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Oral bioavailability, therapeutic efficacy and reactive oxygen species scavenging properties of coenzyme Q10-loaded polymeric nanoparticles

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ABSTRACT

The present investigation consists in the development and characterization of CoQ10 loaded PLGA nanoparticles (CoQ10-NPs, size < 100 nm) by a scalable emulsion-diffusion-evaporation method. Thermal and crystallinity analysis collectively corroborated that CoQ10 was entrapped into the NPs in amorphous form. The lyophilized CoQ10-NPs were found to be stable for a period of 6 months (at room temperature). *In vitro* cell culture studies indicated that CoQ10-NPs significantly quenched ROS with nearly 10 fold higher efficacy than free CoQ10. Further, positively charged CoQ10-NPs were localized in two major sources of ROS generation: mitochondria and lysosomes. CoQ10-NPs showed improved oral bioavailability (4.28 times) as compared to free CoQ10. Finally remarkably higher hepatoprotective and anti-inflammatory activity of CoQ10-NPs as compared to free CoQ10 was observed due to mitigation of deleterious effects associated with the generation of free radicals. As elucidated by live noninvasive animal imaging, the higher anti-inflammatory activity of CoQ10-NPs can be attributed to significant accumulation of these NPs in the inflamed tissues.

1. Introduction

Coenzyme Q10 (CoQ10) is a fat soluble vitamin analog virtually found in every cell including tissue of the heart, liver and skeletal muscles. It functions as an obligatory co-factor (electron carrier) for the suitable transfer of electrons within the mitochondrial oxidative respiratory chain and serves as a modulator of the transition pore [1]. CoQ10 is a well known antioxidant, which acts like a coenzyme in different energy-producing metabolic pathways of cells, scavenges free radicals and inhibits lipid peroxidation [2]. However, CoQ10 level in blood and tissues fall with normal aging [3]. The deficiency of CoQ10 is often implicated by oxidative stress in several disease forms. Cellular mitochondrial functions are impaired by lacking level of CoQ10 in cells, thus producing the different oxidative stresses which are mediated by generation of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), resulting into a deathly vicious oxidative cycle by destabilizing the lysosomal membrane [4]. The exogenous supplementation of CoQ10 is often required to maintain the level of ROS in normal cells [5]. Hence, CoQ10 has been approved by the US Food and Drug Administration (FDA) for treatment of mitochondrial disease and currently marketed products are syrup (LiQ10®) and softgel formulation (Q-Gel® and Q-Nol®). CoQ10 has also shown its potential in promoting cardiovascular health, combating aging, supporting healthy blood glucose levels and improving neurodegenerative diseases [5–7]. Based on all these findings, various biomedical indications are now under clinical trials [8,9].

The systemic availability of CoQ10 by oral route is low due to its high lipophilicity, large molecular weight (863 Da) and thermolability [10]. The literature is flooded with the various reports regarding the modification of physiochemical properties to improve its oral bioavailability by various formulation strategies like a solubilized system with soya lecithin [11], a micellar solution of CoQ10 with polyoxyethylene hydrogenated castor oil [12], a redispersible dry emulsion [13], the complexation of CoQ10 with cyclodextrins [14], nanosupension formulation [15], a solid dispersion formulation [16] and self emulsified drug delivery system and polymeric nanoformulation of CoQ10 [17,18]. Most of the studies concerning these formulations are limited to *in vitro* evaluation of their efficacy. Also *in vivo* of remaining reports, to date, show very low bioavailability. Even this bioavailability problem becomes worse due to limited tissue uptake in animal model at doses ranging from 10 to 123 mg/kg/day [19–21]. Several human studies reported that the supplementation of CoQ10 at very high dose (300–3000 mg/day) is required for several weeks to months to see any significant pharmacological or therapeutic effect

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[19,20] because of limited ability of CoQ10 concentration near to mitochondria. Thus, the amounts needed to afford protection from ROS were very high [22,23]. Overall, the uptake, distribution and ultimately the therapeutic efficacy of CoQ10 from the formulation in the treatment model still remain unanswered.

