A more efficient ocular delivery system of triamcinolone acetonide as eye drop to the posterior segment of the eye

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ABSTRACT
As a leading cause of vision impairment of the retina, macular edema (ME) has one of the highest clinical demands for treatment. Current treatment of ME relies heavily on invasive techniques resulting in complications and poor patient compliance. To enhance the efficiency of triamcinolone acetonide as eye drop to the posterior segment of the eye, we developed and characterized a novel formulation, namely, triamcinolone acetonide chitosan-coated liposomes (TA-CHL), prepared by the calcium acetate gradient method with some modifications. TA-CHL provided the mean particle size of 135.46 ± 4.49 nm and high entrapment efficiency (90.66 ± 3.21%), exhibited a sustained release profile, excellent physical stability, and no significant toxicity on cornea, conjunctiva, and retina. Optical coherence tomography system (OCT) was used to detect pharmacokinetics of CHL \textit{in vivo}, indicating that CHL had good potency for drug delivery. Cellular uptake experiments showed CHL had the higher transduction efficiency into HCEC and ARPE-19 than liposomes. TA-CHL was shown to be potentially effective eye drop to contribute to the posterior segment of the eye.

1. Introduction
Macular edema (ME) is characterized by extravascular edema of the macula resulting from abnormal retinal capillary permeability. ME is related to various underlying diseases, especially diabetic retinopathy and retinal venous occlusion (Herrero-Vanrell et al., 2011).

As a leading cause of vision impairment of the retina, ME has one of the highest clinical demands for treatment. The current treatment options for ME include laser photocoagulation, and anti-angiogenesis or anti-inflammation treatment. Despite its clinical effects, laser photocoagulation is considered to be associated with scotoma, choroidal neovascularization, and subretinal fibrosis (Bahrami et al., 2016). Intravitreal administration of anti-angiogenic or anti-inflammatory regimens could achieve high local drug concentrations, but repeated injections are costly to the patients, and is accompanied by complications including subconjunctival and/or vitreous hemorrhage, increasing intraocular pressure, endophthalmitis, or retinal detachment (Shikari et al., 2014).

Ocular corticosteroid implants, such as Retisert, Ozurdex, have been developed for the sustained release of drugs up to six months (Chang-Lin et al., 2011); however, secondary glaucoma, cataract, and implant movement or division were reported (Reid et al., 2015). Obviously, corticosteroids should be delivered to the posterior segment of eye noninvasively, with sustained and effective vitreous drug levels.

Eye drop application is easy to use and is an alternative and promising way to avoid adverse effects when used to treat ocular diseases. However, conventional eye drop formulations failed to deliver effective drug concentrations to the posterior segment of the eye, because of the various elimination mechanisms and complex penetration barriers of the eye (Ruponen & Urtti, 2015).

In the last decade, liposomes was found to have the benefits of superior corneal penetration, excellent biocompatibility, relative nontoxicity, the ability of maintaining drug activity at the action site and controlling release, and extended half-life for eye drop (Bourlais et al., 1998; Ebrahim et al., 2005; Hironaka et al., 2009), which improved local drug concentrations in the vitreous. To the best of our knowledge, Altamirano-Vallejo et al. is the first report that TA-liposomes topical use can deliver drugs into the posterior segment of the eye efficiently (Altamirano-Vallejo et al., 2018). However, as the only formulation suitable for topical administration, TA-liposomes-formulation has low encapsulation efficiency (48%) and the low ocular bioavailability resulting from the existence of elimination mechanisms and penetration barriers. Obviously, the formulation should be optimized. Thus, we develop a novel topical administration system with high encapsulation efficiency and high ocular bioavailability to deliver corticosteroid to the posterior segment of the eye.

In this study, liposomes were drug carriers and was prepared using the calcium acetate gradient method; Chitosan...
(CH), a cationic polysaccharide, one of the most widely used polymers in ophthalmology, was selected as the surface modification of liposomes for its unique properties of bioadhesion to the corneal surface and penetration-enhancement (Fuente et al., 2010; Cho et al., 2016). Triamcinolone acetonide (TA), an intermediate-acting glucocorticoid in suspension form, was chosen as the model drug, which is applied in a wide field of inflammatory, edematous, and angiogenic ocular diseases, and has low oral absolute bioavailability (23%) and high first-pass metabolism (Rohatagi et al., 1995). Therefore, this study developed and evaluated the potential of triamcinolone acetonide chitosan-coated liposomes (TA-CHL) as a novel drug delivery system enhancing the efficiency of triamcinolone acetonide as eye drop to the posterior segment of the eye. Furthermore, this study was the first time to investigate intuitively the ophthalmic pharmacokinetics of drug using the anterior and posterior segment modules on an OCT system.

