	.0907 ± .0206
	Acetonitrile	164 ± 4.5	.0863 ± .0218
Nonsolvent Type	Water	167 ± 5.2	.0651 ± .0256
	PBS	256 ± 15 *	.1759 ± .0475 *
PLGA mass (mg)	25	126 ± 2.9	.0672 ± .0157
	50	145 ± 2.0 *	.0609 ± .0163
	150	174 ± 6.3**	.0608 ± .0194
Volume ratio (Nonsolvent: solvent)	5	126 ± 5.2	.0549 ± .0159
	10	155 ± 2.7 *	.0504 ± .0189
	30	164 ± 5.0 *	.0455 ± .0173

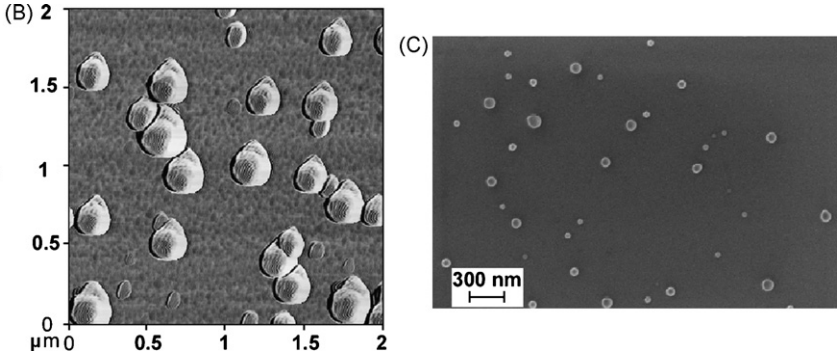


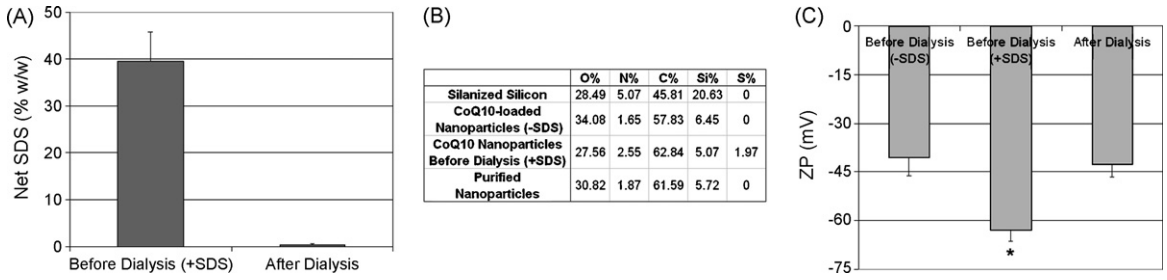
Fig. 1. Synthesis of individual, surfactant-free PLGA nanoparticles with controllable diameters and polydispersities was achieved by nanoprecipitation. (A) Only changing the nonsolvent to PBS, increasing the polymer mass to 50 mg and 150 mg or increasing the volume ratio from 5 had significant effects (mean ± standard error, $n = 3$, $^*p < 0.05$ different from 1st variable, $^{**}p < 0.05$ different from 1st and 2nd variables). (B) Representative phase AFM image of empty, surfactant-free PLGA nanoparticles showed spherical morphology, lack of aggregates and diameters between 100 and 200 nm. (C) Representative SEM image (30,000× magnification) of empty, surfactant-free PLGA nanoparticles confirmed the morphological and size observations from AFM.

