International Journal of Pharmaceutics 372 (2009) 66–75

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Topical use of Coenzyme Q_{10}-loaded liposomes coated with trimethyl chitosan: Tolerance, precorneal retention and anti-cataract effect

Jing Zhang, Siling Wang*

Department of Pharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 5 November 2008
Received in revised form 4 January 2009
Accepted 6 January 2009
Available online 14 January 2009

Keywords:
Coenzyme Q_{10}
Trimethyl chitosan
Liposomes
Ocular tolerance
Precorneal retention time
Anti-cataract effect

ABSTRACT

The effects of the molecular weight (M_W) and concentration of trimethyl chitosan (TMC) on the characteristics of Coenzyme Q_{10}-loaded liposomes coated with trimethyl chitosan were investigated while the efficacy of the antioxidant Coenzyme Q_{10} in delaying selenite-induced cataract was assessed. The existence of a thick polymer layer on the surface of the liposomes nanoparticle system was affirmed by transmission electron microscopy (TEM) analysis and the changes in particle size distribution and zeta potential. The entrapment efficiency was almost the same as when the polymer was added to liposomes. The Draize test and histological analysis demonstrated the excellent ocular tolerance of TMC for topical administration. Gamma scintigraphic data clearly showed that the drug elimination of polymer-coated liposomes is significantly slower than the radiolabelled solution used as a control. An almost 4.8-fold increase in the precorneal residence time was achieved in the presence of TMC with a higher Mw compared with the control group. Furthermore, the anti-cataract effect was evaluated by morphological examination and analysis of biochemical changes. Coenzyme Q_{10} exhibited a markedly anti-cataract effect with the percentage of lens opacity being about 53% at the final examination. The mean activities of superoxide dismutase and reduced glutathione were significantly higher in the Coenzyme Q_{10}-treated group than in the cataract model group, while malondialdehyde was significantly lower. In conclusion, the physical properties and precorneal retention time of liposomes could be modified with TMC and ophthalmic instillation of Coenzyme Q_{10} is able to retard selenite-induced cataract formation.

1. Introduction

Cataract is a progressive opacity of the lens of the eye that impairs vision and becomes the leading cause of blindness around the world. Nowadays, although surgical removal of the natural lens and replacement with a lens made of synthetic polymers is the main treatment for cataract, the incidence of cataracts is so high globally that surgery alone has been found unable to cope with the problem. Moreover, it remains a constant public health problem because of the shortage of surgical facilities, an increasingly older population and the incidence of non-correctable postsurgical complications. Therefore, development of pharmacological means for cataract prevention is highly desirable (Varma and Hegde, 2004).

There is growing evidence that oxidative stress, mediated by active oxygen species (ROS) in the lens and lipid peroxides, produced in the crystalline lens, are responsible for a breakdown of lens homeostasis leading to lens opacification (Yu et al., 2007). This has been supported by the protective effect of physiologic antioxidants, like pyruvate (Devamanoharan et al., 1999), and nutritional antioxidants, such as ascorbate, vitamin E and carotenoids (Gerster, 1989), in the treatment of experimental cataract. High concentrations of selenium have been reported to produce nuclear cataract when injected subcutaneously to suckling rats (Oštádalová et al., 1978), which attributed to the shifting of the oxidative status of lens to the prooxidant side in the developing lens. Although selenite cataract shows no high molecular weight covalent aggregates or increased disulfide formation compared with human cataract, there are still many similarities between them such as vesicle formation, increased insoluble protein and decreased GSH (Shearer et al., 1997). In addition, this model is also extremely rapid and convenient, which make it a useful *in vivo* rodent model for initial drug testing.

During the past two decades, great attention has been paid to the possible roles of physiologic and nutritional antioxidants in cataract, since the disease is a slowly progressing one and patients need to be on life-long treatment. As many of drugs currently used possess one or another drawback such as adverse effects, a short half-life and poor bioavailability, it would be very helpful to find an antioxidant that could be consumed daily as a form of nutrition. Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble vitamin-like substance present in many organisms and it consists of a redox active quinoid

* Corresponding author. Tel.: +86 24 23986348; fax: +86 24 23986348.
E-mail address: silingwang@hotmail.com (S. Wang).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.01.001

moiety and 10 isoprenoid units in the hydrophobic tail. CoQ_{10} functions as a well-known electron carrier in the electron transport chain, thus contributing to energy conservation, and the reduced form of CoQ_{10} (ubiquinol) is capable of scavenging free radical oxygen intermediates (Frei et al., 1990). It efficiently protects membrane phospholipids from peroxidation and also mitochondrial DNA and membrane proteins from free-radical-induced oxidative damage. CoQ_{10} participates in the transfer of electrons from flavoprotein dehydrogenase (NADH CoQ reductase, complex I) and succinate CoQ reductase (Complex II) to $CoQH_2$-cytochrome *c* oxidoreductase (complex III) which is an obligatory process used in protecting the membranes (Echtay et al., 2001; Ishii et al., 2004). The protective effect of CoQ_{10} is independent of exogenous antioxidants, for example vitamin E, and it can both prevent the formation of free lipid radicals and eliminate them either directly or by regenerating vitamin E (Groneberg et al., 2005; Pobezhimova and Voinikov, 2000). Its excellent ability to scavenge free radicals makes CoQ_{10} attractive as a potential anti-cataract agent for the first time. However, instability to light and extreme lipophilicity of CoQ_{10} are hampering its bioavailability as a therapeutic agent. Therefore, a potential ophthalmic technology capable of delivering the drug efficiently is being investigated here for the first time.

Corneal and conjunctival epithelia, along with the tear turnover, serve as biological barriers for the entrance of extraneous substance to eyes. For topical ocular administration, the use of colloidal drug delivery systems, such as liposomes, is a suitable strategy to obtain enhanced bioavailability in comparison with liquid formulations (Monem et al., 2000). Liposomes are preferable because they exhibit unique features by offering easy delivery and no interference with vision and stabilizing the drug as excellent reservoirs for drug loading. However, liposomes are generally rather unstable and tend to degrade or aggregate and fuse, which leads to leakage of entrapped drug during storage or after administration. To minimize these disruptive influences, many attempts have been made, including surface modification of liposomes which is an attractive method to improve liposomal stability both *in vitro* and *in vivo*. The formation of a bioadhesive and polymeric membrane around the liposomes has been studied (Iwanaga et al., 1999).

Chitosan has been used as a pharmaceutical excipient for drug delivery because of its favorable biological properties, for example, it is biodegradable and exhibits low toxicity. Moreover, because of the high affinity of chitosan to cell membranes, several studies have examined chitosan derivatives as coating material for liposomes (Janes et al., 2001). However, a major drawback of the polymer that needs to be taken into account is that it is insoluble at physiological pH. This inconvenience can be circumvented by synthesizing the partially quaternized derivative, N-trimethyl chitosan chloride (TMC), which is soluble over a wide pH range.

In the case of TMC-coated liposomes, the structured adsorbed polymer film stabilizes the particles against interaction between particles, presumably through a mechanism of steric stabilization. Moreover, cationic liposomes obtained by coating the liposomes with positively charged TMC are assumed to reduce rapid precorneal drug loss and improve poor corneal permeability by exploiting the negative charges present at the corneal surface (Rabinovich-Guilatt et al., 2004). In addition, TMC exhibits excellent absorption enhancing effect even at neutral pH values (Thanou et al., 2000) by opening the tight junctions between adjacent cells of epithelial cell monolayers through exploiting the negatively charged sites on the cell membrane through ionic interaction (Zambito et al., 2006). It has been suggested that TMC with a medium to high degree of quaternization is capable of significantly enhancing the permeability of ofloxacin across a stratified epithelium, for example the cornea (Di Colo et al., 2004). Therefore, we chose the polymer with different molecular weight (M_W) but the same high degree of quaternization, about 65%, as a component of the preparation. Taking the above information into account, we hoped that TMC might exhibit an absorption enhancing effect and prolonged precorneal retention time during the ophthalmic application of Coenzyme Q_{10}-encapsulated liposomes.

