Nanodelivery of Triamcinolone Acetonide using PLGA-Chitosan Nanoparticles for the Treatment of Inflammation-related Ocular Cataract

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Yi Xing
Zhengzhou University First Affiliated Hospital

Lijuan Zhu
Zhengzhou University First Affiliated Hospital

Ke Zhang
Zhengzhou University First Affiliated Hospital

Teng Li
Zhengzhou University First Affiliated Hospital

Shaohua Huang
Zhengzhou University First Affiliated Hospital

SpencerNewmanxMsMn@yahoo.com

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Abstract
Impairment of vision is a serious public health emergency, with cataracts responsible for half of the world blindness. Triamcinolone acetonide (TA) is widely indicated in the treatment of several ocular disorders, however, free drug suspension often limits its clinical benefits and commercial compositions often cause adverse effect to eye. In this study, triamcinolone acetonide (TA) was successfully formulated in PLGA-chitosan nanoparticle for the treatment of ocular inflammatory diseases such as cataract. The PLGA-chitosan NP (PLC NP) was characterized in detail with regard to size, shape and stability analysis. PLC NP demonstrated to control the release of drug until 100h compared to that of PLGA NP depicting the importance of chitosan coating for prolonged drug release. Blank PLC NP did not influence the cell viability indicating the excellent biocompatibility of the designed nanocarriers. PLC-TA exhibited an excellent anti-inflammatory activity against HCE cells and significantly decreased the secretion of IL-6 in TNF-α activated cell. In rabbit model, PLC-TA did not show any clinical sign of typical inflammation of eye and significantly alleviated the inflammatory signs compared to that of free TA suspension upon single administration after 24h. Moreover, PLC-TA exhibited a greater %AHT compared to that of either NS or free TA suspension indicating a notable reduction in the fogginess in anterior chamber. Pharmacokinetic analysis in rabbit’s eye revealed that PLC-TA peaked at 6h and significant concentrations of TA was observed for most part of the study until 24h indicating the superior advantage of PLGA-Chitosan based nanocarrier system. Overall, PLGA-chitosan based nanoparticulate formulations present a new approach towards the treatment of ocular inflammatory diseases.

Introduction
Cataract is the main reason behind the vision impairment and is considered to be a major public health concern. According to World Health Organization (WHO) there is an estimated 250 million people with vision impairment especially in underdeveloped and developing nations [1]. As per WHO assessment, cataract was responsible for more than 50% of blindness in the year 2010 worldwide [2]. The cataract collectively inhibits the passage of lights and results in clouding of eye lens. Among all the reason, age-related cataract (lens opacification) is predominant among people and multifactorial
in nature [3]. This multifactorial impairment results from multiple mechanisms that directs several post-translational modifications and abnormal protein signaling in the eye lens that contribute to the racemization of local cellular proteins [4]. Furthermore, lenticular lesions are increased with a reduction in protein solubility due to oxidative stress-related metabolic products in the eye [5]. At present there is no single treatment strategy that could effectively prevent the cataract. Surgery is the only alternative and costly option in most of the developing countries, however, surgery in the eye is always associated with enormous risk and healthcare access is required post-surgery [6]. Corticosteroids such as dexamethasone (DEX) and triamcinolone acetonide (TA) are often indicated in the treatment of several eye disorders including cataract, conjunctivitis, scleritis, uveitis, diabetic macular edema (DME) and diabetic retinopathy (DR) [7, 8]. However, lipophilic nature of TA will create a non-specific distribution across the ocular tissues resulting in lesser bioavailability in the region of interest in the eye [9]. At the same time, it is highly likely for the drug to reach the sensitive tissues and cause unnecessary adverse effects. It is widely reported that topical administration of free corticosteroids damages the structure and functions of local tissues such as canal of Schlemm [10, 11]. Such adverse effects are observed upon the long term administration of corticosteroids [12]. Lack of appropriate ophthalmic delivery system that could control the release of encapsulated drugs specifically in the region of interest while inhibit its accumulation in the sensitive tissues is required [13]. Nanomedicine has shown great potential to improve the therapeutic efficacy of poorly soluble drugs like TA. Several nanoscale carriers including polymer and lipid origin have shown immense potential in controlling the release and biodistribution of encapsulated therapeutics [14]. Polymeric nanoparticles owing to their small size and adjustable surface characteristic offer interesting features for the ophthalmic applications. Contrary to free drug solution that non-selectively distributes into sensitive cells, nanoparticles (NP) could be tuned for its cellular interactions and subsequent internalization in the respective cells [15]. We expected that the corticosteroid loaded in the polymeric nanoparticles could preferentially accumulate in the specific tissues in the eye and minimize the associated side effects compared to molecular dispersions that present supra-
therapeutic concentrations immediately after administration and sub-therapeutic concentrations later on. In the present study, we have selected poly(D,L-lactide-co-glycolide) (PLGA) as a biopolymer to encapsulate the hydrophobic drug [16]. PLGA is known for its salient features including excellent biodegradability, biocompatibility, non-immunogenic and non-toxic properties [17]. PLGA nanoparticles have been reported to degrade by hydrolysis and release the encapsulated therapeutics in a controlled manner. In order to further control the release of drug from PLGA, nanoparticle was surface coated with chitosan (CHT) polymer. CHT is a cationic polymer which is highly biodegradable and biocompatible in nature. The slight positive charge on the CHT-coated NP will enhance the cellular uptake and cellular targeting mechanism [18, 19].