were first optimized for appropriately small and monodisperse nanoparticles. Diameters under 200 nm were desired because smaller nanoparticles are more easily engulfed by cells (Desai et al., 1997; Win and Feng, 2005) for intracellular drug delivery (Panyam et al., 2002; Elamanchili et al., 2004), and narrowly distributed nanoparticles were desired in order to minimize complications in filtering, purification and future characterization of cellular interactions. All tested parameters resulted in presumably individual, nanoscale particles with narrow size distributions (PDI's < 0.1), except with the nonsolvent PBS (Fig. 1A). Aggregation (large diameter and polydispersity) in PBS was attributed to high salt concentrations, which probably neutralized the negative nanoparticle surface charge. Also, nanoparticle sizes were controllable by adjusting various formulation parameters (Fig. 1A). By changing the polymer mass or nonsolvent:solvent volume ratio (with a constant volume of acetonitrile solvent), final nanoparticles of different sizes were obtained. With more PLGA in the solvent, larger initial nanoemulsion droplets were formed during nanoprecipitation, which resulted in larger nanoparticles. Lastly, higher nonsolvent volumes effectively increased the concentration gradient for rapid solvent diffusion and removal, which has been attributed to larger, more porous particles (Soppimath and Aminabhavi, 2002). Based on these experiments, the following optimal nanoprecipitation conditions were chosen for further studies: 50 mg PLGA in acetonitrile (with/without CoQ10) at a volume ratio of 1:10 with DIH_2O. This DLS data demonstrated the versatility and ease of synthesizing narrowly distributed, sub-200 nm nanoparticles by nanoprecipitation.

Surfactants are customarily utilized during nanoparticle synthesis to prevent aggregation despite their numerous disadvantages. During synthesis, the hydrophobic block of amphiphilic surfactants may be intercalated with the PLGA chains, and/or attach to nanoparticle surfaces via hydrophobic bonds (Murakami et al., 1999; Sahoo et al., 2002; Galindo-Rodriguez et al., 2004). In either instance, they cannot be completely removed, even after extensive washing (Sahoo et al., 2002). Residual surfactants affect nanoparticle surface properties and drug release (Sahoo et al., 2002), and they disturb cell membranes, potentially diminishing nanoparticle biocompatibility. In addition, surface modification for targeted delivery is complicated by the presence of surface-integrated surfactants. Because of these numerous disadvantages, this study focused solely on the synthesis of surfactant-free nanoparticles. A primary concern was whether surfactant-free conditions would cause nanoparticle aggregation. DLS data demonstrated nanoscale diameters (Fig. 1A) but it could not unequivocally eliminate the possibility of aggregates. Therefore, surfactant-free nanoparticles were visualized with AFM and SEM, which elucidated single particles and spherical morphologies (Fig. 1B and C). Between DLS, AFM and SEM, it was clearly demonstrated that surfactants were not needed to prevent nanoparticle aggregation. The ZP of these nanoparticles was probably sufficiently negative (−40 mV, "Before Dialysis, SDS" Fig. 2C) to stabilize the surfactant-free nanoparticles due to the carboxylate end groups of PLGA. Therefore, individual PLGA nanoparticles with controllable properties were routinely synthesized via nanoprecipitation without the interference of surfactants.

3.2. Coenzyme Q10 encapsulation

The CoQ10 molecule comprises a quinone ring and a long isoprene side chain, which renders it lipophilic and favorable for encapsulation via nanoprecipitation. CoQ10-loaded nanoparticles were synthesized by nanoprecipitation, collected by centrifugation and unencapsulated CoQ10 was removed with the supernatant. Unencapsulated CoQ10 likely existed as crystals in the aqueous supernatant because CoQ10 is practically insoluble in water. Fortuitously, in preliminary nanoprecipitation experiments with CoQ10 but without PLGA in the solvent, unencapsulated CoQ10 crystals did not pellet at the centrifugal force utilized to pellet CoQ10-loaded nanoparticles (data not shown). Therefore, we were confident that unencapsulated CoQ10 was completely removed by aspirating the supernatant and washing the nanoparticle pellet after centrifugation, so we proceeded to analyze the yield, EE and drug content. Increasing the CoQ10 mass in acetonitrile from 1 to 10 mg had no effect on total nanoparticle yield, which remained approximately 60%, but there was an upward trend in EE from 49% to 72% (Fig. 3A). The drug content significantly increased



	O%	N%	C%	Si%	S%
Silanized Silicon	28.49	5.07	45.81	20.63	0
CoQ10-loaded Nanoparticles (-SDS)	34.08	1.65	57.83	6.45	0
CoQ10 Nanoparticles Before Dialysis (+SDS)	27.56	2.55	62.84	5.07	1.97
Purified Nanoparticles	30.82	1.87	61.59	5.72	0