To our knowledge, there are few reports on the use of TMC in ophthalmology and the application of such formulations should take into account the tolerability of the polymer. So, the first aim of the present study was to assess the potential ocular irritation of the polymer with different M_W or concentrations. Secondly, the physical properties of Coenzyme Q_{10}-loaded liposomes were evaluated with the precorneal residence time of the formulations being investigated by gamma scintigraphy. A further attempt was made to determine the anti-cataract effect of Coenzyme Q_{10} in an experimental *in vivo* setting.

2. Materials and methods

2.1. Materials

Coenzyme Q_{10} (CoQ_{10}) was purchased from Taizhou Kaichuang Chemical Co., LTD., and all the preparations were protected from light since CoQ_{10} is not stable when exposed to light. Soy phosphatidylcholine (SPC) was from Shanghai Taiwei Pharmaceutical Industry Co., LTD. Cholesterol (CH) was purchased from Taizhou Hisound Chemical Co., LTD., and technetium-99m-diethylentriaminepentaacetic acid (^{99m}Tc-DTPA) was prepared in the Department of Nuclear Medicine at the General Hospital of Shenyang Military Command (Shenyang, China).

Two chitosan with similar degree of deacetylation (≥85%) were purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. (Jinan, China) and subjected to further purification: CHI-1 (molecular weight of 100 kDa) and CHI-2 (molecular weight of 450 kDa). TMC601 and TMC602 (TMC prepared from CHI-1 and CHI-2 with a 65% degree of quaternization) were prepared in our own laboratory. N-Methylpyrrolidinone was obtained from Shanghai Chineway Pharmaceutical Tech. Co., Ltd., and methyl iodide was from Shanghai Jinjinle Industry Co., Ltd. Sodium iodide was obtained from Tianjin Kebodi and sodium selenite was purchased from Peking Chemical Works. All other chemicals and solvents were of at least analytical grade.

Eleven-day-old Sprague-Dawley rat pups (20–25 g, body weight) and rabbits (3–5 kg, body weight) were obtained from the animal center of Shenyang Pharmaceutical University and given a commercial diet and water ad libitum and the rat pups were housed with their mother. The animal room was well ventilated and had a regular 12:12-h light/dark cycle throughout the experimental period. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Synthesis and characterization of TMC

TMCs with different degrees of substitution were synthesized by two reductive methylation steps as previously described (Polnok et al., 2004). Commercial chitosan was purified as described (Dung et al., 1994). Briefly, sieved chitosan (60 meshes) was mixed with methyl iodide in a basic solution of N-methylpyrrolidinone at 60 °C. The product was washed with ethanol and ether and the polymeric material underwent a second methylation to yield the final products TMC iodide with a 65% degree of substitution after 90 min. The polymer salts were converted into the chloride form by dialysis against sodium chloride solution and finally dehydrated under vacuum at 40 °C for 12 h.

TMC601 and TMC602 were characterized by ^{1}H-NMR spectroscopy (ARX-600, Bruker, German). The degree of quaternization (DQ) was calculated using the following equation:

$$DQ(\%) = \left[\left(\frac{\int CH_3}{\int H}\right) \times \frac{1}{9}\right] \times 100$$

where $\int CH_3$ is the integral of the chemical shift of the trimethyl amino group at 3.3 ppm and $\int H$ is the integral of the ^{1}H peaks from 4.7 to 5.7 ppm.

2.2.1. Tolerance evaluation

Solutions for tolerance purposes were prepared with increasing concentrations (0.1, 0.2, 0.5 and 1.0% w/v) by dissolving the polymer in isocryoscopic sterile phosphate buffer solution (pH 6.8). All the solutions were adjusted to the final pH using a digital pH meter (PB-10, Sartorius Science Instrument Ltd., Beijing, China). The rheological properties of the solutions were determined by a rheometer (DV-II +Pro. Rheometer, Brookfield, USA). Each measurement was made in triplicate.

Each formulation was tested on six rabbits and the treatment was performed according to the Draize technique (Draize et al., 1944). A single instillation without spillage (0.01 ml) of the solution under test was performed into the lower conjunctival sac of one eye five times a day for one week. Any signs of irritation (redness, inflammation, chemosis or increased tear production) were monitored periodically after instillation. In order to examine the effect on the structure and integrity of the cornea, iris and conjunctivae, the eyes of the rabbits from each group were opened at the cornea–scleral junction, and the cornea, iris and conjunctivae were removed and fixed in formalin solution 8% (w/w) for 24 h. The samples were then dehydrated with an alcohol gradient, wrapped by melted paraffin and solidified in block form. Cross sections (<1 μm) were cut before being stained, and then examined blind under a microscope for any pathological modifications (Olympus BX60, Japan) in co-operation with a pathologist (n = 3).

2.3. Coating of liposomes

Liposomes from the ethanol injection method (SPC liposomes) were obtained as follows. The basic composition of the lipid fraction was 83% (w/w) SPC and 17% (w/w) CH and α-tocopherol was used as an antioxidant to protect the lipids and CoQ_{10} from oxidation. An appropriate amount of absolute ethanol was added to dissolve the aforementioned liposome ingredients. The solution was injected into phosphate buffer (pH 6.8) at 50 °C and the suspension obtained was stirred for 30 min to facilitate the removal of absolute ethanol, before it was sonicated for 4 min under an atmosphere of nitrogen. The liposome dispersion was stored at 4 °C until use.

TMC601 and TMC602 were dissolved in phosphate buffer (pH 6.8) to give a series of concentrations (0.1, 0.2, 0.5, and 1% (w/v)) and these solutions were added dropwise to equal volumes of liposomes prepared as described before under magnetic stirring, and then incubated at 10 °C for 60 min.

Formulations were prepared in a similar way for gamma scintigraphic studies. A ^{99m}Tc-liposome radiolabelling technique was used based on a method described previously (Meseguer et al., 1993) by mixing 50 ml ^{99m}Tc-DTPA with 450 ml liposomal suspensions and votexing.

2.4. Characterization

2.4.1. Transmission electron microscopy (TEM)

Liposomes were viewed under a JEM-1200 EX electron microscope (JEOL, Japan) by conventional negative staining methods using 0.3% phosphotungstic acid buffer (pH 6.0) as a staining agent.

2.4.2. Particle size distribution (PSD) and zeta potentials

PSD and zeta potentials of the liposomes were determined by dynamic light scattering and electrophoretic light scattering, respectively, both using an ELS 800 apparatus (Otsuka, Japan) to evaluate the effect of different molecular weights (A) and concentrations of TMC (B) on liposomes. The surface electric charge of liposomes was determined at 25 °C. The dispersion medium was Millipore-filtered water (pH 7.0) and ions were eliminated to avoid influence on the surface electric charge of liposomes. The equations (Nicoli et al., 1997) used for converting the electrophoretic mobility to the zeta potential are:

$$\mu = \frac{\nu}{E}$$

$$\zeta = \frac{\eta\mu}{\varepsilon}$$

where μ is the electrophoretic mobility, ν the velocity of particles, E the electric field intensity, ζ the zeta potential, ε the dielectric constant of the solvent and η the viscosity.