Polymeric nanoparticles (NPs) have been extensively used for improving the therapeutic efficacy of bio-actives through parenteral and non-parenteral route. In present study oral route has been preferred because of its noninvasive nature and consequently, better patient compliance. Among various polymers, poly (lactic-co-glycolic acid) (PLGA) is a US-FDA approved, biocompatible, biodegradable and safely administered polymer [24]. PLGA NPs have been widely used for the loading of a variety of drugs ranging from hydrophilic drugs [25–27] to hydrophobic ones [28]. Upon oral administration, drug loaded NPs are absorbed predominantly via M-cells in the Peyer's patches (PP) and the isolated follicles of the gut-associated lymphoid tissue (GALT) resulting in enhanced bioavailability and protect the drug from enzymatic degradation and first pass metabolism. Overall, NPs decrease the dose (bio-burden of drug) and ultimately reduce the drug associated toxicity [29].

In the present work, we have prepared and optimized laboratory scalable, freeze dried PLGA NPs of CoQ10 (CoQ10-NPs). CoQ10-NPs were evaluated for crystallinity of drug, stability in simulated gastric intestinal (GI) fluids and accelerated storage stability. Cellular uptake, sub cellular localization and antioxidant activity of CoQ10-NPs was also examined. Finally, oral pharmacokinetic and pharmacodynamic studies i.e. hepatoprotective efficacy and anti-inflammatory activity in tissue compartment were conducted in suitable animal model.

2. Materials and methods

2.1. Materials

PLGA 50/50 (inherent viscosity 0.41 dl/g in chloroform at 25 °C) was used from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (PVA) (MW. 30000-70000), CoQ10, Acetaminophen (APAP), Pluronic F 68 (PF-68), Coumarin-6, Didodecyldimethylammonium bromide (DMAB), 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), Triton X-100, rhodamine 123 (Rh123) and propidium iodide (PI), lipopolysaccharide (LPS), Hexadecyltrimethyl ammonium bromide (HTAB) were obtained from Sigma, USA. Dulbecco's modified Eagle's medium, fetal bovine serum and Hanks's balanced salt solution (HBSS) were purchased from PAA Laboratories, Austria. Tissue culture plates were purchased from Tarsons, India. 8-well culture slides were procured from BD Falcon, USA. Alexa Fluor 647 (AF647) was purchased from Invitrogen, USA. Ethyl acetate, Acetonitrile (HPLC grade), and methanol (HPLC grade) were purchased from Rankem Fine Chemicals (New Delhi, India). Neutral red (NR), Thiobarbituric acid (GR grade), and acetic acid (LR grade) were purchased from Loba chemie, India. Ultra pure water (SG water purification system, Barsbuttel, Germany) was used for all the experiments. All other reagents used were of analytical grade.

2.2. Method of preparation

NPs were prepared by emulsion-diffusion-evaporation technique with slight modification from the reported method [29]. In brief, 50 mg of PLGA (50:50) and 5 mg CoQ10 were dissolved in 2.5 ml of ethyl acetate at room temperature and stirred for 30 min. The organic phase was added drop wise (1.0 ml/min) into 5 ml of an aqueous phase containing stabilizer. The droplet size reduction of resulting emulsion was carried out by homogenization (high-speed homogenizer, Polytron PT 4000, Switzerland) at 10,000 rpm for 15 min. The resulting emulsion was poured into 25 ml of water with constant stirring which resulted in nanoprecipitation and formation of NPs. The NPs suspension was collected in the form of pellet by centrifugation at different speeds (i.e. 15,000 g for 5 min, 25,000 g and 40,000 g for 15 min) and re-suspended in water. This procedure was repeated three times to remove the excess surfactant and then subjected to freeze drying (FD) as mention in subsequent section.

2.2.1. Optimization of process variables

2.2.1.1. Screening of suitable stabilizer and its concentration. CoQ-NPs were prepared by using different type and concentration of stabilizers like DMAB, PVA and PF-68. The best suitable stabilizer was identified based on the particle size, PDI and entrapment efficiency.

2.2.1.2. Droplet size reduction process. The homogenization was used to reduce the droplet size of emulsion and to get the optimum size (below 100 nm). For this, NPs were prepared following the above described process keeping other experimental parameters like aqueous to organic phase ratio 1:2 v/v and final volume of dilution 25 ml. In this method, stabilizer concentration (1% w/v DMAB) was used during the o/w emulsion preparation. Different homogenization speeds were employed for preparation of NPs and optimum speed was selected based on entrapment efficacy.

2.2.1.3. Optimization of drug loading. CoQ10-NPs were prepared using different CoQ10 loading i.e. 5%, 10%, 15%, 20% and 25% w/w of PLGA polymer and its effect on particle size and entrapment efficiency was studied. The other experimental parameters like homogenization (at 15000 rpm for 15 min), stabilizer concentration (1% w/v DMAB) and aqueous to organic phase ratio 1:2 v/v were kept constant.