Fig. 2. SDS removal from purified CoQ10-loaded nanoparticles was confirmed with three methods. (A) The methylene blue active substances assay detected a dramatic decrease in surface-associated SDS from 40% (w/w) before centrifugation and dialysis to 0.45% (w/w) after dialysis ($n = 6$). (B) XPS showed changes in nanoparticle chemical composition. Before dialysis and centrifugation, surface-adsorbed SDS was detected by the presence of sulfur atoms, but dialysis completely removed sulfur (and SDS) from the nanoparticle surfaces. (C) Zeta potential measurements showed that CoQ10-loaded nanoparticles with adsorbed SDS were considerably more negative (−63.0 mV) than unpurified CoQ10-loaded nanoparticles (−40.4 mV). The surface charge was recovered in purified CoQ10-loaded nanoparticles (−42.5 mV) after dialysis, suggesting total SDS removal ($n = 4$, $^*p < 0.05$, different from other two conditions).

with each increase in CoQ10 mass from 1% at 1 mg CoQ10 to 19% at 10 mg CoQ10 ($^*p < 0.05$). For subsequent experiments, nanoparticles loaded with 5 mg CoQ10 (65% yield, 50% EE, 7% drug content) were utilized because they exhibited the highest drug content with sub-200 nm diameters. Additionally, these 7% CoQ10-loaded nanoparticles also maintained the spherical, non-aggregated morphology characteristic of empty nanoparticles (Fig. 3B). The yield and CoQ10 EE agreed with published results for hydrophobic drug encapsulation by nanoprecipitation (Fonseca et al., 2002; Liu et al., 2005; Zeisser-Labouebe et al., 2006) and the one report of CoQ10 EE in wax core nanoparticles (Hsu et al., 2003). The drug content for our particles was much greater than most other reports (Govender et al., 1999; Zeisser-Labouebe et al., 2006) but reasonably close to testosterone-loaded, surfactant-free nanoparticles (Jeong et al., 2002). A high drug content was expected because of hydrophobic interactions during nanoprecipitation; CoQ10 molecules were strongly associated with PLGA chains in the nanoemulsion and were not likely to diffuse into the aqueous nonsolvent during nanoprecipitation (Choi et al., 2002). To our knowledge, this is the first demonstration of CoQ10 encapsulation within surfactant-free PLGA nanoparticles, and the therapeutic promise of these biodegradable CoQ10-loaded nanoparticles may surpass that of the nondegradable CoQ10 nanoparticles.