2.4.3. TMC coating efficiency

To calculate the coating efficiency of liposomes, the phosphor content was subsequently determined by Stewart's method (Stewart, 1974). The coating efficiency of liposomes by TMC was obtained as the phosphor content within the polymer-coated liposomes versus the total phosphor content of liposomes which was determined by following the same procedure without centrifugation.

2.4.4. Determination of entrapment efficiency

Liposome encapsulation efficiency (EE%) was determined using the ultrafiltration technique for separating the non-entrapped drug from liposomes (Lei et al., 2006). For this, 5 ml drug-loaded liposomal dispersion was placed in a stirred cell (Millipore 8010, Millipore Corporation, Bedford, USA) which was fitted with a filter membrane (molecular weight cut off: 50 000 M_W) under nitrogen. The ultrafiltrate was collected, and the drug content in the ultrafiltrate (c_{free}) was determined by HPLC with an L-7420 UV detector at a wavelength of 275 nm (HITACHI, Japan). Then 2 ml liposomal suspension was diluted with mobile phase to determine the total CoQ_{10} (c_{total}) by HPLC. The entrapment efficiency (EE%) was calculated by:

$$EE\% = \frac{c_{total} - c_{free}}{c_{total}} \times 100$$

2.4.5. Assessment of the precorneal clearance of liposomal dispersions

Precorneal drainage of liposomes was evaluated by gamma scintigraphy after instillation of 25 μl of each formulation, the activity of which ranged from 1 to 2 MBq per 25 μl dose. The precorneal clearance was measured using a gamma camera (Hawkeye VG5 SPECT, General Electric, USA) adjusted to detect radiation of ^{99m}Tc (140 keV) and fitted with a 4 mm pinhole. The rabbit was held stationary and its head was supported by the hand of the experimenter to keep the eye 6 cm from the collimator aperture. Recording was started 5 s after instillation and the dynamic protocol (36 × 5 s frames followed by 15 × 10 s frames and 15 × 20 s frames) was employed over a period of 10.5 min using a 128 × 128 pixel matrix. Each formulation was tested on three rabbits.

In image analysis, five regions of interest (ROIs) are defined: the position reference, the background, the cornea, the inner canthus, and the lacrimal duct (Fig. 1). The parameters are the $t_{1/2}$ (half-life of elimination), $t_{10.5}$ (remaining activity on the corneal surface at the end of 10.5 min) and $AUC^{(0,\ 10.5\,min)}$ (area under the curve

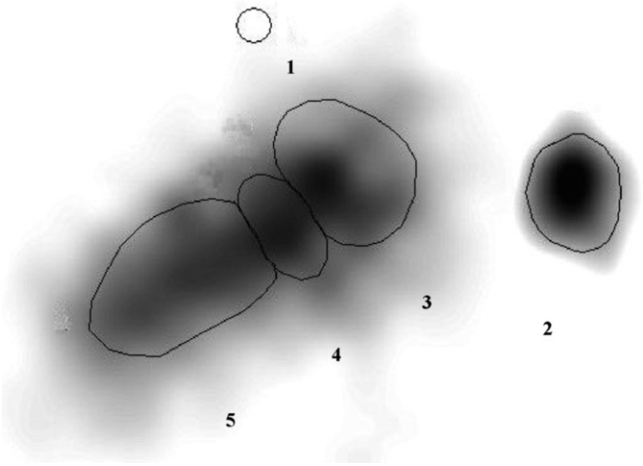

Fig. 1. Typical scintigraphic images divided into five regions of interest (ROIs): (1) The background; (2) the position reference; (3) the cornea; (4) the inner canthus; and (5) the lachrymal duct.

of the percentage activity remaining in the precorneal ROI versus time), which represents the residence time of the formulation tested. ^{99m}Tc-DTPA solution was used as a standard for comparison (Nagarsenker et al., 1999; Felt et al., 1999).

2.5. Animal treatment protocol

The pups were allocated to three groups. The blank control group ($n = 8$) received no injection and was treated with normal saline (5 μl/20 g, body weight). The model control group ($n = 8$) received a subcutaneous sodium selenite injection (19 μmol/kg, body weight) and was administrated with normal saline (5 μl/20 g, body weight). The CoQ_{10}-treated group ($n = 8$) received the same amount of sodium selenite along with ophthalmic administration of TMC-coated CoQ_{10} liposomes (5 μl/20 g, body weight) on postpartum day 10, then all the instillations were repeated three times a day for eight days.

2.5.1. Morphological examination of lenses

The development of cataract was assessed every two days by slit lamp illumination. The pupils were dilated with tropicamide 0.5% and phenylephrine hydrochloride 2.5% before taking slit images with a photo slit lamp microscope and an anterior eye segment analysis system, EAS-1000 (Nidek, Aichi, Japan) equipped with a CCD camera, as described previously (Ito et al., 1999). The observer did not know the identity of the animals beforehand.

The area of lens opacity, expressed as pixels, was analyzed by a computer with image analysis software connected to EAS-1000 and calculated by the following equation:

$$\text{Pixels within opacity} = \text{pixels within outline} - \text{pixels within transparent area}$$

The percentage of lens opacity of each group was determined by the following equation:

$$\text{Percentage of opacity\%} = (\text{Area}_{\text{opacity in the treated group}} / \text{Area}_{\text{opacity in the model group at the 8th day}}) \times 100$$

2.5.2. Superoxide dismutase, reduced glutathione and malondialdehyde analysis

The litters were killed at the end of the study and the lenses were removed intracapsularly using a posterior approach. The lenses from each group (four lenses at a time) were homogenized in 10 times their mass of 10 mM phosphate buffer (pH 6.8) and a clear supernatant was obtained by centrifugation at 12 000 rpm for 15 min at 4 °C (HC-3018R, USTC Chuangxin Co., Ltd. Zonkia Branch).

Superoxide dismutase (SOD) activity was determined by the xanthine oxidase method (hydroxylamine method) with an SOD

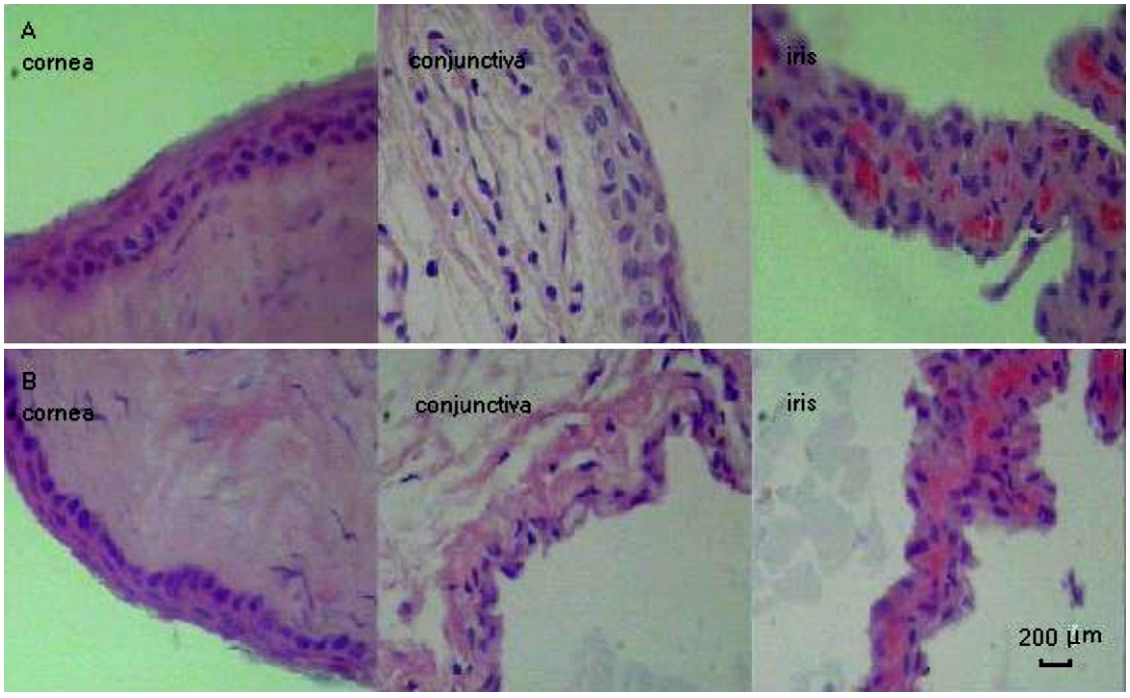

Fig. 2. (A group) Light micrographs of control group ocular tissue treated with PBS (pH 6.8); (B group) light micrographs of the ocular tissue from the 1% TMC 602-treated group.

assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The difference in absorbance was read at 550 nm (UV-2000 spectrophotometer, UNICO, USA) against a blank and the enzyme activity was presented as units/g tissue.

Reduced glutathione (GSH) content was measured by the method previously described (Moron et al., 1979) in which a yellow color was read spectrophotometrically at 412 nm following the addition of 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and expressed as μmol/g tissue.

The level of lipid peroxide was expressed as malondialdehyde (MDA) calculated by thiobarbituric acid chromatometry with an MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and expressed as nmol/g tissue.

2.6. Statistical analysis

The mean value of each parameter in each individual group of rats was calculated from at least four individual values. Statistical analysis was using a one-way ANOVA with SPSS software. *P*-values <0.05 were considered significant.

3. Results and discussion

3.1. Tolerance evaluation

The physical properties of every formulation tested are summarized in Table 1. Depending on the synthesis conditions, such as reaction time, different types of TMC may have a different viscosity which could give information about the molecular weight and molecular weight distribution of the polymer. The pH values and osmolalities were within the physiologically compatible range, from which we can presume that any irritation induced after instillation of the solutions could be mainly ascribed to the polymer itself rather than the physicochemical characteristics of the solutions.

Table 1
The physical properties of TMC solutions.

TMC type	TMC concentration (%)	pH value	Osmolality (mmol/kg)	Viscosity (mPa s)
TMC601	0.1	6.71	264.8	0.62
	0.2	6.67	274.6	1.34
	0.5	6.76	285.4	3.79
	1.0	6.81	316.1	9.48
TMC602	0.1	6.71	261.4	2.70
	0.2	6.74	279.8	7.10
	0.5	6.78	289.5	11.3
	1.0	6.73	323.0	28.9

According to the observations on the rabbits' eyes, excellent ocular tolerance was noted as average scores of irritation for each solution was zero. The results of the ocular irritation studies indicated that TMC was non-irritant at lower concentrations of 0.1–1.0% (w/v), as far as the cornea (disperse opacity), iris (hyperaemia) and conjunctiva (redness of palpebral conjunctivae, swelling and discharge with moistening of the lids) were concerned.

Histological results showed that there were only very minor ocular tissue lesions due to the instillation of formulations containing TMC. A phosphate buffer (pH 6.8) was used as a control. More precisely, the corneal cross sections after instillation of various preparations were used to evaluate their effect on the corneal cell structure and tissue integrity. The cornea of the control group showed a normal structure of the three layers: the epithelium layer composed by superficial flattened cells, the Bowman's layer and the anterior layer of stroma which consists of collagenous lamellae running parallel to each other with keratocytes in between (Mahmoud et al., 2006). The microscopic examination of the cornea of rabbits

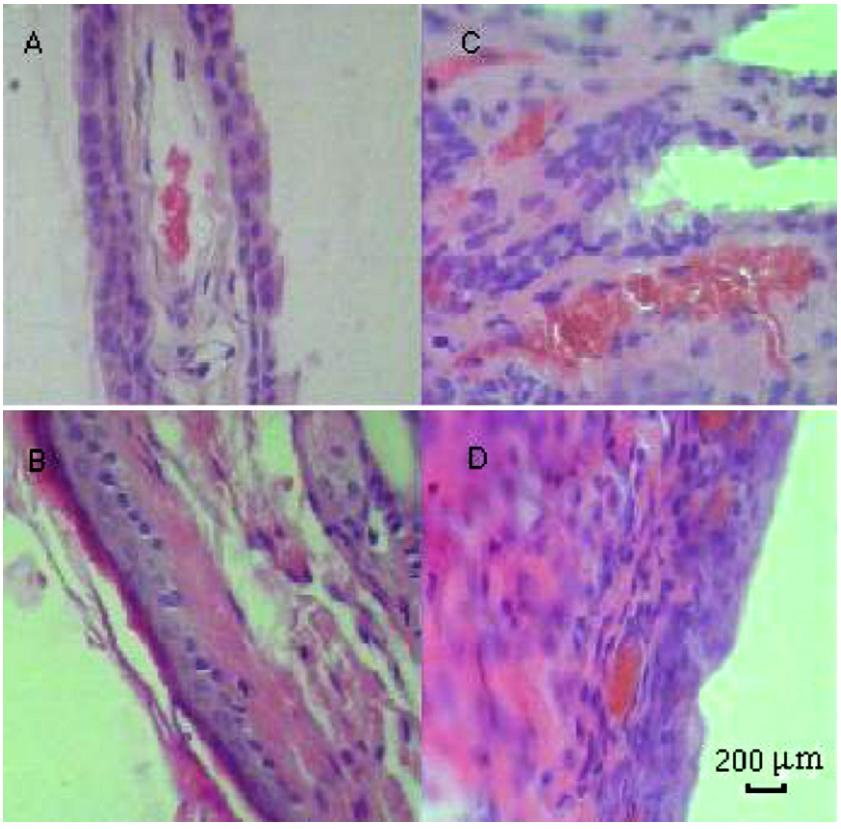

Fig. 3. (A) Light micrograph of the control rabbit iris; (B) light micrograph of the control rabbit palpebral conjunctiva; (C) light micrograph of the rabbit iris from the high dose level of the 1% TMC 601-treated group; (D) light micrograph of the rabbit palpebral conjunctiva from the high dose level of the 1% TMC 602-treated group.

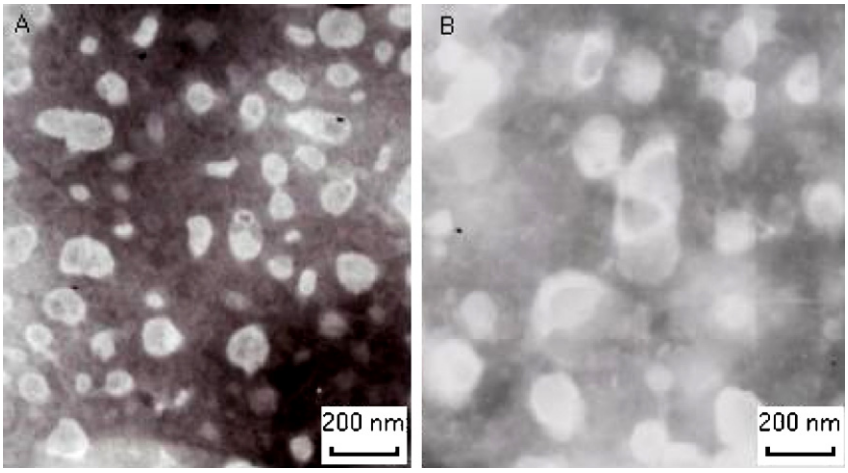

Fig. 4. Transmission electron micrographs: (A) SPC liposomes; (B) TMC-coated liposomes.

