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**Triamcinolone acetonide Loaded-Cationic Nano-lipoidal
Formulation for Uveitis: Evidences of improved biopharmaceutical
performance and anti-inflammatory activity**

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Abstract

Topical administration of corticosteroids is the cornerstone treatment of anterior uveitis, but poor corneal penetration and retention cause hindrance in their therapeutic utility. The conventional eye drops are less valuable in conditions where inflammation reaches deeper regions of the eye. Therefore, there is a clear need for an effective drug delivery system, which can increase corticosteroid penetration after topical application. To address this, cationic nanostructured lipid carriers of the drug triamcinolone acetonide (cTA-NLC) were prepared. The cTA-NLC were prepared by a hot microemulsion method and evaluated for drug release, permeation, cell uptake, cytotoxicity, anti-inflammatory activity and ocular irritancy. The cTA-NLC are nanometric in size (< 200 nm), with a zeta potential of about +35 mv and % drug EE of 88%. The nanocarriers exhibited slow and sustained release of around 84% in 24 h and transcorneal drug permeation of 51% in 8 h. The nanocarriers exhibited no cytotoxicity (% cell viability of >90%). The cell uptake study showed that nanocarriers could retain inside the cells for 24 h. The developed formulation could significantly reduce the TNF- α level in LPS induced inflamed cells. The studies indicated that cTA-NLC could be a promising option for the topical treatment of uveitis.

Keywords: Uveitis, triamcinolone acetonide, nanostructured lipid nanocarriers, corneal permeation, human corneal fibroblast cells (HCF)

1. Introduction

The term 'uveitis' refers to a group of conditions characterised by intraocular inflammation and may arise from infectious or non-infectious causes. Uveitis can occur as an isolated ocular disease or be associated with a systemic syndrome [1]. In Western countries, uveitis is responsible for about 10-15% of cases of preventable blindness [2]. A vital part of the treatment strategy for all forms of uveitis is to control the inflammation, favouring the use of formulations able to effectively deliver therapeutic drug concentrations while minimising ocular and systemic side-effects. In the case of non-infectious uveitis, the aetiology is presumed to be auto-immune or auto-inflammatory and treatment require the administration of corticosteroids alone or in combination with other immunosuppressants. In contrast, infectious uveitis requires antibiotic therapy as a first-line option, which may need to be supplemented by appropriate corticosteroid [3]. Therefore, corticosteroids represent the cornerstone of treatment for most forms of uveitis.

Traditionally, triamcinolone acetonide (TA), a water-insoluble corticosteroid has been the preferred choice; for treating posterior uveitis. The TA injections are given as intravitreal or subtenon. Both of these injections can lead to complications, such as increased intraocular pressure, endophthalmitis and cataract formation. Additionally, intravitreal administrations themselves come with an increased risk of intraocular haemorrhage, retinal detachment, endophthalmitis and secondary ocular infection [4]. Therefore, there is a necessity to develop a topical dosage form to avoid these types of complications. However, the conventional eye drops have the limitations, such as poor corneal uptake and retention due to nasolacrimal drainage. Further, the strong defensive barriers like corneal epithelial layer, aqueous-vitreous humour, blood-aqueous humour barrier and blood-retinal barrier limit the entry of drug molecules through topical administration into the eyes, which in turn results in very low bioavailability (<5%) of drugs [5,6]. Hence, there is a necessity to develop the dosage form based on the novel drug delivery system, which can help in achieving increased ocular bioavailability of the administered drugs.

Scientists worldwide are taking an interest in lipid nanocarriers (LNs) for ocular drug delivery. LNs offer many advantages for ocular delivery including improved

drug permeation/retention, increased delivery to posterior segments of the eye and prolonged drug release onto the ocular mucosa [7]. Lecithin (Phosphatidylcholine) is the most abundant groups of phospholipids found in the cell membrane and it can help in achieving high drug loading while encapsulating hydrophobic molecule like. Along with increasing drug loading, lecithin also prevents drug leakage and acts as a drug penetration enhancer in ocular drug delivery [8,9]. Further, introducing a positive charge on the surface of the particles may increase residence time and drug uptake, through increased ionic interaction between the cationic lipid nanocarriers and negatively charged mucus layer of the ocular surface [10]. Hence, nowadays there is an increasing interest in inducing positively charge to the nanocarriers through the coating of a cationic moiety, e.g. with chitosan [9,10], L-Arginine[13] and other cationic lipids (e.g. stearylamine and cetyltrimethylammonium bromide)[14,15].

In the present study, TA loaded cationic, nanostructured lipid carriers (NLC) have been tested as a potential nanomedicine for topical uveitis treatment. We hypothesized that the encapsulation of TA in cationic lipid nanocarriers would increase corneal penetration without increasing toxicity, and hence increase the drug's bioavailability within the eye.

2. Materials and Methods

2.1 Materials

Triamcinolone acetonide was provided as a gift by Lupin Ltd, (Pune, India). Transcutol[®]P (diethylene glycol monoethyl ether), Capmul MCM C10 (Glyceryl monocaprate) and Captex 200 P (Propylene glycol dicaprate) were provided as gift samples by ABITEC Corporation, (Columbus, Ohio, USA). Lecithin was supplied as a generous gift by Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 was purchased from Fischer Scientific Pvt Ltd, (Mumbai, India). Stearylamine was purchased from Sigma-Aldrich, USA. All other chemicals and reagents were of analytical grade, while HPLC solvents were of HPLC grades.