The main aim of this work was to optimize the design of CHT-coated PLGA NP to encapsulate TA to improve the therapeutic efficacy in cataract. Detailed physicochemical characterizations were performed and therapeutic efficacy was proved by performing IL-6 assay and clinical inflammation score of rabbit.

Conclusions
In summary, triamcinolone acetonide (TA) was successfully formulated in PLGA-chitosan nanoparticle for the treatment of ocular inflammatory diseases such as cataract. The PLGA-chitosan NP (PLC NP) was characterized in detail with regard to size, shape and stability analysis. PLC NP demonstrated to control the release of drug until 100 h compared to that of PLGA NP depicting the importance of chitosan coating for prolonged drug release. Blank PLC NP did not influence the cell viability indicating the excellent biocompatibility of the designed nanocarriers. PLC-TA exhibited an excellent anti-inflammatory activity against HCE cells and significantly decreased the secretion of IL-6 in TNF-α activated cells. In rabbit model, PLC-TA did not show any clinical sign of typical inflammation of eye and significantly alleviated the inflammatory signs compared to that of free TA suspension upon single administration after 24 h. Moreover, PLC-TA exhibited a greater %AHT compared to that of either NS or free TA suspension indicating a notable reduction in the fogginess in anterior chamber. Pharmacokinetic analysis in rabbit’s eye revealed that PLC-TA peaked at 6 h and significant concentrations of TA was observed for most part of the study until 24 h indicating the superior
advantage of PLGA-Chitosan based nanocarrier system. Overall, PLGA-chitosan based nanoparticulate formulations present a new approach towards the treatment of ocular inflammatory diseases.

Materials And Methods
Preparation of TA-loaded Chitosan-PLGA nanoparticles
The TA-loaded PLGA-Chitosan nanoparticle was prepared by emulsion-evaporation method. Briefly, 50 mg of PLGA and 10 mg of TA was dissolved in dichloromethane (DCM, 3 ml) and stirred for 5 min. The organic phase was added slowly in aqueous phase consisting of 1% polyvinyl alcohol (PVA) and immediately homogenized at 13000 rpm using Ultra-Turrax T-25 homogenizer forming an oil-in-water emulsion which was then sonicated for 5 min at 100 W employing a probe-sonicator. The organic solvent was gradually evaporated using a rotaevaporator at room temperature. The TA-loaded PLGA NP was dialyzed briefly for 12 h to remove unencapsulated drugs. The concentrate of PLGA NP was collected and chitosan solution (pH 4.0) was added at a weight ratio of 10/1 w/w (PLGA/Chitosan) and incubated for 30 min at room temperature. The nanoparticle was centrifuged at 10000 rpm for 10 min and the final PLGA-CH NP was collected and stored until further use. The particle size distribution, PDI and surface charge was evaluated using ZetaSizer Nano ZS90 (Malvern Instruments Inc., Worcestershire, UK) at 25 °C and particle morphology was evaluated by FEI Morgagni Transmission Electron Microscope (TEM) (FEI Co., Hillsboro, OR, USA) at 100 kV. The entrapment and loading efficiency of TA was evaluated by HPLC method. Stability analysis of PL NP and PLC NP was evaluated at 4 °C for 45 days. Throughout study period, change in particle size was considered as a means of stability analysis.