Table 2
The PSD, zeta potentials and coating efficiency of TMC-coated liposomes with different polymer concentrations ($n = 3$).

TMC type	Concentration (%)	PSD		Zeta potential (mV)	Coating efficiency (%)
		D1 (nm)	D2 (nm)		
	0	136		−8.7	0
TMC601	0.1	71.3	193.1	4.12	81.5
	0.2	84.1	228.8	9.36	95.8
	0.5	91.2	217.1	22.2	97.7
	1.0	99.2	331.6	24.1	98.1
TMC602	0.1	78.7	245.1	8.16	85.7
	0.2	87.3	256.9	12.3	93.4
	0.5	91.9	307.4	24.6	95.9
	1.0	102.3	354.7	25.1	96.1

that received the highest dose of TMC 602 showed no significant histological changes (Fig. 2).

With regard to the high dose of TMC with a higher molecular weight, it appeared that the integrity of some tissues was destroyed. Iris congestion was found in the 1% TMC 601-treated group compared with the control group (Fig. 3A and C). A similar result was obtained with the 1% TMC 602-treated group (Fig. 3B and D), where palpebral conjunctiva vascular congestion was observed. When the cornea was exposed to a higher concentration of TMC solution, previously narrow intercellular spaces were widened with deformed cells and nuclei and superficial epithelial cells were detached from tissue assembly. Nevertheless, independent of the molecular weight and of the concentration of TMC tested, all the solutions used were considered as being very well tolerated following examinations carried out under the supervision of a professional oculist.

3.2. Characterization of polymer-coated liposomes

3.2.1. Morphology

The morphological characterization of SPC liposomes and TMC-coated liposomes are shown (Fig. 4). In all cases, spherical-shaped particles were found. As to the polymer-coated lipsomes, the presence of polymer layers surrounding the liposomes led to the appearance of larger vesicles.

3.2.2. Size distribution

Conventional phospholipid vesicles are supposed to be spherical, self-closed structures. The liposome suspensions after being coated with TMC exhibited a bimodal size distribution and were somewhat larger than SPC liposomes (Table 2). In the light of Masayukl Hara's theory (Masayukl et al., 1989), it was estimated that the population of smaller liposomes were partially polymerized. The larger liposomes were fully coated liposomes, as well as the aggregation of liposomes caused by TMC chains. Compared with conventional liposomes, the increased particles size of the TMC-coated liposomes was ascribed to the electrostatic force repulsion between positively charged TMC chains supposed to be mainly on the outer leaflet of the layer membrane. This would increase the curvature of the liposome particles, indicating an increase in the thickness of the coating layer. TMC602-coated liposomes exhibited a greater mean size than TMC601, which can be explained by the presence of rather long chain molecules of TMC602 leading to a higher viscosity and more absorbed polymer.

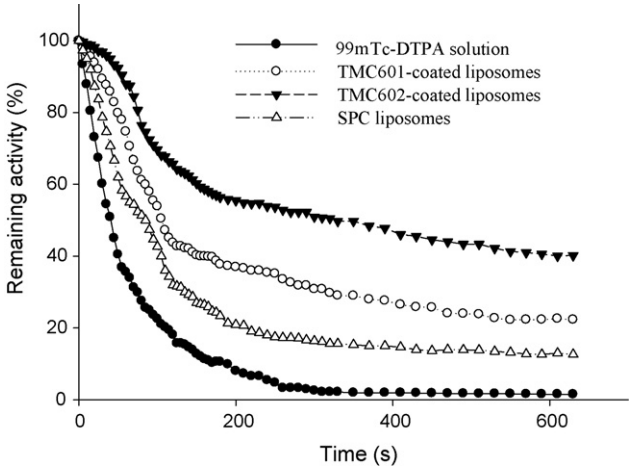

Fig. 5. Pre-corneal drainage of ^{99m}Tc-DTPA in preparations.

Table 3
Clearance half-life ($t_{1/2}$), remaining activity after 10.5 min ($t_{10.5}$) and area under the curve values ($AUC^{(0,\ 10.5\ min)}$) for formulations and ^{99m}Tc-DTPA solution ($n = 3$).

Formulations	$t_{1/2}$ (s)	$t_{10.5}$ (%)	AUC (% s)
TMC601-coated liposomes (0.5%)	$288.05^{\S **\P} \pm 42.76$	$22.319^{*\P} \pm 11.95$	$4953.4^{\S\S **\P\P} \pm 32.66$
TMC602-coated liposomes (0.5%)	$466.27^{\S\S **} \pm 82.15$	$40.194^{\S\S **} \pm 4.87$	$5307.0^{\S\S **} \pm 31.47$
SPC liposomes	209.70 ± 16.33	12.667 ± 7.32	4435.8 ± 25.82
^{99m}Tc-DTPA solution	97.52 ± 10.21	1.5363 ± 0.56	3607.3 ± 12.43

$^{*}P < 0.05$ and $^{**}P < 0.01$ versus ^{99m}Tc-DTPA solution; $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ versus SPC liposomes; $^{\P}P < 0.05$ and $^{\P\P}P < 0.01$ versus TMC602-coated liposomes.

3.2.3. Zeta potential

The zeta potential gives us information about the charge beyond the hydrodynamically stagnant layer and a high positive charge may play an important role in flocculation and coagulation resistance. The movement of a charged surface with respect to an adjacent liquid phase is the basic principle underlying this technique (González-Rodríguez et al., 2007). The effects of factors A and B of TMC coating on the characteristics of the liposomes are shown in Table 2. It was observed that coating the negatively charged liposomes with TMC moved the sheared plane into the bulk solution side caused by the increase in thickness of the polymer layer formed on the surface of liposomes and shifted the zeta potential from a negative value to a positive value, an indicator of surface charge. The higher the concentration of TMC (≤0.5%), the higher the zeta potential. Then it reached a relatively constant value. The molecular weight of the polymer played a comparably weaker role as far as the zeta potential of the preparation was concerned.

3.2.4. TMC coating efficiency

The coating efficiency of polymer-coated liposomes increased with increasing the concentration of TMC used in the preparation with the optimal coating efficiency reaching at least 96% at a concentration of 0.5%, and remained at this level at higher concentrations (Table 2). The polymer acts as both a polycationic agent and a participant in hydrophobic interactions owing to its acetyl groups. Since the free liposomes were negatively charged, hydrophobic interactions between TMC and liposomes should be of minor importance, while the main interaction between positively charged polymer and the phospholipid was an electrostatic attraction.

3.2.5. Encapsulation efficiency (EE)

Lipid based formulations including liposomes offer the potential for enhancing the absorption of poorly soluble and/or poorly permeable compounds. CoQ_{10}, the yellow colored crystalline powder with a melting point of 48 °C, is practically insoluble in water. In the case of the CoQ_{10}-loaded liposomes, the drug was incorporated into the hydrophobic compartment of vesicles with 98% encapsulation efficiency. Since the polymer forms a thick layer on the surface of vesicles instead of changing the rigidity of the liposomal bilayer, the EE of the polymer-coated liposomes was nearly the same as before.

3.3. Precorneal retention time

The curve of the remaining activity on the corneal surface as a function of time (10.5 min dynamic imaging) is shown in Fig. 5 and the precorneal clearance parameters are listed in Table 3.