2.2 Methods

2.2.1 Preparation of cationic TA-loaded NLC

TA loaded NLC were prepared by hot microemulsion method [16,17]. NLC compositions were selected based on a pseudoternary phase diagram and solubility studies of the drug in different excipients [17,18]. The lipid phase consisted of a mixture of Capmul[®] MCM C10, soya lecithin (solid lipids) in 1:1 ratio and Captex[®] 200 P (liquid lipid). The ratio between the solid lipids and liquid lipids were kept as (75:25). The total solid lipid content was 0.5% of total formulation, i.e. 200 mg, while the oil content was 0.125% i.e. 50 mg. Transcutol[®] P and polysorbate 80 were used as the surfactant mixture ($S_{mix} = 1:1$, total 100 mg), while the cationic charge was provided by stearylamine. The amount of stearylamine was 10 mg taken in 1 ml of ethanol, dissolved by slight heating. Briefly, a weighed amount of drug (40 mg) and lipid mixture i.e., Capmul MCM C10, lecithin (solution in dichloromethane, 100 mg/ml) and Captex 200 were taken in a small beaker at 1:6 w/w of the drug: lipid mix. The drug and lipid mix was then heated at 60°C, followed by the addition of the S_{mix} (Transcutol P and tween 80 in 1:1 ratio). Once a homogenous mixture was obtained, stearylamine (solution in ethanol) was added followed by a small amount of hot water (200 mg) (50-60°C) to yield a microemulsion. After 5 min of stirring, the microemulsion was added dropwise to cool water (10-15°C). The above mixture was then subjected to high shear homogenisation (16,000 rpm) for 10 min (Heidolph, Silent Crusher M, Germany). The homogenized mixture was left to stir at room temperature (700 rpm) (25°C) for 3-4 h before use [18,19].

2.2.2 Characterization of cTA-NLC

a) Size, polydispersity index (PDI) and zeta potential.

Triamcinolone acetonide-loaded NLC (cTA-NLC) were characterized with respect to (w.r.t) to their particle size, PDI and % drug entrapment efficiency (% EE). The size and PDI of the formulations were determined on a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, Worcestershire, UK). An aliquot of NLC suspension was diluted 10 times with distilled water for size measurements while undiluted samples were used for surface charge analysis [20].

b) Powder X-Ray diffraction (PXRD) studies

The PXRD studies were carried out on pure drug sample (TA), blank NLC and cTA-NLC. Briefly 15-20 mg of the respective samples of drug, blank NLC and cTA-NLC were subjected to X-ray diffraction analysis on X-ray powder diffractometer (Pananalytical's X'PertPro Almelo, Netherlands) at 2θ angles from 2 to 40° using Cu Ka radiation ($\lambda=1.54 \text{ \AA}$) at 40 kV, 40 mA passed through nickel filter [21].

c) Surface morphology by FE-SEM imaging

The surface morphology of cTA-NLC was studied using Field Emission Scanning Electron Microscopy (FE-SEM). Briefly, an aluminium stub was taken and a piece of carbon tape adhered to it. After that, a drop of NLC suspension sample was placed on the carbon-coated stub and air-dried. Then, the sample was subjected to platinum coating with MC1000 ion sputter (HITACHI, Singapore). The sample was then loaded into FE-SEM instrument (HITACHI, SU8010, Japan) for imaging [22].

c) Drug entrapment efficiency

A direct lysis method was used to determine the encapsulation efficiency. Briefly, the cTA-NLC dispersion was centrifuged at 28,000 g for 40 min at 4°C . The supernatant was discarded and the pellet was washed twice with double distilled water: N-Methyl-2-Pyrrolidone (NMP) (9:1) to remove any un-entrapped drug. The pellet was separated and lysed with a mixture of chloroform: dichloromethane (1:1)[17,23]. The lysed samples were then filtered ($0.22 \mu\text{m}$) and diluted with the mobile phase. This solution was analyzed for drug content by HPLC (Shimadzu LC-2010C HT ver. 3.01 system, M/s Shimadzu Inc., Tokyo, Japan). The separation was done with isocratic elution on Thermo Scientific Hypersil (M/s Thermo Fisher Scientific Inc., USA) C_{18} column (250 mm x 4.6 mm, $5 \mu\text{m}$). The mobile phase consisted of a mixture of acetonitrile: potassium dihydrogen phosphate buffer (20 mM, $\text{pH}=4.2$) (50:50 % v/v). The detection wavelength was 240 nm. Injection volume was kept at $20 \mu\text{l}$ with the flow rate of 1 mL/min at room temperature. The % entrapment efficiency was calculated by using the following equation [17].

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Amount of drug in NLCs}}{\text{Total amount of drug added}} \times 100$$

2.2.3 *In vitro* drug release study

In vitro drug release was conducted by using a dialysis bag method. Briefly, the NLC dispersion (1 mL), was introduced in a dialysis membrane (MWCO 10–12 kDa, Himedia, India) and the content was dialysed against 30 mL of an 80:20 mixture of simulated tear fluid (pH= 7.4) and N-methyl pyrrolidine (NMP). Simulated tear fluid (STF) comprised of sodium chloride (0.67%), sodium bicarbonate (0.2%), and calcium chloride (0.008%) in water, while NMP was taken to maintain sink condition [24,25]. Drug release was studied under gentle stirring (400 rpm) and a constant temperature of $37 \pm 0.5^\circ\text{C}$. At predefined time intervals, 1 mL of sample was withdrawn and replaced by the same volume fresh release medium. The samples were then filtered (0.22 μ membrane) and analyzed by using HPLC at 240 nm. The HPLC analyses were done as described previously in section 2.2.2 c. The release profile from the developed formulation was then fitted into different release kinetic models such as zero order, first order, Hixon-Crowell, Higuchi, and Korsmeyer-Peppas to study the release behaviour from the formulations, and regression analysis was implemented [23].

2.2.4 *Ex-vivo* corneal permeation study

The *ex vivo* transcorneal permeation study of the developed formulation was carried in freshly excised goat cornea, which is a well-established method and reported in various research articles [26–30]. The transcorneal permeation of the developed formulation was compared with that of the free drug suspension. The free drug suspension contains 0.1% of the drug, 0.5% of carboxymethyl cellulose, 0.1% of tween 80.

Goat Cornea Preparation: Freshly excised whole eyeballs from goats were transported from the abattoir to the laboratory and immediately placed in saline solution upon enucleation. The corneas were dissected along with 2–4 mm of surrounding scleral tissue rim from the enucleated eye and washed with saline solution to remove any adhering pigments. The washed corneas were then stored in simulated tear fluid until the start of the experiment.