In vitro drug release studies
The TA-loaded PL NP and PLC NP was freeze-dried using 1% Trehalose and 2 mg equivalent of TA containing nanoparticle was used for the release study. Briefly, freeze-dried nanoparticle was suspended in 1 ml of physiological buffer (pH 7.4) and packed in a dialysis tube (MWCO – 5000) and placed in a tube containing 25 ml of release buffer. The samples were shaken at 100 rpm per minute at 37 °C and samples were collected time to time periodically. The amount of drug released was calculated using HPLC method. Shimadzu Prominence LC-2030C 3D liquid chromatograph with diode array detector was used. Altima C18 RP18 HP column (250 x 4.6 mm2, with 5 µm particles) was used
as drug separating column. The mobile phase consisted of methanol/water at a ratio of 72/28 at a flow rate of 1 ml/min and detected at 254 nm. The mobile phase was filtered and degassed before use. The samples were filtered using 0.45 µm filter and 10 µl was used for the analysis.

**Cell culture and cell viability assay**

The human corneal epithelial cells (HCE) were employed to perform the in vitro cell viability assay. The HCE cells were grown in DMEM/F-12 medium containing 15% of FBS and 1% antibiotic mixture. The medium was additionally supplemented with 0.5% DMSO, 10 ng/ml and 5 mg/ml of insulin. The cells were maintained at ambient conditions in incubator. For the cell viability assay, HCE cells were platted in 96-well plate (1 × 10^4) and allowed overnight for attaching the cells. The cells were incubated with 3 different formulations; blank PLC, PLC-TA (0.1%) and PLC-TA (1%) respectively for 24 h. The medium was replaced with fresh medium containing MTT reagent and incubated for 4 h and then 100 µl of 0.1 N HCl in isopropanol anhydride and absorbance was read at 570 nm using a microplate reader.

**In vitro anti-inflammatory assay**

The anti-inflammatory activity of free TA and PLC-TA was evaluated by the method established by Enríquez-de-Salamanca, et al [20]. The HCE cells were plated in 24-well plate (4 × 10^4) and incubated for 36–48 h in the regular medium as described above. Before the actual study, cells were added with serum-free media for 4 h and incubated with 20 ng/ml of TNF-α for additional 48 h. The HCE cells were then treated with free TA and PLC-TA for 1 h and the supernatant medium was collected for the quantification of secretion of IL-6 using ELISA kit. The cells treated with only TNF-α were used as a positive control and the experiment was performed in triplicate.

**In vivo therapeutic efficacy in animal model**

Endotoxin-induced uveitis (EIU) rabbit model was used for this analysis. Briefly, male albino rabbits were subjected to topical anesthesia using 0.5% proparacaine hydrochloride eye solution. The male albino rabbit was administered with 50 µl of LPS (1 ng/ml) via intravitreal injection. The rabbits were divided into three groups with 4 rabbits in each group and treatment was initiated after 1 h of LPS injection. The animals were administered with 100 µl of TA (1%) suspension or PLC-TA (equivalent to 250 µg/ml) or normal saline (NS) subconjunctival manner. The clinical signs after the respective
treatment were observed through binocular indirect ophthalmoscopy employing a 20D lens at 24 h, 48 h and 72 h, respectively after the treatment. The normal saline groups were used for baseline response. The severity of treatment was observed in terms of inflammation score determined by corneal damage, anterior chamber cell flares, vitreous haze and iris vessels. In a separate study, fogginess of the aqueous humor was photographed, and image was processed through Image J software and difference between the corneal reflections between each animal group was recorded. The change in fogginess between different animal group was quantified in terms of aqueous humour transparency (AHT%) which is calculated by %AHT = D/E x100 in which D represents the fogginess in the aqueous humor of EIU rabbit while E represents the fogginess in the normal rabbit’s eye. All animal study protocols were duly approved by Institutional Care and Use Committee.