As shown by the AUC and $t_{1/2}$ values, the presence of TMC resulted in a significant increase ($P < 0.01$) in the mean precorneal retention time of the polymer-coated liposomes in comparison with ^{99m}Tc-DTPA solution when about a 1.5-fold increment in AUC was observed by adding TMC with a higher Mw. Similarly, the $t_{1/2}$ values show that the elimination the polymerized formulation from the corneal surface was significantly delayed in the presence of TMC, being 3–5-fold superior to control solution. Moreover, 40% of the activity remained at the end of 10.5 min for TMC602-coated liposomes, nearly 26 times as much as that of the control solution.

As far as the molecular weight was concerned, the data in Table 3 indicate that the higher Mw leads to significantly higher precorneal retention times ($P < 0.05$) and AUC values ($P < 0.01$) with respect to two groups of polymer-coated liposomes. Furthermore, changing the Mw of the TMC coating around the vesicles significantly altered the radioactivity remaining after 10.5 min, increasing from 22.3% to 40.2% ($P < 0.05$).

The marked improvement in the retention times for liposomes coated with polymer revealed that the positive charge induced by TMC and the increment in viscosity (Table 1) resulted in higher AUC values. In fact, especially for TMC602 with a higher optical viscosity, there were higher AUC value; longer retention times and higher remaining activity on the corneal surface over 10.5 min (Table 3).

In fact, the corneal surface and the conjunctiva were covered by mucin. The mucin layer is secreted by goblet cells of the conjunctiva, and it consists of a protein or polypeptide core with carbohydrate side chains branching off the core (Qi et al., 2007). Accordingly, at physiological pH, the corneal surface is negatively charged and in the case of positively charged TMC-coated liposomes, electrostatic attraction plays an importance role in enhancing absorption. The ability of TMC-coated vesicles to exhibit prolonged precorneal clearance through absorption to the corneal surface, would contribute to the enhanced absorption.

3.4. Evaluation of anti-cataract effect

3.4.1. Opacity assessment of changes in lenses

The slit images of the three groups are shown in Fig. 6. Our experiments involving the treatment of suckling rats with selenite alone resulted in the formation of nuclear cataract within four days in the model group and the cataracts developed rapidly over six days to a mature dense opacity involving the entire lens. With regard to the control group, lenses remained clear throughout the experiment. Concerning the CoQ_{10}-treated group, the onset of cataract was later than in the model group and the percentage of opacity was lower and the size was smaller.

Fig. 7 shows the percentage of opacity after the pups were treated with CoQ_{10}. Compared with the blank control group, the opacity of the model control group reached 95% at the end of the examination, while that of the treated groups was 52%. Following treatment with liposomes, an increase in the percentage opacity began from the third day, when there was barely any change in transparency in the CoQ_{10}-treated groups. This difference among groups was statistically significant ($P < 0.01$).

3.4.2. Superoxide dismutase, reduced glutathione and malondialdehyde analysis

3.4.2.1. Superoxide dismutase. Superoxide dismutase, as a chain-breaking antioxidant, exists in three forms including mitochondrial MnSOD, cytosolic CuZnSOD and the extracellular secreted enzyme which is anchored to proteoglycans and the cell surface (Carlsson et al., 1995). SOD is one of the key enzymes that detoxify the reactive superoxide anion through dismutation to form H_2O_2, which hints a more serous damage to cell. From Table 4, we can see that the

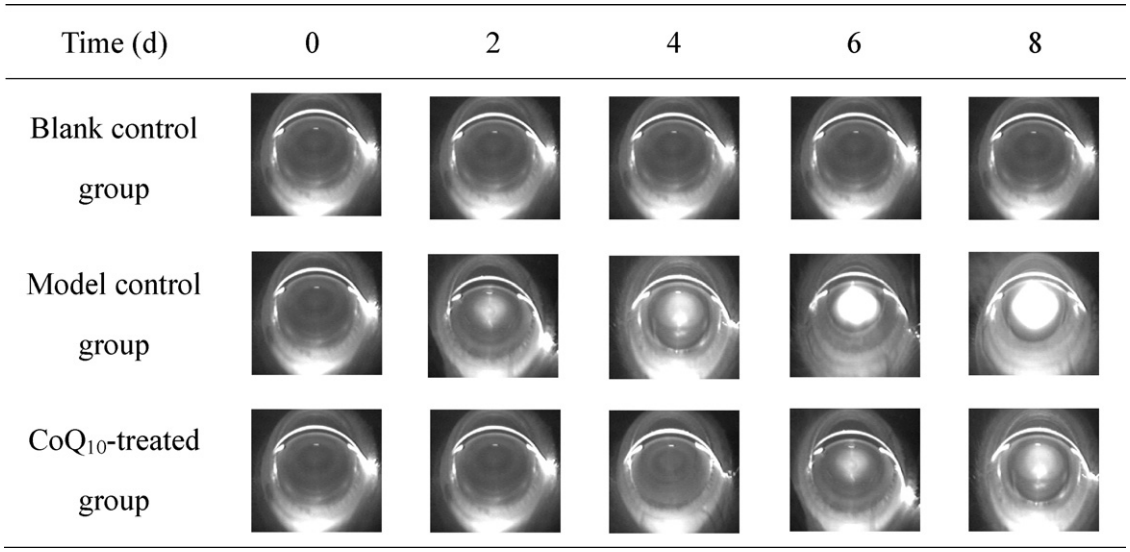

Fig. 6. The slit images of eyes from selenite cataract rats, with or without Coenzyme Q_{10} treatments.

Table 4
Levels of biomarkers in blank control group, model group and CoQ_{10}-treated group ($n = 4$).

Parameter	Blank control group	Model group	CoQ_{10}-treated group
SOD (U/g tissue)	4.83 ± 0.28	2.38 ± 0.08*	4.15 ± 0.16§*
GSH (μmol/g tissue)	12.20 ± 0.24	5.65 ± 0.16*	6.88 ± 0.31§*
MDA (nmol/g tissue)	4.90 ± 0.80	10.47 ± 0.63*	8.50 ± 0.52£*

*$P < 0.001$ versus blank control group; §$P < 0.001$ versus model group; £$P < 0.01$ versus model group.

SOD level in the model control group (2.38 ± 0.08 U/g tissue) was significantly lower than in the blank control group (4.83 ± 0.28 U/g tissue, $P < 0.001$). In the CoQ_{10}-treated group, there was a significant restoration of the SOD level (4.15 ± 0.16 U/g tissue) compared with the model group ($P < 0.001$), in spite of it still being lower compared with control group lenses. This suggests that CoQ_{10} positively modulates the antioxidant enzyme in the presence of selenite and improves the antioxidant defense mechanism of the normal lens, which is in accordance with the previous finding involving its oxygen-quenching activity.

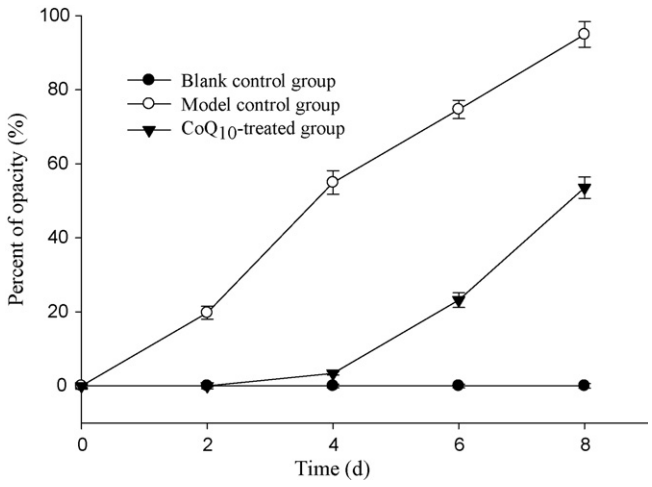

Fig. 7. The effects of Coenzyme Q_{10} on the opacification of selenite cataract in rat eye lenses.