Goat Cornea Permeation experiment: Fresh cornea obtained by the above procedure was mounted in the Franz diffusion cell apparatus between the clamped donor and the receiver chamber. Care was taken to ensure that the epithelial surface of the cornea was placed facing the donor side. The receiver chamber was filled with 30 mL of the release medium containing STF: NMP (80:20). The receiver fluid was maintained at $37 \pm 1^\circ\text{C}$ and kept under stirring with the help of magnetic beads. An aliquot (1 mL) of the developed cTA-NLC formulation/free drug suspension was placed on the epithelial surface of the cornea in the donor chamber. At regular time intervals, 1 mL of sample was withdrawn via the sampling port and replaced with the same volume of fresh media. The samples were then filtered ($0.22 \mu\text{m}$) and suitably diluted with mobile phase and analyzed on HPLC instrument [26,31].

2.2.5 Corneal permeation and distribution study with dye loaded formulations

In order to assess the permeation behaviour and distribution of the formulation, dye loaded (Nile red) NLC were evaluated for corneal permeation. Dye loaded NLC were prepared by the same procedure as described in section 2.2.1, only difference is that drug is replaced by Nile red (NR) dye. Briefly, freshly excised pig eyes were collected and the corneas were dissected out. Biopsy punches of the cornea were made and placed between 96 well inserts and the whole assembly was put into the 96-well plate with Hank's Balanced Salt Solution (HBSS) buffer placed in the wells under the insert. 3 different assemblies were prepared and mounted. The dye loaded formulations (20 μl) were added and the plates were placed in an incubator at 32°C for 1 h and 2 h. At each time point (1 h and 2 h), the plates were removed and the corneas were then fixed in 4% formaldehyde, dehydrated in sucrose and sectioned on the cryostat. The nuclear stain DAPI was added to the sections and then fluorescence was assessed using the Axio-vision fluorescence microscope using Axio-vision 4.8 software (Carl Zeiss Microscopy, Germany).

2.2.6 Cell line studies

2.2.6.1 Human Corneal Fibroblast culture

Primary Human Corneal Fibroblast (HCFs) cells generated from redundant transplant corneal scleral tissue (REC 06/Q2702/44, CI Rauz, Sponsor RG06-152) as previously described [32,33] were used in all of the cell line studies. Cell culture medium was prepared by adding 10% (v/v) of Fetal Calf Serum (FCS), 1% (v/v) Glutamax and 1% (v/v) Penicillin-Streptomycin to RPMI-1640 media. The cells were maintained in this medium and grown under standard cell culture conditions (5% CO₂; 37°C) until they reached 80% confluency at which point they were harvested using Tryple Express reagent (Gibco, MA, USA) [32,33].

2.2.6.2 MTT assay

The cell viability study was carried out using an MTT assay [34]. In a first instance, cells were seeded in a 96-well plate at a density of 5,000 cells per well, for a 6 point calibration curve of 0 to 20,000 cells per well in triplicate (0, 1000, 2000, 5000, 10000, 20000 cells/ well), while the other wells were seeded at 5000 cells/well. The 96-well plate was then incubated under normal cell culture conditions for 24 h. After 24 h, the cells were treated with plain drug solution (in DMSO+ Dulbecco's phosphate-buffered saline (D-PBS), cTA-NLC and blank NLC. The concentrations for plain drug solution and cTA-NLC were taken as 1, 0.5, 0.2 and 0.1 mg/mL (equivalent to drug), while the lipid concentrations of blank-NLC were 5, 2.5, 1 and 0.5 mg/mL. Untreated cells were used as negative control. After 24 h, the treatments were removed and replaced with 100 µl fresh phenol-red free cell culture medium to which 10 µl of MTT solution in D-PBS was added. The cells were incubated for 4 hours at 37°C/ 5% CO₂. After 4 h, 85 µl of media+ MTT mix from each well was removed, leaving 25 µl of media+ MTT mix., in which 50 µl of DMSO was added and pipetted up and down to solubilise the formazan. The plate was again incubated at 37°C for 10 minutes and the absorbance was then measured on a microplate reader (TECAN, Switzerland) at 540 nm [35,36].

2.2.6.3 Cell uptake study

For cell uptake study, HCF cells were seeded at 25,000 cells/ well in a 96 well plate and incubated for 24 h. After 24 h, the cells were treated with Nile red solution (NR-Solution), Nile red loaded NLC (NR-NLC) (at conc. of 50 µg/mL of NR). The NR-NLC formulations were prepared as discussed in section 2.2.1, only

replacing the drug with dye. The dye solutions were prepared by dissolving the dye in a minimum amount of ethanol (1 mL) and further diluting it 10 times with cell culture media. After 2 h of treatment, the cells were washed with D-PBS buffer to remove any free particles and the plate was then put inside the confocal microscope (Yokogawa, Japan) for imaging using CQ1 software. After, imaging, the plate was placed back into the CO₂ incubator. After 24 h, further images were captured to assess the retention of formulations inside the cells as previously described [35,36].

2.2.6.4 Human TNF alpha assay

Human TNF alpha Elisa assay was conducted to assess the anti-inflammatory activity of nanocarriers. The ELISA assay was performed according to the instructions provided by the manufacturers (Human TNF alpha Elisa kit, RAB1089, Sigma Aldrich, USA)[37,38]. The tested compounds were TA solution (in DMSO: cell culture media in 1:10 ratio), cTA-NLC and blank NLC. The concentration of drug in solution and NLC was kept as 1 mg/mL, while the conc. of lipid in blank NLC was 5 mg/mL. The inflammation was induced by Lipopolysaccharide (LPS) of *E. Coli* (Sigma Aldrich, USA), after treating cells with LPS for 24 h. All the formulations were sterilized by membrane filtration before use.

2.2.7 Ocular irritancy test

For evaluation of ocular irritancy of the developed formulation (cTA-NLC), the HET-CAM (hen's egg-chorioallantoic membrane test) test was carried out. Briefly, freshly collected fertilized hen's eggs were incubated at 37 ± 0.5 °C and $65.0 \pm 5.0\%$ relative humidity for 3 days. The eggs were kept horizontally and rotated manually thrice a day during incubation. On day nine, eggs were removed for the assay. The region of air cell was cut and the membrane was pared off. The membrane was then moistened using a 0.9% NaCl. Eggs were placed in the incubator again for 30 min. The inner membrane was then carefully removed with forceps. Afterwards, 200 µL of each undiluted formulation was placed on the membrane (n=3)[39]. The treatments were applied as **A**) Positive control i) Sodium hydroxide solution (0.5 M) as a strong irritant ii) 1% Sodium dodecyl sulphate (SDS) as a moderate irritant **B**) NaCl solution (0.9 %) as a

negative control (non-irritant) **C**) cTA-NLC formulation. After application of each treatment, blood vessels were investigated for any vascular changes including haemorrhage, hyperemia, and blood coagulation at different times post-application (0.5, 2 and 5 min), and the images were captured. The sum scores for all three irritant responses were made to yield a single numerical value. The scoring of the irritant responses was done as a) < 0.9 =Non-irritant b) 1-4.9 as slight irritant, 5-8.9 moderately irritant, 9-21- severe irritant [40,41].