2. Materials and methods

2.1. Materials

TA was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Soybean phosphatidylcholine (PC), containing ≥80% phosphatidyl, was purchased from Aikang Chemical Co., Ltd. (Shanghai, China). CH was purchased from Haidebei Biochemical Corp (Shandong, China). Coumarin-6(C6) and cholesterol were obtained from Aladdin Industrial Corporation. (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of TA-liposomes/C6-liposomes

TA-loaded liposomes were prepared by the calcium acetate gradient method described by Hironaka et al. with some modifications (Hironaka et al., 2011). The mixture of PC, cholesterol (8:1 molar ratio) was formed as a thin lipid film, and the film was freeze-dried overnight. Next, a calcium acetate solution (120 mM) was added. The resulting multilamellar vesicles were freeze-thawed and passed through a micro-membrane filter (pore size: 0.22 μm) (Tianjin, China) for 10 cycles under nitrogen pressure (≤200 psi). The external liposome medium was replaced with the distilled water in two dialysis steps. TA/C6 was subsequently added into the liposome suspension and incubated at 37°C. The final TA concentration in the resultant liposomal suspension was 1.5 mg/mL.

2.3. Preparation of TA/C6 chitosan-coated liposomes (TA-CHL/C6-CHL)

Chitosan solution, 0.5% (w/v) was added dropwise to the TA-liposomes/C6-liposomes under a controlled stirring rate at room temperature, respectively. The obtained formulations were stirred for another 30 min to obtain homogeneous suspensions.

2.4. Characterization of the formulations

2.4.1. Morphology

TA-loaded liposomes were observed using a transmission electron microscope (TEM; JEM-1200EX, JEOL, Tokyo, Japan). The sample was diluted with distilled water (1:100) and dropped onto a copper grid, and the 2% (w/v) phosphotungstic acid was added. After drying for 10 min at room temperature, the sample was examined by TEM.

2.4.2. Particle size and zeta potential

The particle size and zeta potential of liposomes were measured using a Zetasizer Nano ZS (Malvern, UK).

2.4.3. Entrapment efficiency (EE)

The measurement of the EE of the TA-loaded liposomes and TA-CHL was carried out using a centrifugation ultrafiltration method. The free TA was removed using centrifugal filter tubes (Amicon Ultra-4, Millipore, Ireland) at 4000 rpm for 30 min. The amount of free TA was measured using high-performance liquid chromatography (HPLC) equipped with a persil-ODS2 column at a UV wavelength of 240 nm with a flow rate of 1.0 ml/min. The mobile phase consisted of methanol:water (70:30). The EE in the liposomes was calculated using the following equation:

\[
EE\% = \frac{W_{total\ amount\ of\ TA} - W_{free\ TA}}{W_{total\ amount\ of\ TA}} \times 100\%
\]

where W is the weight.

2.4.4. Differential scanning calorimetry (DSC) analysis

All samples (about 5–6 mg) were sealed in an aluminum pan and scanned using a differential scanning calorimeter (CDR-4P, Shanghai Tianping Instrument Ltd., Shanghai, China) from 30 to 400°C at a rate of 10°C/min under a dry nitrogen atmosphere at a flow rate of 0.2 mL/min.

2.4.5. In vitro release study

One milliliter of each sample was placed in a dialysis bag (Spectrum Laboratories, Rancho Dominguez, CA, USA; molecular weight cut off (MWCO)=3500 Da) and immersed in 35 mL of phosphate-buffered saline (PBS) (pH 7.4, containing 0.5%, w/v Tween 80) at 37°C in a water bath and shaken at 100 rpm. At the predetermined time point, 1 ml of the release medium was withdrawn and an equal volume of fresh buffer was added. The amount of TA in the samples was measured using HPLC as described in section 2.4.3. Each sample was assayed in triplicate.