3.4.2.2. Reduced glutathione (GSH). GSH is one kind of small peptides for scavenging O_2^-, H_2O_2 and LOOH, which consists of methoinine, glycine and cysteine. As the substrate of glutathione peroxidase and glutathione s-transferase, GSH is necessary for decomposing hydroperoxides, stabilizing thiol enzymes and protecting hemoglobin and other accessory factors from oxidative damage. It is reported that GSH helps vitamin E recover its reduced state. The toxic effect of sodium selenite has been shown to be due to the depletion of tissue glutathione, which is the result of conversion of GSH to GSSG and simultaneous formation of selenodiglutathione induced by selenite. Selenodiglutathione is converted to GSSG rapidly, generating selenium dioxide which restarts the thiols' depleting reaction (Varma and Hegde, 2004). The data on GSH levels here are in accordance with this reaction theory (Table 4). The mean activity of GSH in the model group (5.65 ± 0.16 μmol/g tissue) was significantly lower than in the blank control group (12.20 ± 0.24 μmol/g tissue, $P < 0.001$). A significant increase (22%) in GSH was observed in the treated group compared with the model group ($P < 0.001$).

3.4.2.3. Malondialdehyde. The superoxide anion free radical is the main source of ROS generated by enzyme and non-enzyme systems, which can attack the unsaturated fatty acids in the biomembrane and induce lipid peroxidation. The resulting peroxides eventually damage the cells. The MDA level reached 10.47 ± 0.63 nmol/g tissue in the model group of rat pups but was reduced to 8.50 ± 0.52 nmol/g tissue in the CoQ_{10}-treated group, and both were significantly higher than in the control group (4.90 ± 0.80 nmol/g tissue, $P < 0.01$, Table 4).

With aging, the compromised function of the lens is exacerbated by depleted or diminished primary antioxidant reserves, antioxidant enzyme capability, and diminished secondary defense systems, such as proteases. Actually, it is very limited for superoxide to be generated in the aqueous milieu of cells during normal respiration because of the tight binding of the reducing electrons to the cytochromes. However, the binding decreases with age with about 5% of the respired O_2 for production of ROS (Varma and Hegde, 2004), accompanied by a loss of lens transparency and impaired enzyme and non-enzyme defense systems. Although cataract is a multifactorial disease associated with several risk factors, oxidative stress has been suggested as a common underlying mechanism of cataractogenesis. The augmentation of the antioxidant defenses

of the lens has been shown to prevent or attenuate experimental cataract (Spector, 1995).

Coenzyme Q_{10} is a lipid redox agent that is endogenously synthesized in mammals. Deficiencies in CoQ_{10} have been found under many pathological conditions suggesting its therapeutic value, such as in chronic heart failure (Folkers, 1993) and myocardial infarction (Verma et al., 2007). Under our experimental conditions, CoQ_{10} has been found to defend the lenses from oxidative attack significantly for the first time. In our study, the mean activity of SOD in the blank control group was found to be significantly higher than in the model group, however, the higher mean activity of SOD in the CoQ_{10}-treated group compared with the model group highlight the potential anti-cataract effect of CoQ_{10}. Also, this protective effect was manifested morphologically as a decrease in the intensity of lenticular opacification. It has been reported that selenite induces a significant depletion of GSH and increases membrane damage as indicated by the level of MDA (Gupta et al., 2002). We observed that restoration of GSH and MDA levels were significantly improved in the CoQ_{10}-treated groups, with higher mean GSH levels and lower mean MDA concentrations compared with the model control group. These data indicate that the positive protective action of CoQ_{10}, as manifested in the gross morphological study, may also be related to the increased GSH and decreased MDA levels. This could be interpreted in two ways. First, it functions as an electron carrier in the respiratory chain which produces a proton-motive force for synthesizing ATP. Administration of CoQ_{10} has also been demonstrated to increase H^+ transport (Echtay et al., 2001). Its reduced form is also a powerful antioxidant which can react with oxygen radicals and lipoperoxides to prevent damage to biomolecules in different tissues and cell compartments (Turunen et al., 2004). Second, CoQ_{10} might influence the generation of superoxide anion production by reducing the uncoupling of reactions during electron transport that could otherwise result in superoxide anion production (Ishii et al., 2004). Furthermore, it has been demonstrated that CoQ_{10} is responsible for the recycling mechanism of α-tocopherol in respiring mitochondria (Lass and Sohal, 1998).

4. Conclusions

In this study, TMC601 and TMC602 were synthesized and found to be well tolerated when topically administered to the cornea for the first time by using the Draize test and histological analysis. Ophthalmic formulations based on TMC were prepared and characterized. The micrographs showed the spherical shape of SPC liposomes and polymer-coated liposomes, the vesicle dimensions of which increased and the zeta potentials shifted to positive values, indicating that the polymer was anchored on the liposomal surface. Both polymerized liposomes have similar encapsulation efficiency with SPC liposomes at about 98%. The differences in size distribution, zeta potential and coating efficiency between the two forms of polymer-coated liposomes were related to their properties, such as molecular weight and viscosity.

The presence of TMC with two different Mw values both significantly prolonged the retention time of the formulations on the corneal surface as shown by gamma scintigraphy compared with the ^{99m}Tc-solution as a control. This could be explained by the interaction between the polymer and the mucus layer covering the corneal surface involving electrostatic forces. In addition, TMC602 with the higher Mw allowed the liposomes to achieve better contact with the cornea offering a suitable viscosity and enabling easy manipulation for instillation.

We also have demonstrated for the first time that CoQ_{10} can be used as a prophylactic measure to combat the onset and progression of cataract in rats. This effect is associated with delayed lens opacity, improved enzyme defense system, increased GSH and decreased MDA levels, which emphasize the anti-cataract effect of CoQ_{10} as an antioxidant and free radical scavenger by attenuating oxidative stress in selenite-induced cataract. Interest in CoQ_{10} has increased recently, mainly because of its antioxidant function and its dietary supplement; unfortunately, the exactly molecular mechanisms of its functions have not been elucidated so far. Although our preliminary results are encouraging, further studies are desirable at biochemical and pharmacological levels.

Acknowledgement

The authors thank Dr. David Jack for commenting and suggesting to our manuscript.

References

Carlsson, L.M., Jonsson, J., Edlund, T., Maklund, S.L., 1995. Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. Proc. Natl. Acad. Sci. U.S.A. 92, 6264–6268.

Devamanoharan, P.S., Henein, M., Ali, A.H., Varma, S.D., 1999. Attenuation of sugar cataract by ethyl pyruvate. Mol. Cell. Biochem. 200, 103–109.

Di Colo, G., Burgalassi, S., Zambito, Y., Monti, D., Chetoni, P., 2004. Effects of different *N*-trimethylchitosans on in vitro/in vivo ofloxacin transcorneal permeation. J. Pharm. Sci. 93, 2851–2862.

Draize, J.H., Woodard, G., Calvery, H.O., 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 82, 377–390.

Dung, P.L., Milas, M., Rinaudo, M., Desbrieres, J., 1994. Water soluble derivatives obtained by controlled chemical modifications of chitosan. Carbohydr. Polym. 24, 209–214.