2.2.8 Stability study

The stability studies of the cTA-NLC formulations were conducted at accelerated conditions (40°C/75% RH) for a period of 6 months. The formulations were evaluated for time to time w.r.t drug crystallization, size, PDI and % entrapment efficiency (% EE).

3. Results and Discussion

3.1 Characterization of cTA-NLC

a) Particle size, PDI and % EE

The average particle size of the optimized formulation was found to be 198.95 ± 12.82 nm. The PDI of the particles was found to be 0.326 ± 0.04 , which indicated the homogenous particle size distribution. The zeta potential value of the NLC was found to be positive at 35.8 ± 1.94 . The positively charged NLC were prepared so that they can adhere to the negatively charged corneal surface. The drug entrapment was found to be 88.14 ± 3.03 % w/w, indicating high drug entrapment inside the NLC, this might be attributed to the lipophilic nature of the drug ($\log P = 1.94$)[18,42].

b) PXRD analysis

The results of the PXRD analysis is shown in fig 1 a. The XRD spectra of the drug TA shows crystalline peaks at 2θ positions 9.87° , 14.50° , 14.97° , 15.87° , 17.18° , 17.59° , 19.86° , 24.68° and 26.92° . The blank NLC show their characteristic peaks at 2θ positions of 5.39° , 10.78° , 18.78° , 19.28° , 19.65° , 20.01° , 20.42° , 20.91° and 23.65° , 16° . In the XRD spectra of cTA-NLC, most of the peaks of blank NLC have reappeared, with a slight shift at 2θ positions of

5.41°, 10.72°, 10.87°, 19.20°, 19.60°, 20.36°, and 23.61°, which confirmed the complete entrapment of the drug inside the nanoparticles (**Fig. 1a**) [16].

b) Surface morphology

The surface morphology showed the spherical shaped particles with smooth surface and round edges (**Fig. 1b**). The particles were found to be nanometric in size and most of the particles were found to be in uniform size, with uniform size less than 200 nm. There were no drug crystals seen, which confirmed absence of any free drug or crystallization in NLC. The smooth surface, round edges can also indicate absence of grittiness, which further promises non-irritant nature of NLC [43,44].

3.2 *In vitro* drug release study

The *in vitro* drug release profile of cTA-NLC demonstrated biphasic drug release behaviour, with the initial fast release of nearly 42% in 2 h, followed by a slow and sustained release about 84% at the end of 24 h. The initial fast release can be attributed to the drug adhering to the surface of the NLC, while the later sustained release can be attributed to the slow diffusion of the drug inside the core of NLC (**Fig 1c**)[45].

-Space for Figure 1-

Release kinetic models were used to assess the mechanism of drug release. The release kinetic study allowed us to measure the release profile from the formulation. The highest correlation coefficient (r^2) value was used as criteria to choose the best release kinetic model. The r^2 values are given in table 1. From the results, it was apparent that the drug release followed the Higuchi model (r^2 values of 0.9155) as well as the Korsmeyer-Peppas model ($r^2= 0.9271$). The Higuchi model demonstrated that the drug release mimicked Fick's first law of diffusion. Korsmeyer-Peppas model (which is developed mainly for polymeric matrix) demonstrated that for spheres, the value of $n < 0.43$ corresponds to drug release by Fickian diffusion, while the value of ' n ' in the range of 0.43–0.85 indicates a combination of diffusion-controlled and swelling controlled drug release [46,47]. In our case, the ' n ' value for the Korsmeyer-Peppas model was

0.519, which suggests that the drug release is by diffusion-controlled as well as swelling controlled drug release.

-Space for Table 1-

3.3 *Ex vivo* transcorneal permeation study

The *ex vivo*, transcorneal permeation profile of cTA-NLC was found to be biphasic, similar to that of its *in vitro* drug release; however, the extent of the drug permeation was found to be lower than the drug release (**Fig 1d**). The fig showed around 39% of the drug having permeated within the first 4 h, while the remaining drug penetrated slowly for the next 4 h i.e., the total drug permeation was found to be around 51% at the end of 8 h. The initial fast permeation could be advantageous as the drug level would be available immediately to treat the inflammation, while the latter half of the drug release can maintain the sustained drug level at the site of action. On the other hand, the drug permeation from the free drug suspension (TA-suspension) was found to be low and rapid. The highest drug level was achieved in 4 h i.e. around 28%, however after that, the drug level found to be decreased and at the end of 8 h, the drug permeation was observed to be around 22%. Hence, around a 2-fold increase in drug permeation was found in case of cTA-NLC, as compared to a free drug suspension.

3.3 Corneal permeation and distribution study with dye loaded formulations

The results of the porcine corneal permeation and distribution study is shown in **Fig 2**. Results show that at the 1 h time point, no fluorescence (red) was observed, while at 2 h, red fluorescence was observed surrounding the blue (DAPI) stained nuclei. This confirmed that the formulation requires at least 2 h, to permeate through the cornea. Faint red staining was found, as the dye loaded formulation was permeated through the epithelium, corneal stroma and the endothelium after 2 h.