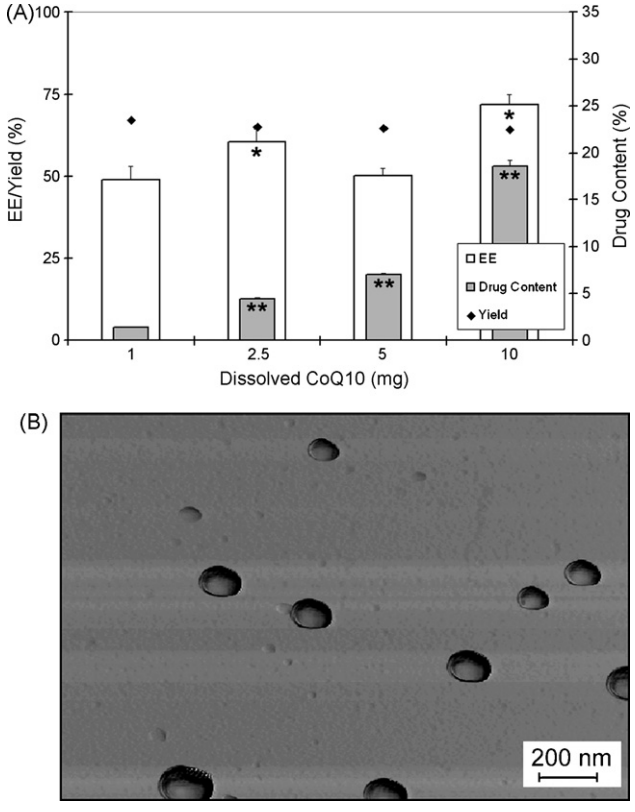


Fig. 3. CoQ10 was successfully incorporated within surfactant-free PLGA nanoparticles by nanoprecipitation. (A) Total nanoparticle yield did not change from 65%, CoQ10 EE generally increased and the drug content significantly increased with every addition of CoQ10 in acetonitrile. ($n = 3$, $^*p < 0.05$, significantly greater than EE at 1 mg, $^{**}p < 0.05$, significantly greater than lesser dissolved CoQ10). (B) Representative phase AFM image showed that surfactant-free CoQ10-loaded PLGA nanoparticles maintained the spherical, non-aggregated, sub-200 nm diameter characteristics of empty nanoparticles.

3.3. Coenzyme Q10 release from nanoparticles

In order to function as drug delivery devices, the CoQ10 payload must be released from the nanoparticles over time. Nanoparticle mass and CoQ10 release into PBS (quantified as the unencapsulated CoQ10) at 37 °C was measured for 2 months (Fig. 4). There was a prolonged release of CoQ10, lasting about 2 weeks, followed by a plateau with little to no release for six more weeks. At the same time, the steady decrease in nanoparticle mass indicated a slow degradation of the nanoparticles. Total CoQ10 release after 2 weeks was approximately 950 μg, which was equivalent to about 45% of the encapsulated CoQ10. Interestingly, about 55% of encapsulated CoQ10 remained within the nanoparticles for at least 2 months, even as the nanoparticles apparently degraded. Thus, if CoQ10-loaded nanoparticle degradation is accelerated by cells or other biological mechanisms, CoQ10 may be released for many weeks in vivo. Common drug release profiles from nanoparticles (with surfactants present) exhibited a short and considerable 'burst' lasting 1–2 days followed by slower, but nearly 100% drug release (Fonseca et al., 2002; Hans and Lowman, 2002; Budhian et al., 2005). Our CoQ10-loaded nanoparticles exhibited a similar profile, with two important differences. The 'burst' was substantially longer

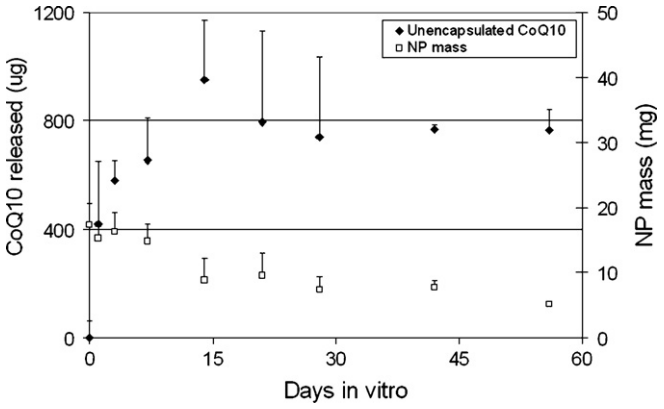


Fig. 4. Total CoQ10 release into PBS and CoQ10-loaded nanoparticle mass was followed for 2 months. CoQ10 release steadily increased for 2 weeks to a total of 950 μg (about 45% of total encapsulated CoQ10) and considerable CoQ10 remained encapsulated for 2 months. CoQ10-loaded nanoparticle mass slowly decreased over 2 months ($n = 4$, up to 1 month, $n = 2$ afterwards).

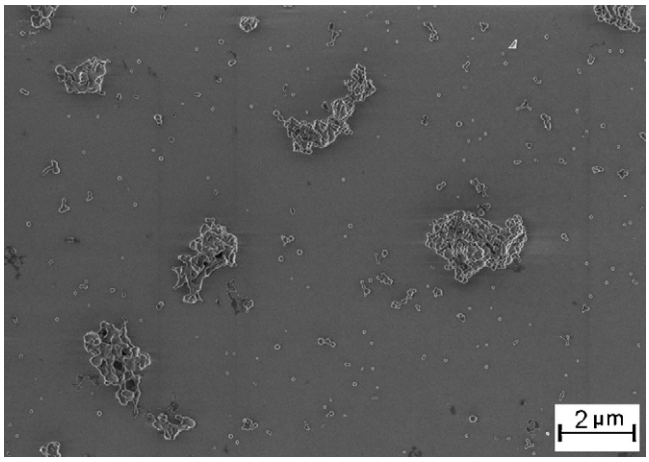


Fig. 5. Representative SEM image (6000× magnification) showed large surfactant-free PLGA nanoparticle aggregates of various sizes and morphologies. This remarkable aggregation motivated the development of our novel purification protocol for drug-loaded, surfactant-free nanoparticles.