Echtay, K.S., Winkler, E., Frischmuth, K., Klingenberg, M., 2001. Uncoupling proteins 2 and 3 are highly active H^+ transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). Proc. Natl. Acad. Sci. U.S.A. 98, 1416–1421.

Felt, O., Furrer, P., Mayer, J.M., Plazonnet, B., Buri, P., Gurny, R., 1999. Topical use of chitosan in ophthalmology: tolerance assessment and evaluation of precorneal retention. Int. J. Pharm. 180, 185–193.

Folkers, K., 1993. Heart failure is a dominant deficiency of coenzyme Q_{10} and challenges for future clinical research on CoQ_{10}. Clin. Invest. 71, 51–54.

Frei, B., Kim, M.C., Ames, B.N., 1990. Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. Proc. Natl. Acad. Sci. U.S.A. 87, 4879–4883.

Gerster, H., 1989. Antioxidant vitamins in cataract prevention. Z. Ern&hrungswiss 28, 56–75.

González-Rodríguez, M.L., Barros, L.B., Palma, J., González-Rodríguez, P.L., Rabasco, A.M., 2007. Application of statistical experimental design to study the formulation variables influencing the coating process of lidocaine liposomes. Int. J. Pharm. 337, 336–345.

Groneberg, D.A., Kindermann, B., Althammer, M., Klapper, M., Vormann, J., Littarru, G.P., Döring, F., 2005. Coenzyme Q_{10} affects expression of genes involved in cell signalling, metabolism and transport in human CaCo-2 cells. Int. J. Biochem. Cell Biol. 37, 1208–1218.

Gupta, S.K., Halder, N., Srivastava, S., Trivedi, D., Joshi, S., Varma, S.D., 2002. Green tea (*Camellia sinensis*) protects against selenite-induced oxidative stress in experimental cataractogenesis. Ophthal. Res. 34, 258–263.

Ishii, N., Senoo-Matsuda, N., Miyake, K., Yasuda, K., Ishii, T., Hartman, P.S., Furukawa, S., 2004. Coenzyme Q_{10} can prolong *C. elegans* lifespan by lowering oxidative stress. Mech. Ageing Dev. 125, 41–46.

Ito, Y., Cai, H., Koizumi, Y., Nakao, M., Terao, M., 1999. Correlation between prevention of cataract development by disulfiram and fates of selenium in selenite-treated rats. Curr. Eye Res. 18, 292–299.

Iwanaga, K., Ono, S., Narioka, K., Kakemi, M., Morimoto, K., Yamashita, S., Namba, Y., Oku, N., 1999. Application of surface-coated liposomes for oral delivery of peptide: effects of coating the liposome's surface on the GI transit of insulin. J. Pharm. Sci. 88, 248–252.

Janes, K.A., Calvo, P., Alonso, M.J., 2001. Polysaccharide colloidal particles as delivery systems for macromolecules. Adv. Drug Deliv. Rev. 47, 83–97.

Lass, A., Sohal, R.S., 1998. Electron transport-linked ubiquinone-dependent recycling of α-tocopherol inhibits autoxidation of mitochondrial membranes. Arch. Biochem. Biophys. 352, 229–236.

Lei, G.F., Chen, L., Deng, Y.J., Zhong, H.J., Hao, A.J., Zhang, L.N., 2006. Determination of entrapment efficiency for liposomal formulation of breviscapine using ultrafiltration-HPLC method. J. Shenyang Pharm. Univ. 23, 237–243.

Monem, A.S., Ali, F.M., Ismail, M.W., 2000. Prolonged effect of liposomes encapsulating pilocarpine HCl in normal and glaucomatous rabbits. Int. J. Pharm. 198, 29–38.

Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim. Biophys. Acta 582, 67–78.

Mahmoud, A.A., Karam, S.H., Abdel-Wahhab, M.A., 2006. Chromium-picolinate induced ocular changes: protective role of ascorbic acid. Toxicology 226, 143–151.

Masayukl, H., Masso, M., Qing, Y., 1989. Interaction between a novel amphiphilic polymer and liposomes. Supermol. Sci. 5, 777–781.
Meseguer, G., Gurny, R., Buri, P., Rozier, A., Plazonnet, B., 1993. Gamma scintigraphic study of precorneal drainage and assessment of miotic response in rabbits of various formulations containing pilocarpine. Int. J. Pharm. 95, 229–234.
Nagarsenker, M.S., Londhe, V.Y., Nadkarni, G.D., 1999. Preparation and evaluation of liposomal formulations of tropicamide for ocular delivery. Int. J. Pharm. 190, 63–71.
Nicoli, D.F., Wu, J.S., Chang, Y.J., Ovod, V., Hasapidis, K., 1997. Zeta potential and particle size analysis of colloids using ELS and DLS. Am. Lab. 29, 12.
Oštádalová, I., Babický, A., Obenberger, J., 1978. Cataract induced by administration of a single dose of sodium selenite to suckling rats. Experientia 34, 222–223.
Pobezhimova, T.P., Voinikov, V.K., 2000. Biochemical and physiological aspects of ubiquinone function. Membr. Cell Biol. 13, 595–602.
Polnok, A., Borchard, G., Verhoef, J.C., Sarisuta, N., Junginger, H.E., 2004. Influence of methylation process on the degree of quaternization of *N*-trimethyl chitosan chloride. Eur. J. Pharm. Biopharm. 57, 77–83.
Qi, H.Y., Chen, W.W., Huang, C.Y., Li, L., Chen, C.M., Li, W.M., Wu, C.J., 2007. Development of a poloxamer analogs/carbopol-based in situ gelling and mucoadhesive ophthalmic delivery system for puerarin. Int. J. Pharm. 337, 178–187.
Rabinovich-Guilatt, L., Couvreur, P., Lambert, G., Dubernet, C., 2004. Cationic vectors in ocular drug delivery. J. Drug Target. 12, 623–633.
Shearer, T.R., Ma, H., Fukiage, C., Azuma, M., 1997. Selenite nuclear cataract: review of the model. Mol. Vis. 3, 8.
Spector, A., 1995. Oxidative stress induced cataract: mechanism of action. FASEB J. 9, 1173–1182.
Stewart, D.J., 1974. Sensitive automated methods for phosphate and $(Na^+ + K^+)$-ATPase. Anal. Biochem. 62, 349–364.
Thanou, M., Verhoef, J.C., Marbach, P., Junginger, H.E., 2000. Intestinal absorption of octreotide: N-trimethyl chitosan chloride (TMC) ameliorates the permeability and absorption properties of the somatostatin analogue in vitro and in vivo. J. Pharm. Sci. 89, 951–957.
Turunen, M., Olsson, J., Dallner, G., 2004. Metabolism and function of coenzyme Q. Biochim. Biophys. Acta 1660, 171–199.
Varma, S.D., Hegde, K.R., 2004. Effect of α-ketoglutarate against selenite cataract formation. Exp. Eye Res. 79, 913–918.
Verma, D.D., Hartner, W., Levchenko, T.S., Torchilin, V.P., 2007. Coenzyme Q_{10}-loaded liposomes effectively protect the myocardium in rabbits with an acute experimental myocardial infarction. J. Mol. Cell. Cardiol. 42, 190–218.
Yu, X.L., Wang, W., Yang, M., 2007. Antioxidant activities of compounds isolated from *Dalbergia odorifera T*. Chen and their inhibition effects on the decrease of glutathione level of rat lens induced by UV irradiation. Food Chem. 104, 715–720.
Zambito, Y., Zaino, C., Di Colo, G., 2006. Effects of N-trimethylchitosan on transcellular and paracellular transcorneal drug transport. Eur. J. Pharm. Biopharm. 64, 16–25.