-Space for Figure 2-

3.4 Cell line studies

a) Biocompatibility of the formulation

The % cell viability of all the formulations was found to be satisfactory (> 85%) and the formulations were found to be non-toxic to human corneal fibroblasts. The results of the cell viability study are given in fig 3. At lower conc., the cell viability of drug solution, cTA-NLC and blank-NLC after 24 h was more than 90%. The % cell viability of cTA-NLC at higher conc. (1 mg/ml) was 94.5%, while the % cell viability of TA solution at higher conc. (1 mg/ml) was 89.76%. However, at lower conc. (0.2 and 0.1 mg/mL) the % cell viability of drug solution was slightly higher than the cTA-NLC. Hence, very negligible amount of cytotoxicity found with cTA-NLC. In the case of blank-NLC, a similar trend was observed, i.e., at higher lipid conc. the % viability is less than lower conc. ($5 < 2.5 < 1 < 0.5$ mg/mL). However, the % cell viability is > 95 % at all the conc. studied. At higher conc. (5 mg/ml of lipid) the % cell viability of blank NLC was 97%. Hence, from the results, it was confirmed that our formulation and all the formulation components are non-toxic, non-irritant and biocompatible in nature.

-Space for Figure 3-

b) Cell uptake study

Cell uptake of dye solution (NR solution), as well as a dye, loaded NLC (NR-NLC) are given in **Fig 4**. From the figure, it is clearly visible that the cell uptake of NR-NLC is higher than the NR solution at 2 h. The intracellular retention assessed after 24 h shows that the cellular retention was very less in case of dye solutions i.e., NR solution. Conversely, a sufficient amount of dye was retained in case of NR-NLC treated cells after 24 h. The NR-NLC treated cells have retained the dye in almost every cells, while very few cells treated with NR-solution have retained the dye. This confirmed the higher intracellular uptake and retention of the NR-NLC than the NR-solution [36, 46].

-Space for Figure 4-

c) Anti-inflammatory effects of formulations

The anti-inflammatory activity of the developed formulation was assessed using TNF- α assay, as this is the principle mediator of inflammation. The cTA-NLC shows lowest TNF- α values i.e., least inflammatory expressions, as compared to other treatments (**Fig 5**). As expected, only LPS treated cells show the highest

levels of TNF- α (\sim 2100 pg/mL), followed by blank-NLC (\sim 1600 pg/mL), TA-solution (\sim 1450 pg/mL) and at last cTA-NLC (\sim 1100 pg/mL). Hence, it was confirmed that our formulation possesses anti-inflammatory activity. The higher anti-inflammatory activity than plain drug solution might be due to higher corneal cell uptake and retention of the formulation (cTA-NLC), than plain drug solution [38]. The high corneal uptake and retention of the cTA-NLC formulation than the plain drug solution could be ascribed to nanosized particles as well as an ionic interaction between positively charged nanoparticles and the negatively charged sialic acid residues in mucus, which increased the mucoadhesion [6,49,50].

-Space for Figure 5-

3.5 Ocular irritancy test

HET-CAM assay was done to assess the ocular irritancy of the developed formulation i.e., cTA-NLC. The results of the HET-CAM assay are given in fig 6, while the irritancy score of various animal treated groups are given in table 2. From the **Fig 6**, it can be clearly seen that the strong irritant (0.1 N NaOH) shows the highest amount of haemorrhage, hyperemia and blood coagulation, while the moderate irritant 1% SDS shows slightly fewer signs of irritation i.e., haemorrhage, hyperemia, etc. The irritation starts early (at 30 s after treatment) and the signs can be visible within 2 min after treatment. The -ve control i.e., normal saline, didn't show any signs of irritation and the vasculature structure and integrity remain the same as original even after 5 min. of treatment. cTA-NLC also shows a similar kind of results with no signs of irritation [41]. The results are in agreement with the cell viability studies, which confirm the non-irritant and biocompatible nature of cTA-NLC.

-Space for Figure 6-

The sum of irritancy score (IS) for haemorrhage, hyperemia and blood coagulation was calculated and are depicted in **Table 2**. As seen in tabulated results, the highest IS was found with strong irritant i.e., 0.1 N NaOH, which is 14.63 ± 1.004 , followed by a moderate irritant (1% SDS), which is 5.37. The least or negligible IS (0.07) was found with negative control (normal saline). cTA-NLC formulation showed nearly similar IS to negative control i.e., 0.103.

The IS results again proved that our formulation is non-irritant and biocompatible in nature.

-Space for Table 2-

3.6 Stability studies

After 6 months of stability studies, no drug crystallization/grittiness was observed in the formulations. No lipid aggregation was observed in the formulation. No significant change in the particle size or PDI was observed in the formulations kept at room temperature. At the accelerated conditions, the drug content decreased only slightly i.e. upto 5% at the end of 6 months, which demonstrated good physicochemical stability of the formulation.

Conclusion

TA loaded positively charged lipid nanocarriers (cTA-NLC) of size around 198 nm and % EE of 88% were developed using biocompatible lipids and surfactants. The developed lipid nanocarriers of TA exhibited slow and sustained *in vitro* release of 84 % in 24 h and *ex vivo* transcorneal permeation of 51 % over a period of 8 h. The cell viability studies showed that the developed nanocarriers exhibited good biocompatibility with human corneal cells with over 95% of cell viability at each tested dose. The biocompatible nature of the formulation is again reflected in the ocular irritancy test, which confirmed the non-irritant nature of the formulation with negligible IS i.e., 0.103. The cell uptake study showed that the dye loaded nanocarriers can get access inside the cells within 2 h and retain inside the cells for over 24 h, however, in the case of plain dye, the retention inside the cells was very less in 24 h. The *ex vivo* permeation in porcine cornea confirmed that the time required for the formulation to deposit into the cornea is 2 h. All the above results suggested that our formulation can penetrate into the deeper eye layers in 2 h and can retain there up to 24 h. Conclusively, our developed formulation (cTA-NLC) worked as a biocompatible carrier for sustained release and transcorneal permeation of drug and exhibited enhanced ocular bioavailability and anti-inflammatory response.

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6. Declaration of interest

All the authors declare that this article content has no conflicts of interest.