2.4.6. Stability studies

The stability of TA-CHL was evaluated by determining the particle size, zeta potential, and EE of the samples during storage at 4°C for 30 or 60 days.
2.5. Animal studies

C57BL/6 mice (25–30 g) were purchased from the Experimental Animal Center of Shandong University. All protocols for the animal experiments complied with the requirements of the Institutional Authority for Laboratory Animal Care of Shandong University. A total of 15 C57BL/6 mice were randomly allocated into three groups that received drops of 5 μl C6 solution, 5 μl C6-liposomes, or 5 μl C6-CHL onto the surface of the left eye, respectively. The right eye was used as the control and received no treatment. Under anesthesia, their pupils were dilated with 0.5% tropicamide (Santen, Osaka, Japan) and topically applied polyvinyl alcohol eye drops to prevent eye dehydration. The eyes of mice were imaged by the anterior and posterior segment module on the Heidelberg Spectralis OCT system (Heidelberg Engineering, Heidelberg, Germany) at 0, 10, 20, 30, 40, and 50 min, and at 1, 2, 3, 4, 6, 8, 10, 12, and 24 h. The relative signal intensity of the OCT images indicates the value of a treated sample when the signal intensity of the control sample is estimated as 1 (Daruich et al., 2017).

2.6. Cellular uptake studies

Corneal epithelial (HCEC) cells and retinal pigment epithelial (ARPE-19) cells were obtained from Shanghai Cell Bank, the Institute of Cell Biology, China Academy of Sciences (Shanghai, China). HCEC and ARPE-19 were seeded in 12-well plates at 2.5 × 10^3 cells/well, respectively. The culture medium was removed after the cells reached 80% conflu-ence and the cells were treated with fresh serum-free medium containing 10 μM C6-liposomes and C6-CHL for 4 h at 37°C with 5% CO2 in an incubator. Sequentially, the cells were examined under a fluorescence microscope (BX40, Olympus, Japan).

2.7. Ocular histology study

The potential ocular irritancy and/or damaging effects of the formulations were evaluated by studying the histology of the eye tissues. Five microliter of the formulations (TA suspension, TA-liposomes, and TA-CHL) were instilled into the lower conjunctival sac of the left eye of each mouse five times a day for a period of seven days, while the right eye used as a control. At the end of the seven days, all the mice were euthanized, and the eyes were isolated and fixed in 4% paraformaldehyde. Sections were cut along sagittal sections of 3 μm per eye from the paraffin blocks and stained with hematoxylin and eosin (HE). In addition, the terminal deoxy-nucleotidyl transferase nick-end-labeling (TUNEL) assay was performed in tissue sections using a TUNEL kit (Roche, Switzerland). The sections were examined by inverted microscopy (Olympus, Japan). The number of TUNEL-positive cells per visual field was counted and expressed as percent of the total number of cells.

2.7. Data analysis and statistics

All data were expressed as means ± standard error. Statistical analysis was performed using an independent-samples t-test or one-way analysis of variance (ANOVA). In all cases, statistical significance was set at \( p < .05 \).

3. Results and discussion

3.1. Characterization of the formulations

3.1.1. Morphological studies

The morphology of the obtained liposomes was estimated by TEM. As shown in Figure 1, the TA-liposomes were uniform, spherical particles with a relatively smooth surface and

![TEM images of TA-liposomes (a) and TA-CHL (b).](image)

Table 1. Particle size (PZ), Zeta Potential (ZP), Polydispersity Index (PDI), Entrapment Efficiency (EE) % of TA-liposomes, TA-CHL, C6-liposomes, and C6-CHL.

| Sample      | PZ(nm)   | ZP(mv)   | PDI    | EE(%)   |
|-------------|----------|----------|--------|---------|
| TA-liposomes| 108.48 ± 5.59 | -10.17 ± 1.71 | 0.18 ± 0.04 | 84.04 ± 3.89 |
| TA-CHL      | 135.46 ± 4.49 | 17.98 ± 3.21 | 0.21 ± 0.03 | 90.66 ± 3.21 |
| C6-liposomes| 126.72 ± 6.01 | -12.83 ± 2.19 | 0.24 ± 0.15 | 75.13 ± 4.67 |
| C6-CHL      | 145.21 ± 5.38 | 15.61 ± 3.77 | 0.27 ± 0.32 | 81.27 ± 5.13 |