Same optimized parameters have been utilized to prepare the Alexa 647 and CoQ10 co encapsulated NPs. Similarly, both coumarin-6 and CoQ10 were also loaded in the NPs. A 0.08% w/w Alexa 647 and 0.2% w/w coumarin-6 with respect to polymer was prepared for the cell culture experiments. These loading showed less than 1% of dye released from the NPs in 24 h.

2.3. Characterization of NPs

2.3.1. Particles size and zeta potential measurement

CoQ10-NPs were evaluated for their mean particle size and PDI by using Zeta Sizer (Nano ZS, Malvern Instruments, UK). All the values were taken by the average of 6 measurements. Zeta potential was estimated on the basis of electrophoretic mobility under an electric field, as an average of 20 measurements. Zeta potential was also determined by using Zeta Sizer (Nano ZS, Malvern Instruments, Malvern, UK).

2.3.2. Entrapment efficiency

The percentage of CoQ10 in NPs was determined by using a validated high-performance liquid chromatography (HPLC) method [30] with slight modifications. Briefly, freeze dried NPs were dissolved in minimum amount of acetonitrile and further diluted with absolute ethanol and analyzed by Shimadzu HPLC system consisting of 996 Photodiode Array Detector and dVR Agilent Technologies. Separation was achieved using a reversed phase C18 Nova Pack (15 cm × 4.6 mm, 5 μm. Waters, USA) column fitted with Nucleosil, C18 (Macherey–Nagel, Germany) guard column. Ethanol: methanol (90:10% v/v) were used as the mobile phase at a flow rate of 1 ml/min with UV detection at 275 nm.

2.3.3. Morphology of NPs

The surface morphology of NPs was analyzed by atomic force microscope (Veeco Bioscope II, USA). A small drop of the NPs suspension was placed on the silicon wafer and allowed to dry in air. The microscope was vibration damped and measurements were made using commercial pyramidal Si_3N_4 tips (Veeco's CA, USA). The cantilever used for scanning was having length 325 μm and width 26 μm with a nominal force constant 0.1 N/m. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded.

2.3.4. Freeze drying of NPs

CoQ10-NPs were freeze dried using Vir Tis, Wizard 2.0, New York, USA freeze dryer. The optimized batch CoQ10-NPs were freeze dried with 5% w/v concentration of different type lyoprotectants (sucrose, dextrose, trehalose and mannitol). Using a previously an optimized freeze drying cycle [29,31]. After freeze drying the CoQ10-NPs were characterized for the appearance of the cake, reconstitution time, particle size, entrapment efficiency after freeze drying.

2.3.5. DSC analysis

DSC thermogram of the pure CoQ10, PLGA, freeze dried CoQ10-NPs, physical mixture of drug free freeze dried NPs and pure CoQ10 (PMN) and trehalose was carried out using a Mettler Toledo differential scanning calorimeter calibrated with indium standards. Measurements were carried out at heating rate of 10 °C/min from 0 to 200 °C.

2.3.6. XRD analysis

The XRD patterns of pure CoQ10, PLGA, freeze dried CoQ10-NPs, physical mixture free freeze dried NPs and pure CoQ10 (PMN) and trehalose were obtained using the X-ray diffractometer (Bruker D8 advance, Bruker, Germany). Measurements were carried out at a voltage of 40 kV and 25 mA. The scanned angle was set from $3^\circ \leq 2\theta \geq 40^\circ$, and the scanned rate was 2° min^{-1}.

2.3.7. pH dependent stability of freeze dried CoQ10-NPs

Freeze dried CoQ10-NPs were evaluated for their stability in various simulated GIT fluids pH 1.2, pH 4.5, pH 6.8, simulated gastric fluids (SGF), and SIF to assess the stability of NPs under various GIT pH and enzymatic conditions that could influence their particle size. Briefly, 10 ml of simulated fluids were added to 2 ml of reconstituted freeze dried CoQ10-NPs (which contain 1.5 mg/ml CoQ10). An incubation time of 2 h was employed for pH 1.2, pH 4.5 and SGF while 6 h for pH 6.8 [29,32]. Particle size, PDI and entrapment efficiency were determined after the incubation of freeze dried CoQ10-NPs with different simulated fluids.