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Table 1: Model fitting for the drug release kinetics for cTA-NLC

Formulation	r ² value Release kinetic models				
	Zero order	First order	Higuchi	Korsemeyer-Peppas	Hixon-crowell
cTA-NLC	0.7247	0.4806	0.9155	0.9271	0.8581

Table 2: Irritancy sum scores of various treatment groups

	Treatments with different formulations/irritants			
	Negative control	Positive control		cTA-NLC
		Strong irritant (0.1 N NaOH)	Moderate irritant (1% SDS)	
IS (mean ± SD)	0.07 ± 0.01	14.63 ± 1.004	5.37 ± 0.66	0.103 ± 0.015

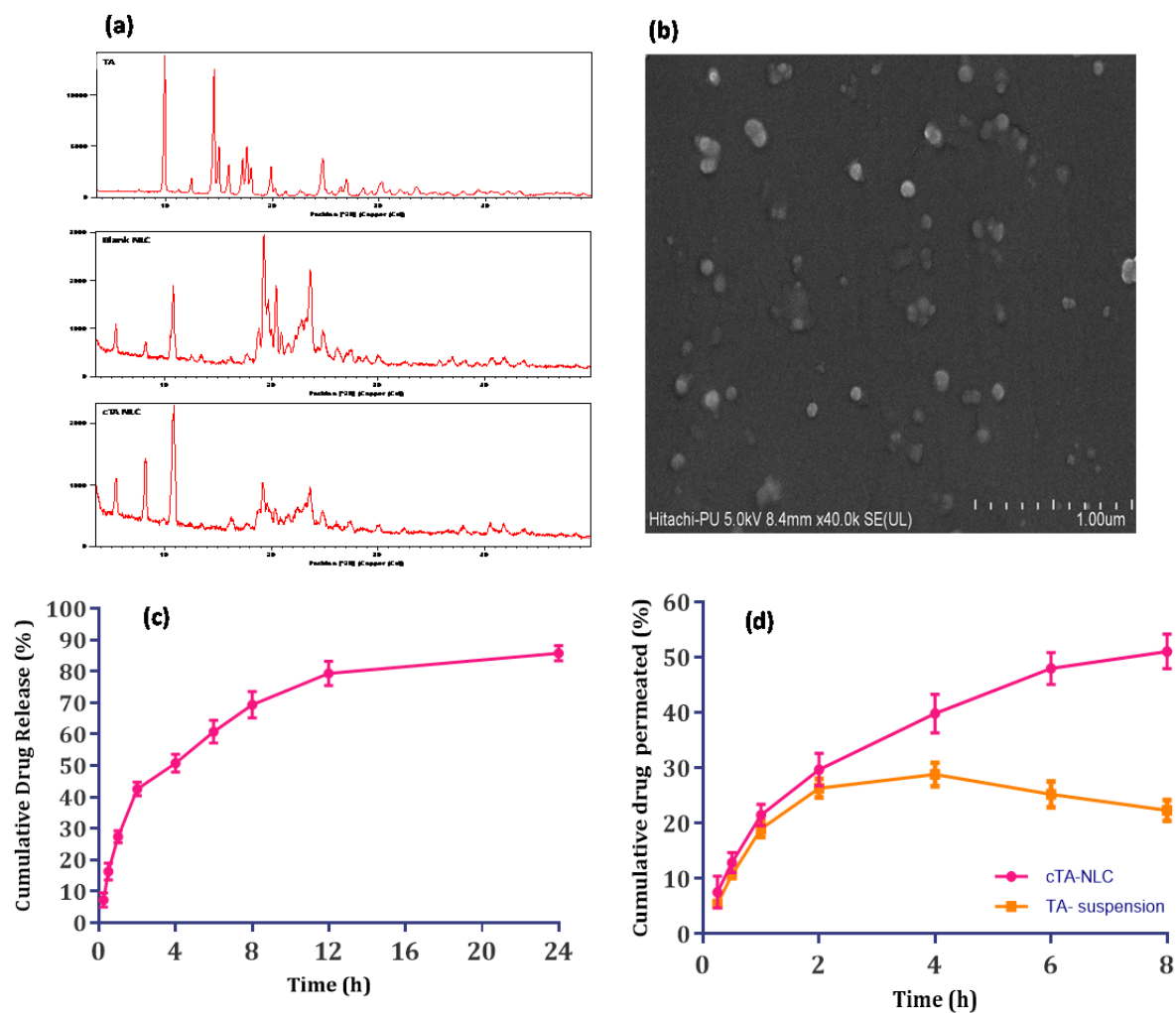


Fig 1: (a) XRD spectra of Drug (TA), Blank NLC and cTA-NLC; (b) FE-SEM image of cTA-NLC; (c) *In vitro* drug release profile of cTA-NLC formulation; and (d) *Ex vivo* transcorneal permeation profile of cTA-NLC formulation

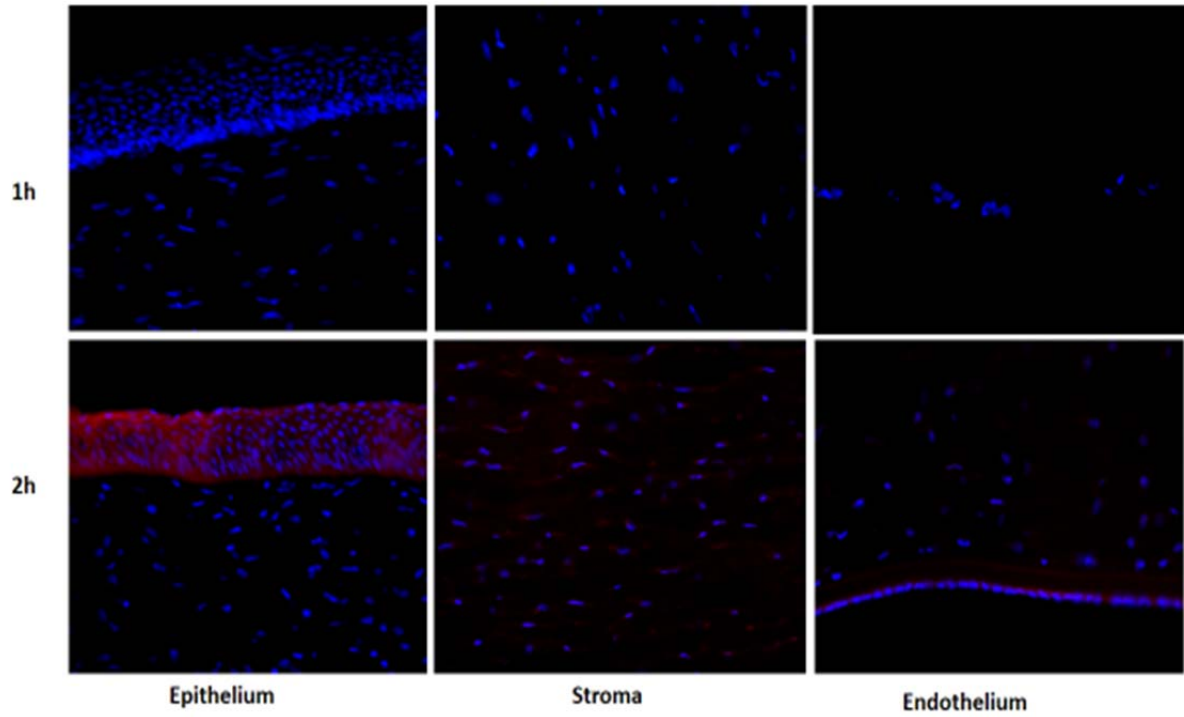


Fig 2: Corneal permeation and distribution study with dye loaded formulation (NR-NLC)