(about 2 weeks), indicating a more sustained drug release characteristic of these nanoparticles. In terms of sustained nanoparticle drug delivery applications, weeks-long drug release is preferable to hours-long drug release. Second, CoQ10 remained encapsulated for up to 2 months. Very little CoQ10 would partition near the nanoemulsion surface during nanoprecipitation due to hydrophobic interactions with PLGA, so the typical short and substantial 'burst' was not observed. Additionally, as the PLGA chains hydrolytically degraded, the hydrophobic interactions between the polymer and CoQ10 remained robust, thus preventing 100% drug release. This slow 'burst' and possibility of biologically mediated CoQ10 release are exciting indicators for sustained antioxidant drug delivery.

3.4. Nanoparticle purification

Unencapsulated drug molecules are typically separated from drug-loaded nanoparticles after centrifugation by simply aspirating free drug within the supernatant. This is facilitated by the presence of surfactants in the aqueous phase during nanoparticle synthesis and their subsequent irreversible incorporation at nanoparticle surfaces. However, centrifugation can cause nanoparticles to agglomerate into unacceptably large clusters (Bilati et al., 2005). In this study, even though individual particles were obtained after the initial synthesis, surfactant-free nanoparticles could not be resuspended into individual nanoparticles after centrifugation at 17,200 × g; larger aggregates were plentiful (Fig. 5). This phenomenon was probably due to the lack of hydrophilic surfactants acting as steric barriers. To our knowledge, there are no reports of nondestructive separation of drug-loaded, surfactant-free nanoparticles from unencapsulated drugs after nanoparticle synthesis. However, purification is critical for cellular drug delivery studies because potential therapeutic effects must be attributed to drug-loaded nanoparticles and not unencapsulated drug molecules. Therefore, a novel protocol was developed to nondestructively purify the CoQ10-loaded, surfactant-free nanoparticles from unencapsulated CoQ10 without altering their unique properties. First, SDS was passively adsorbed to nanoparticle surfaces after nanoprecipitation but before centrifugation. It was hypothesized that SDS would decrease the ZP of the nanoparticle surfaces and introduce a physical barrier layer, which would stabilize the nanoparticles and prevent aggregation during centrifugation. Because unencapsulated CoQ10 does not pellet during centrifugation, it would be aspirated with the supernatant before resuspending the nanoparticle pellet into individual CoQ10-loaded nanoparticles. Next, SDS would be desorbed from the nanoparticles during dialysis because it was not physically integrated with the particles during synthesis. The remaining suspension would be purified of unencapsulated CoQ10 but the CoQ10-loaded nanoparticles would retain their original, surfactant-free surface characteristics. A major advantage of surfactants (facile resuspension of nanoparticle pellets) was exploited but none of the surfactant disadvantages were realized.

To demonstrate the nondestructive nature of this protocol, the physical and drug encapsulation properties of purified CoQ10-loaded nanoparticles were examined. There was no significant difference in the mean diameter (194 nm) or PDI (0.10) between purified and unpurified CoQ10-loaded nanoparticles (Fig. 6A). Also, the purified CoQ10-loaded nanoparticles were not aggregated (Fig. 6B). As hypothesized, the anionic amphiphilic nature of SDS prevented nanoparticle agglomeration during centrifugation. Next, the yield, EE and drug content of purified CoQ10-loaded nanoparticles were compared to unpurified, initial CoQ10-loaded nanoparticles. As expected, the total nanoparticle yield significantly decreased from 66% to 46% because of nanoparticle loss during the several purification steps (Fig. 6C). The CoQ10 EE decreased, but not significantly, by purification. However, the drug content was significantly higher in purified (9%) than in unpurified nanoparticles (7%), and nanoparticle loss during purification (decrease in yield) was manifest in this unexpected increase in drug content. In detail, empty nanoparticles without encapsulated CoQ10 likely existed in the suspension after nanoprecipitation since CoQ10 encapsulation was not 100% efficient. Therefore, nanoparticle loss