Figure 1. TEM images of TA-liposomes (a) and TA-CHL (b).
Figure 2. DSC thermograms of pure TA (a), pure CH (b), blank liposome (c), TA-liposome (d) and TA-CHL (e).
monodispersed with no aggregations. The particles’ mean particle size was about 100–150 nm in TEM imaging, which was in consistent with the expected result. TA-CHL appeared spherical or almost spherical, but there was a rough shell surrounding the vesicle structure, which further confirmed the formation of TA-CHL from the morphological characteristics.

3.1.2. Particle size and zeta potential

The most severe limitation of liposomes for ophthalmological application is that they are quickly cleared by various elimination mechanisms and complex penetration barriers, particularly for negatively and neutral charged liposomes (Abdelbary, 2011). Interestingly, the binding affinity of cationic liposomes to the corneal surface is high, which can interact intimately with the negative charge of ocular surface, and show bio-adhesion to the corneal surface and penetration-enhancing properties, previous studies indicated that CH would increase the absorption capacity and retention time of the drug, and improve the local drug concentration (Ludwig, 2005). Therefore, we designed the TA-CHL with the aim of achieving high encapsulation efficiency and high ocular bioavailability by means of CH-coated liposomes. Considering the influence of particle size and zeta potential on EE, we selected 0.5% (w/v) CH as the optimal concentration because of its relatively smaller particle size and higher positive zeta potential (Tan et al., 2017). The mean particle size and zeta potential of the liposomes were shown in Table 1. Our findings indicated that the mean particle size of TA-CHL increased with the CH coating on the surface. The zeta potential of TA-liposomes was $-10.17 \pm 1.71$ mV, while that of TA-CHL was $17.98 \pm 3.21$ mV. The inversion of the result might have resulted from electrostatic interactions between the positively charged chitosan as the surface modifier and the negative phosphate groups of PC, and further confirmed that the surface of liposomes was coated by the positively charged polymer.

3.1.3. Entrapment efficiency (EE)

EE is one of the important indicators for selection of drug carrier. Low EE is not only one of the reasons for the limited application of liposomes but also inhibits the industrialization of liposomes. As shown in Table 1, the EEs for TA-liposomes and TA-CHL were $84.04 \pm 3.89\%$, $90.66 \pm 3.21\%$, respectively, which was higher than the result of Altamirano-Vallejo et al (48%). The increase in the EE for TA-CHL might have been caused by the calcium acetate gradient method with some modifications and by the ionic effect between the polymers and the anionic segment in the core particles after CH coating.

The calcium acetate gradient method successfully overcomes the problem of low entrapment efficiency of liposomes. Active drug loading using a calcium acetate gradient can significantly improve the EE of drugs, given that transmembrane gradients of ions should be the driving force to load drugs into liposomes.

3.1.4. DSC analysis

DSC was used to investigate the physicochemical state of TA in the formulations. The DSC thermograms of pure TA, pure CH, blank liposomes, TA-liposomes, and TA-CHL were shown in Figure 2. The presence of a sharp endothermic peak at $328.27 ^\circ C$ was shown in the thermogram of TA. However, the
Figure 4. OCT images of the anterior segment (a) and posterior segment (b) of the mouse eye after eye drop application of C6-liposomes/C6-CHL at different times.
endothermic peak at 328.27 °C had disappeared from the thermograms of TA-liposomes and TA-CHL, meaning that TA in the formulations was not in the crystalline state, which proved that TA was successfully entrapped into the TA-liposomes and TA-CHL (Tian et al., 2013; Abdelkader et al., 2016).

3.1.5. In vitro release study
TA release from the liposomes in PBS at 37 °C is depicted in Figure 3. The formulations exhibited a sustained release profile without an initial burst, suggesting that TA was entrapped predominantly inside the liposome, instead of being simply physically adsorbed. Moreover, TA was almost completely released from TA liposomes and TA-CHL in 10 h and 12 h, respectively. TA-CHL exhibited a more prolonged and sustained release profile, which showed that the CH coating exerted stronger control over the release of the drug. This not only reduces the frequency of eye drop, but also improve the compliance of patients. It was attributed to the strong CH coating limiting the release of drug by attaching to the surface of the liposomes (Knudsen et al., 2015).