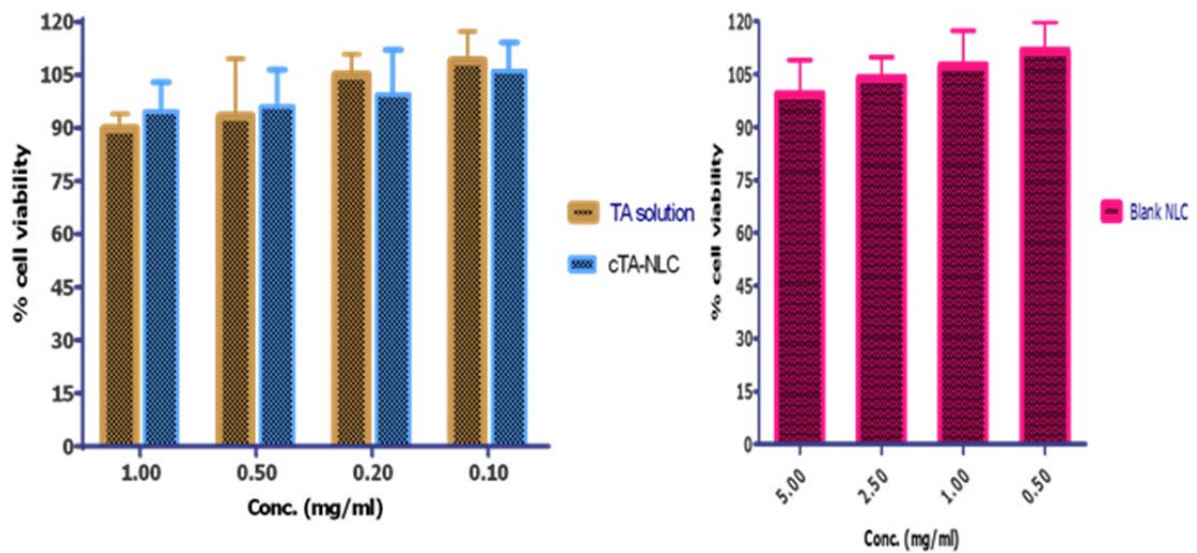


Fig 3: % Cell viability of TA-solution, cTA-NLC and blank NLC formulations at different concentration

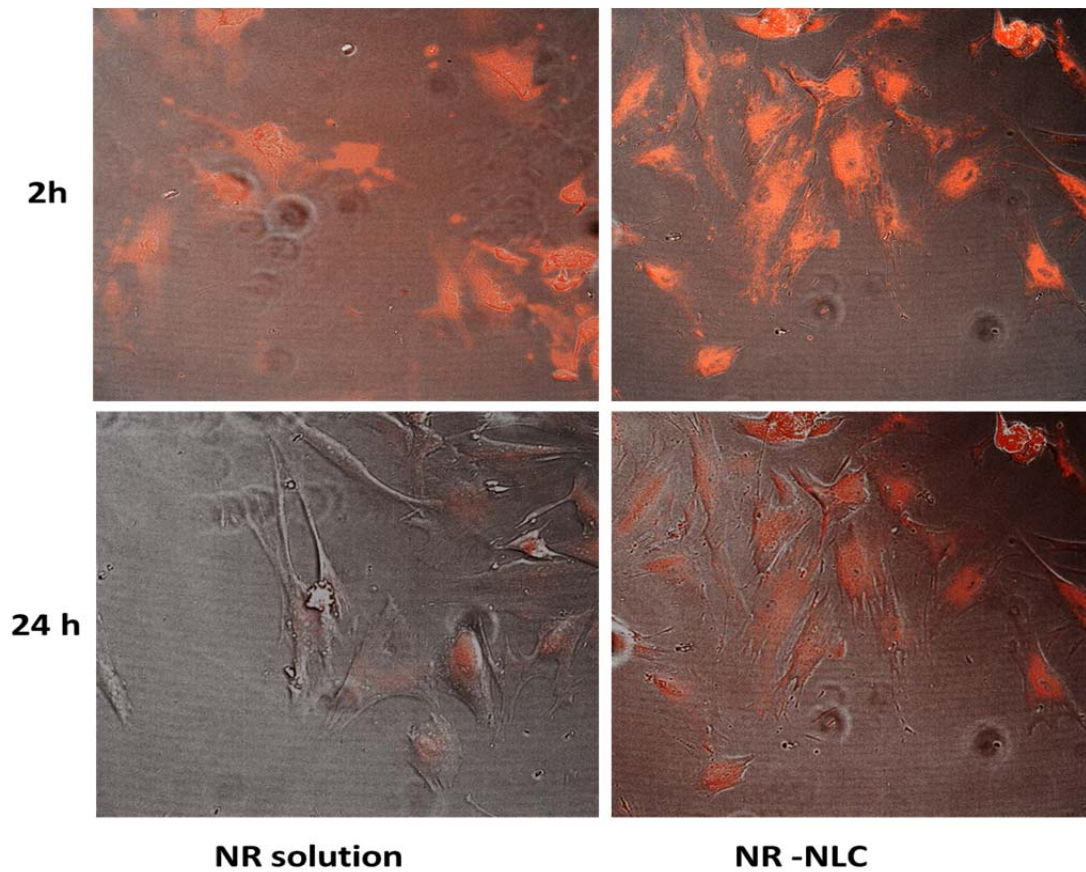


Fig 4: Human corneal fibroblast cells (HCF) uptake images of dye loaded formulations (NR-solution and NR-NLC)