**Pharmacokinetic analysis**
The pharmacokinetic analysis was performed on New Zealand Albino Rabbits (n = 5, 2.5-3 kg). A single dose of free TA or PLC-TA (1 mg/ml equivalent) was administered to the lower conjunctival sac of one of each rabbit. The formulations were freshly prepared on the same day of experiment. Followed by, samples were collected at predetermined time intervals (0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h). A 20 µl of sample was collected from the aqueous humor and diluted with 80 µl of mobile phase (as mentioned above) and filtered through 26 G needle, centrifuged and 50 µl of supernatant sample was used for the drug analysis using HPLC as mentioned above.

**Statistical analysis**
Statistical analysis was performed using SPSS 14.0. Statistical significance was set at 0.05 and data are presented as mean ± standard deviation.

**Results And Discussion**
**Characterization of TA-loaded PLGA-Chitosan nanoparticles**
TA has been shown to possess excellent anti-inflammatory and immunomodulatory effect in the several eye disorders such as cataract. Regardless of the excellent pharmacological actions of TA, administration of TA suspensions often presents clinical limitations. Besides, lipophilic nature of TA will create a non-specific distribution across the ocular tissues resulting in lesser bioavailability in the region of interest in the eye. At the same time, it is highly likely for the drug to reach the sensitive
tissues and cause unnecessary adverse effects. Nanomedicine-based TA administration presents several advantages including sustained release of encapsulated therapeutics in the eye and targeting to the most preferable region of interest. In the present study, we have synthesized chitosan-coated PLGA nanoparticle as a stable delivery system for the ophthalmic applications. TA-loaded PLGA nanoparticles (PL NP) exhibited average particle size distributions of 120 nm with a narrow PDI (0.124) and negative surface charge of -22.5 mV. In order to further stabilize the PLGA NP, it was surface coated with chitosan. The negatively charged PLGA NP will electrostatically interact with the positively charged chitosan particles. Several concentrations of CHT were investigated from 0.1–10 parts w/w of PLGA NP. As shown, particle size gradually increased with the increase in the concentration of CHT; however, higher concentrations of CHT resulted in bigger particle size and were not stable. It is worth noting that slight increase in particle size was observed until 10/1 ratio however steep increase in particle size was observed after 10/1 ratio and bigger particles were observed. It is possible that during the initial addition of CHT, the polymers were non-continuous on the PLGA surface and formed a stable consistent layer at 10/1 ratio. After that, bulky mass of CHT increased and particle size and might formed aggregate and agglomerate leading to big and unstable particles. The addition of CHT was further confirmed by zeta potential analysis. The zeta potential was 22.5 mV for PL NP and started reversing with every incremental in the CHT concentration. At 10/1 ratio, charge reversed to 20 mV and kept increasing above 40 mV for higher concentrations of CHT. We have optimized 10/1 of PLGA to CHT as an ideal concentration for the stable nanoparticles with a final size of 165 nm with a net positive charge of 20 mV.

The particle morphology was investigated by TEM. As shown, PL NP showed a small particle size compared to PLC NP which was relatively bigger as shown by DLS analysis. Importantly, coating of CHT on PLGA was clearly visible through the presence of a thin greyish layer surrounding a black core (PLGA). Nevertheless, a small particle size will allow the suitable distribution in the eye chamber.

Stability analysis and In vitro drug release study
The stability analysis was performed in order to compare the stability of PL NP or PLC NP. As shown, PL NP exhibited size variation from 120-160 nm during 45 days of stability analysis. A significant
increase in the particle size might be attributed to the aggregation of particles. On the contrary, PLC NP exhibited a size variation between 165–176 nm indicating the excellent stability of CHT-coated PLGA NP. A strong surface charge of PLC NP might be responsible for the excellent stability of nanoparticles. Simulated physiological medium (pH 7.4) was used to evaluated the release kinetics of PL NP and PLC NP. After 12 h of incubation, approximately 10% of TA released from both the nanoparticles and after 24 h incubation, > 20% of drug released from both the nanocarrier system indicating that the carriers are stable in the physiological medium and could control the release of drug over a consistent time period. Interestingly, drug release pattern were different for PL NP and PLC NP by 48 h and significant different in release was observed by 60 h. For example, 45% of drug release was observed from PL NP compared to 32% from PLC NP, respectively. The significant difference in release was observed throughout the study period after 36 h through 100 h. Slower drug release in PLC NP was attributed to the stable presence of outer CHT layer and increased path length. Overall, controlled release of drug from the nanoparticle core could be beneficial for the long-term ophthalmic treatment with reduced side effects.