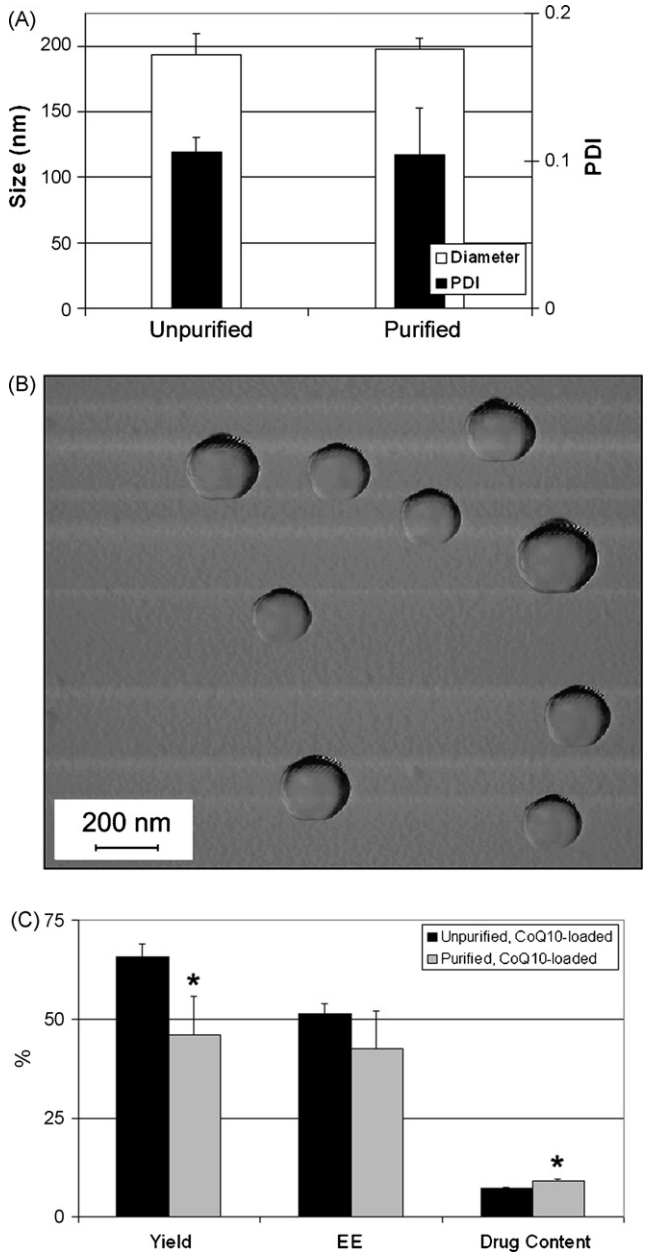


Fig. 6. Purification by transient SDS adsorption, centrifugation and dialysis did not alter the physical or drug encapsulation characteristics of surfactant-free CoQ10-loaded nanoparticles. (A) DLS data showed no difference in mean particle diameter or PDI between unpurified and purified CoQ10-loaded nanoparticles ($n = 5$, mean ± S.D.). (B) Representative phase AFM image confirmed the nondestructive nature of our purification protocol since purified CoQ10-loaded nanoparticles displayed non-aggregated nanoparticles with diameters <200 nm. (C) A significant decrease in total nanoparticle yield, increase in drug content and no change in CoQ10 EE was detected when CoQ10 encapsulation properties of purified and unpurified CoQ10-loaded nanoparticles were compared ($n = 3$, $^*p < 0.05$).