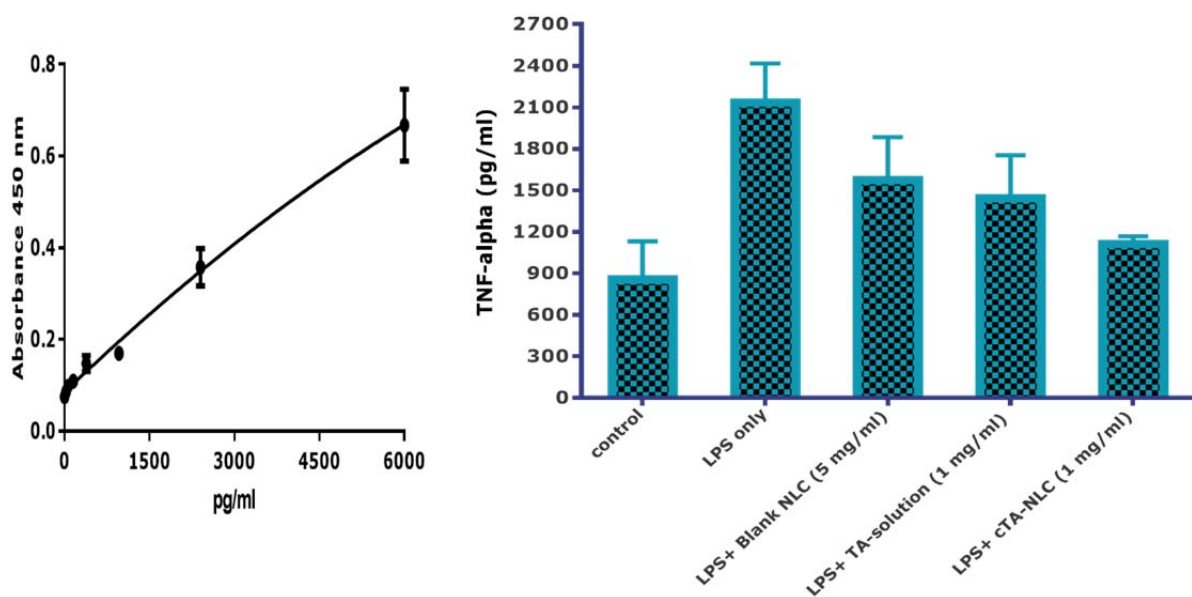


Fig 5: Human TNF alpha assay results of different treatments (LPS only, LPS+ Blank NLC, LPS+ TA solution and LPS+ cTA-NLC)

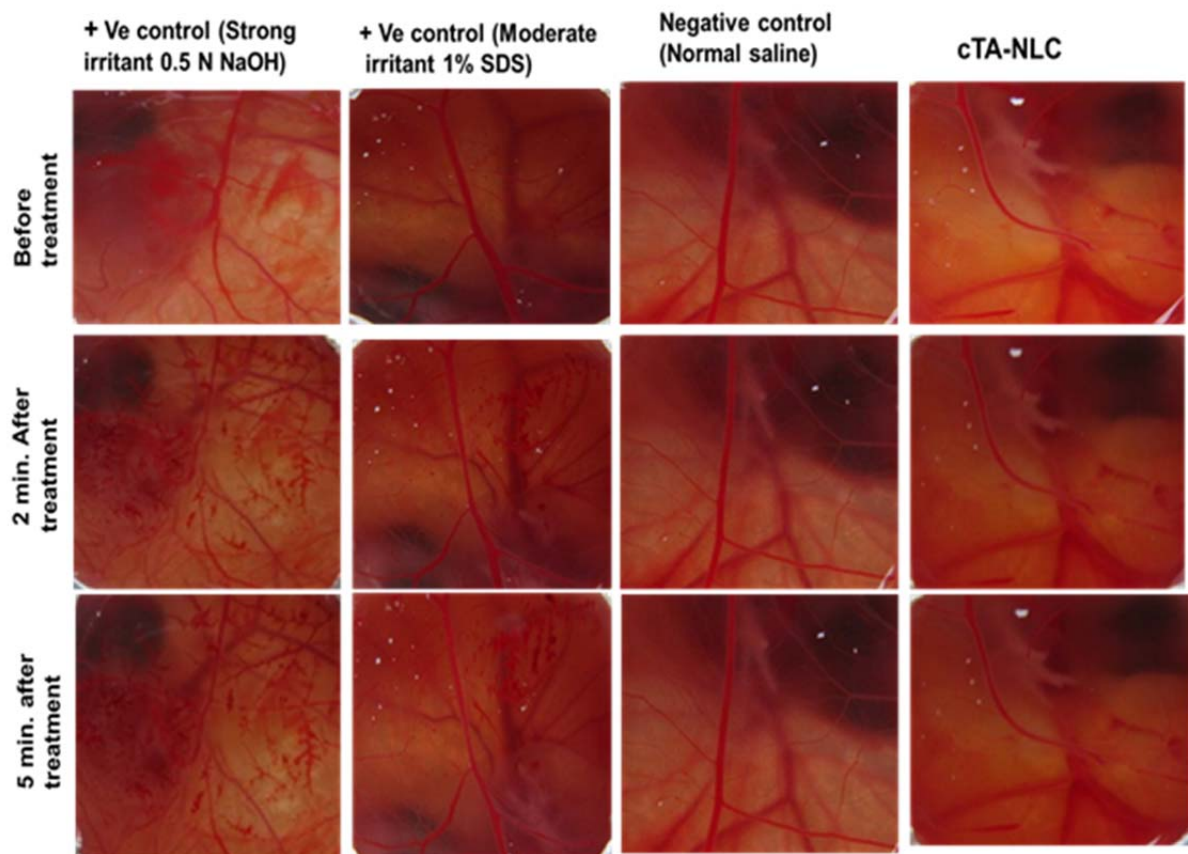


Fig 6: HET-Cam test images of the various treatment groups *viz.* Positive control (0.1 N NaOH- strong irritant, 1% SDS -moderate irritant), Negative control (normal saline) and cTA-NLC