In vitro cell viability assay
It is well known that cells behave in different manner at in vitro conditions and in vivo conditions, nevertheless, cell experiments offer first hand information of cytotoxicity and biocompatibility. The in vitro toxicity of blank and drug-loaded nanoparticles was investigated by cell viability assay. The cells were treated with blank PLC NP and TA (0.1 & 1%)-loaded PLC NP at 3 different concentrations of 1 µg/ml, 10 µg/ml and 100 µg/ml, respectively. The cell viability was not affected significantly with any concentrations of blank PLC NP indicating the excellent biocompatibility of the designed nanocarriers. However, notable decrease in the cell viability was observed for PLC-TA (1%) compared to that of PLC-TA (0.1%) possibly due to the dose-dependent toxic effect in the endothelial cells. It should be noted that free TA is reported to exhibit much toxicity compared to that of the one loaded in nanoparticles. The toxicity in case of TA suspension was attributed to the presence of several excipients while TA-loaded PLC NP are demonstrated to be safe for the ophthalmic applications [21]. In published literature, authors showed a higher toxicity for the commercial formulations of
Fortcinolona® 40 with low cell viability for the equivalent concentrations.

**Anti-inflammatory assay at in vitro conditions**

At first, therapeutic efficacy of nanoformulations was evaluated based on a in vitro model. The study depends on the release of cytokines (IL-6) upon the exposure of proinflammatory cytokine that caused the inflammation of ocular tissue. This in vitro inflammation model was selected owing to its ability to evaluate the anti-inflammatory efficacy of different formulations. The cells were treated with free TA suspension and PLC-TA formulations at two different concentrations. As shown, blank PLC NP did not result in any decrease in the IL-6 compared to that of TNF-α activated control cells indicating the lack of therapeutic efficacy. Although, TA (0.1%) did not show any notable effect, TA (1%) exhibited a significant reduction in the IL-6 secretion. Importantly, maximum anti-inflammatory effect was observed in nanoparticles-based PLC-TA (1%) formulation with a significant reduction in the IL-6 levels compared to any other groups. A higher anti-inflammatory response of PLC-TA (1%) might be attributed to the controlled release of encapsulated therapeutics from the nanosized particles and better presentation of TA to the TNF-α activated cell. The results further highlight the fact that loading of TA in the nanoparticles did not affect its biological activity.

**In vivo therapeutic efficacy in rabbit model**

EIU rabbit model was employed to investigate the therapeutic efficacy of PLC-TA formulations. Study relies on the clinical signs of intraocular inflammation after 24 h of intravitreal lipopolysaccharide administration. The animals administered with normal saline (NS) showed tremendous signs of conjunctival redness and congestion. Typical fibrin deposits were noted in cornea with crystalline lens and reduced intraocular pressure (compared to baseline) [22]. Besides, inflammation of iris and ciliary body attributes to the Tyndall effect that results in the fogginess in the anterior chamber of the eye indicating a typical inflammation of eye. These inflammatory signs were significantly lower in PLC-TA formulations after 24 h of administration compared to that of free TA (1%) suspension of the equivalent concentrations. The results clearly indicated that PLC-TA could effectively alleviate the inflammatory reaction in the eye. Ophthalmic administration of free TA suspension at the comparable dose did not alleviate the clinical inflammation sign and showed insignificant result to that of the NS
treated control. Additionally, PLC-TA did not cause any adverse effect after the ophthalmic administration further confirming the safety of the designed carriers.