3.1.6. Stability studies
The physical stability of TA-liposomes and TA-CHL was evaluated at 4 °C for 30 and 60 days. There was no difference in the visual physical appearance of TA-liposomes and TA-CHL over 30 and 60 days compared with that before storage.
Additionally, as shown in Table 2, no significant change was observed in the particle size, zeta potential, and EE of TA-CHL. The EE of the TA-liposomes decreased slightly after stored for a 30 and 60 days. These results indicated TA-CHL had excellent stability during storage at 4 °C for 60 days. The relatively stable structure might be attributable to the electrostatic attraction between chitosan and phosphate groups of PC.

3.2. Animal studies

To study the intraocular behavior of the formulations after eye drop application, the time-course observation was imaged using the anterior and posterior segment modules on an OCT system. The application of this technology is more intuitive and can be observed repeatedly in vivo, representing a breakthrough in this field. Although we did not perform a quantitative analysis of C6 reaching the posterior segment, we could estimate the relative intensity of the delivery by recording the amount of fluorescence emission. The fluorescence emitted by C6 in the posterior segment of the eye started at 10 min, and increased with time, reaching a peak at 6 h after eye drop application. The fluorescence peak of OCT images in the anterior segment and the posterior segment of the eye after eye drop application were shown in Figure 4. Then the fluorescence emission gradually decreased and almost disappeared at 10 h and 12 h, respectively, after application of C6-liposomes and C6-CHL. Changes in time-course of the relative fluorescence intensity were shown in Figure 5. From the results, C6-CHL showed better potency for drug delivery compared with C6-liposomes as shown in Figure 4 and Figure 5. Therefore, the results demonstrated that CH coating of liposomes was a more efficient ocular delivery system of triamcinolone acetonide as eye drop to the posterior segment of the eye. This might be because the positive charge on the CHL interacted with the negative charge on the surface of the eye to increase the retention time of the drug, providing a long-lasting action, and enhancing drug permeability.

3.3. Cellular uptake studies

To estimate whether the transduction efficiency into HCEC and ARPE-19 could be enhanced, cellular uptake studies were performed. As shown in Figure 6 and Figure 7, an obvious time-dependent increase in uptake amount was
observed from 10 min to 4 h. At given time points, both groups showed high fluorescence in cells after 10 min. The cellular uptake of C6 loaded in CHL was significantly greater than that exposed in liposomes at given time points. The results showed that CHL had the higher transduction efficiency into HCEC and ARPE-19 than that of liposomes, there might be the reason that positively charged chitosan facilitated ionic interaction with the negatively charged HCEC and ARPE-19 cells membrane.

3.4. Ocular toxicity test

The biocompatibility of CH is one of important indicator for a drug delivery system for ocular administration. The conjunctiva, cornea, and retina are the common areas of ocular irritation after eye drop application. The results of HE staining in Figure 8 for conjunctival and corneal cells showed no significant difference in their structures between the control group and the TA-liposomes or TA-CHL-treated groups, indicating that both formulations produced no significant ocular irritation. As shown in Figure 8, the TA-liposomes and TA-CHL displayed no significant difference in the number of TUNEL-positive cells of retina compared with that in the control group, indicating that the formulations did not increase the amount of apoptotic DNA fragmentation.

These results suggest that TA-CHL has no significant ocular toxicity and good biocompatibility, making it an excellent option as a novel formulation for eye drops.

4. Conclusion

To enhance the efficiency of TA as eye drop drug delivery to the posterior segment of the eye, we successfully developed and characterized the novel formulation TA-CHL. TA-CHL provided high entrapment efficiency, exhibited a sustained release profile, and showed excellent physical stability. Importantly, TA-CHL was proven to be a more efficient drug delivery to the posterior of segment of the eye via eye drop application and had no significant toxicity toward ocular tissue, which might greatly avoid the complications caused by invasive operation and provide a new way for the wide application of TA. Therefore, we believe that TA-CHL will be a powerful and promising approach to improve the drug treatment of posterior segment diseases.
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Disclosure statement

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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