due to purification (measured by the yield) included the mass of both empty and CoQ10-loaded nanoparticles. With respect to the drug content equation, the loss of both empty and CoQ10-loaded nanoparticles decreased the denominator proportionally more than the loss of encapsulated CoQ10 mass decreased the numerator. Therefore, higher drug content was calculated even though the yield and EE decreased. The drug content of individual CoQ10-loaded nanoparticles did not literally increase with purification; instead this increase was an arithmetic artifact due to the loss of empty nanoparticles. Importantly, the purification protocol did not alter the size, morphology, polydispersity or drug encapsulation of CoQ10-loaded nanoparticles. Therefore, the physical and drug encapsulation properties of surfactant-free nanoparticles may be optimized quickly before initiating this purification protocol without concern over altering the characteristics of drug-loaded nanoparticles.

It was critical to ensure SDS removal from the nanoparticle surface since residual SDS could affect CoQ10 release and cellular interactions, like any surfactant. After dialysis, the nanoparticles were concentrated and analyzed for residual SDS by the sensitive methylene blue active substances assay. SDS comprised only 0.45 ± 0.25% (w/w) of the purified nanoparticles after dialysis (Fig. 2A). Other groups who have synthesized nanoparticles with surfactants in the aqueous nonsolvent have reported residual surfactant levels between 2% and 13% (w/w) (Murakami et al., 1999; Galindo-Rodriguez et al., 2004; Zeisser-Labouebe et al., 2006). Therefore, the current method resulted in nanoparticles with at least an order of magnitude less residual "surfactant". Next, XPS was utilized to measure the atomic chemical composition of different nanoparticles. The silicon and nitrogen atomic compositions decreased after nanoparticle adsorption to silanized silicon because the nanoparticles masked surface silane groups (Fig. 2B). Sulfur atoms were detected (1.97%) in CoQ10-loaded nanoparticles before dialysis because SDS was passively adsorbed on their surfaces. Notably, no sulfur, and thus no SDS, was detected on purified CoQ10-loaded nanoparticles after dialysis. Thus, XPS data demonstrated complete SDS removal by dialysis and confirmed the methylene blue assay conclusions. It was also important to characterize a practical physicochemical property of the purified nanoparticles, so the surface charge of purified CoQ10-loaded nanoparticles was analyzed via ZP measurements. Anionic SDS was evident in CoQ10-loaded nanoparticles with passively adsorbed SDS before dialysis since their ZP was −63.0 mV (Fig. 2C). After dialysis, the surface charge of purified nanoparticles, −42.5 mV, was not significantly different from unpurified CoQ10-loaded nanoparticles without SDS, −40.4 mV, which further suggested efficient SDS removal. These quantitative and practical observations clearly demonstrated SDS removal from nanoparticle surfaces after dialysis and preservation of the surfactant-free nature of our purified CoQ10-loaded nanoparticles. Therefore, these purified, CoQ10-loaded nanoparticles should behave like unmodified nanoparticles in our future work. Considering the acceptance of irreversible surfactant incorporation at nanoparticle surfaces (Sahoo et al., 2002; Galindo-Rodriguez et al., 2004), the current demonstration of transient surfactant adsorption followed by complete removal represents a significant breakthrough.

4. Conclusions

Nanoprecipitation was a robust and versatile protocol for synthesizing CoQ10-loaded nanoparticles without the inter-

ference of surfactants. Control over the physicochemical and drug encapsulation properties was achieved simply by varying the nanoprecipitation formulation parameters, which may motivate other researchers to customize drug-loaded nanoparticles by nanoprecipitation. The extremely hydrophobic nature of CoQ10 facilitated steady CoQ10 release for 2 weeks along with long-term encapsulation of CoQ10 for 2 months. In a novel demonstration, passively adsorbed SDS protected CoQ10-loaded nanoparticles from aggregation during centrifugation and allowed the separation of unencapsulated CoQ10. It was shown that SDS was completely desorbed from the nanoparticle surfaces during dialysis, and thus the surfactant-free nature and advantages of the original CoQ10-loaded nanoparticles were maintained. These purified, surfactant-free CoQ10-loaded nanoparticles are exciting sustained drug delivery platforms for antioxidant therapy.

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