The mean grey difference between corneal light reflection and anterior chamber was quantified in terms of fogginess and calculated as %AHT. The %AHT effect of NS, free TA and PLC-TA was compared with relative to normal eye. As shown, PLC-TA exhibited a greater %AHT compared to that of either NS or free TA suspension indicating a notable reduction in the fogginess in the eye. On the contrary, free TA suspension did not show any significant difference with NS. Consistent with the lower clinical inflammation score, PLC-TA exhibited a greater transparency of the eye signifying the superior therapeutic efficacy in the animal model.

Pharmacokinetic analysis

The TA concentration in aqueous humor of rabbit’s eye was measured at predetermined time points as mentioned in the method section. The plasma concentration of TA following the administration of free TA and PLC-TA is presented in Fig. 8 and pharmacokinetic parameters are WinNonlin software. As shown, free TA showed peak concentration in 1 h after ophthalmic administration and subsided gradually and observed insignificant level in the aqueous humor after 6 h indicating the rapid clearance of the free drug. On the contrary, PLC-TA peaked at 6 h and significant concentrations of TA were observed for most part of the study until 24 h indicating the superior advantage of PLGA-Chitosan based nanocarrier system. The pharmacokinetic parameters are presented in Table 1. PLC-TA exhibited a 6-fold higher AUC value and nearly 3-fold higher MRT compared to that of free TA suspension indicating the superior pharmacokinetic performances of nanocarrier system. The encapsulation of drug in the core of the NP allowed the sustained release of TA gradually over a period of time in the aqueous humor of the rabbit’s eye.

| Parameters                     | Free TA          | PLC-TA         |
|--------------------------------|-----------------|----------------|
| T_{max} (h)                    | 1               | 6              |
| C_{max} (µg/L)                 | 15.8 ± 0.568    | 43.1 ± 0.568   |
| AUC_{0–t} (µg/L/h)             | 36.8 ± 3.685    | 214.2 ± 6.598  |
| MRT (h)                        | 2.541 ± 0.135   | 6.35 ± 0.346   |

Abbreviations

TA - Triamcinolone acetonide
PLC NP - PLGA-chitosan NP
PLC-TA - Triamcinolone acetonide-loaded PLC NP
CHT - Chitosan
PLGA - poly(D,L-lactide-co-glycolide)

Declarations

**Author’s contributions**

YX and LZ were involved in formulations characterization of nanoparticles. KZ and TL carried out the cell work and animal works. SH secured the grant and written the entire manuscript.

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**Availability of Data and Materials**

All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Competing Interest**

The authors report no declarations of interest.

**Author details**

Yi Xing,1 Lijuan Zhu,1 Ke Zhang,1 Teng Li,2 Shaohua Huang,1*

1Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China, 450001

2Department of Urology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China, 450001.

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Figures

Figure 1

Schematic presentations of preparation of triamcinolone acetonide-loaded PLGA-Chitosan polymer nanoparticles. The PLGA was prepared by solvent-evaporated method and chitosan was coated by electrostatic interactions.
Figure 2

(a) Change in particle size and polydispersity index; (b) change in zeta potential; (c) optimization of PLGA-Chitosan NP in terms of particle size; (d) optimization in terms of zeta potential.
Transmission electron microscope-based morphology analysis of PL NP and PLC NP.

(a) Stability analysis of PL NP and PLC NP over 45 days at 4°C. The stability was performed in terms of particle size; (b) In vitro release characteristics of triamcinolone acetonide from PL NP and PLC NP at 37°C. The release was quantified using HPLC technique.
Figure 5

MTT assay of blank PLC NP, PLC-TA (0.1%) and PLC-TA (1%) in HCE cells. The experiments were performed in triplicate.
Elisa based quantification of IL-6 secretion by TNFa activated HCE cells after treatment with free TA suspensions and PLC-TA formulations.

(a) Clinical inflammatory score of rabbit treated with normal saline, TA suspension and PLC-TA formulations at 0.1 and 1% concentrations; (b) The percentage of aqueous humour transparency (AHT%) in the EIU model after 24-72 h.
Pharmacokinetic analysis of plasma concentration of TA after ophthalmic administration of free TA suspension and PLC-TA. The study was conducted until 24h and analyzed using HPLC technique.