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**Ex vivo and In vivo Evaluation of Chitosan Coated Nanostructured Lipid Carriers for Ocular Delivery of Acyclovir**

Title: (***Ex vivo* and *In vivo* Evaluation of Chitosan Coated Nanostructured Lipid Carriers for Ocular Delivery of Acyclovir**.)

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**Abstract:** Herpes keratitis is the most common infectious cause of blindness in the developed world. It may be treated by acyclovir (ACV), however this antiviral drug is poorly soluble with low ocular bioavailability requiring high and frequent dosing. Nanostructured lipid carriers (NLCs) were investigated to improve the ocular bioavailability of ACV by enhancing corneal penetration as well as prolonging the exposure of infected cells to the antiviral agent. NLCs were fabricated by the hot microemulsion technique and coated with 0.5% w/v chitosan. Cell uptake studies, *ex vivo* tolerance and cell uptake efficacy as well as *in vivo* corneal permeation of the developed lipid based formulations were investigated. NLCs were capable of increasing the cell uptake of encapsulated fluorescein and ACV as examined by fluorescence microscopy and high performance liquid chromatography (HPLC) respectively. When entrapped in NLCs, the antiviral efficacy of ACV was increased by 3.5 fold after 24 hrs of exposure. The *in vivo* corneal permeation of the formulation was studied on Albino rabbits with NLCs capable of increasing the corneal bioavailability by 4.5 fold when compared to a commercially available ACV ophthalmic ointment. In conclusion, NLCs enhanced the ocular bioavailability and antiviral properties of ACV through cell internalization, sustained release, and increased corneal permeation.

**Keywords:** NLCs; Ocular drug delivery; Acyclovir; SLNs.

## 1. INTRODUCTION

Herpes simplex virus (HSV) is a large complex DNA virus from the family herpesviridae, which is ubiquitous and contagious. Herpes simplex type 1 (HSV-1) and 2 (HSV-2) infections are common viral infections seen in patients of every age and ethnicity and can affect almost any organ at any time of the year. A primary ocular infection is often followed by a secondary infection due to the establishment of HSV-1 latency in neurons of the trigeminal ganglion [1]. Once reactivated, HSV-1 can infect the posterior eye via axonal transport and spread to the anterior eye resulting in corneal disease, stromal scarring and blindness.

Acyclovir (ACV) is a potent antiviral agent against HSV-1 but due to its poor bioavailability, frequent administration of high doses is often necessary to attain the minimum effective concentration. Five daily administrations are required when given orally as 200-400 mg tablets while the IV dose is usually 5-10 mg/kg 8 hourly for one week. Topically, a 5% ointment is typically applied on the affected area up to six times a day for one week, often resulting in poor patient compliance.

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Therefore, there is a need to develop a sustained release ocular formulation capable of improving ocular bioavailability as well as reducing the required dose and frequency of administration. We have previously reported that Solid Lipid Nanoparticles (SLNs) and their latest structural modified derivatives, Nanostructured Lipid Carriers (NLCs), are capable of providing sustained ACV release [2]. The resultant particles had satisfactory pharmaceutical characteristics but were negatively charged and exhibited a rather fast drug release profile. As corneal epithelial cells bear negative charges, conversion of NLCs to positively charged particles is expected to significantly increase the precorneal residence time of the formulation and subsequently increase corneal penetration [3]. This approach has also been used for other biomedical applications such as transdermal drug delivery as well as encapsulation of DNA and other bioactive substances that require ionic interactions [4-8]. Moreover, in theory, coating of nanoparticles with a natural polymer should further reduce the drug release rate. Chitosan has previously been used to prepare vesicular drug delivery systems, as permeation enhancer and as coating polymer in the preparation of surface modified ophthalmic formulations. Several ocular drug delivery systems such as microspheres, colloids and nanoparticles have been developed to deliver a wide range of drugs and bioactives [9-14], with the second generation of colloidal drug carriers coated with bioadhesive polymers to improve the transport

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of the encapsulated drugs [14]. Chitosan coated nanoparticles have been found to be more effective in terms of the amount of the drug permeated through the cornea compared to uncoated particles [15]. As well as being a pH-responsive, the amine groups of this polymer can interact with the negatively charged sialic acid groups of the mucin present on the corneal surface, thus resulting in prolonged residence time [16]. Despite the versatility of nanoparticulate drug delivery systems coated with chitosan, only those with an optimal release rate are suitable for ophthalmic use since carrier contact time with the absorption site (corneal epithelium) is short and limited [17]. The emergence of newer preparation techniques such as self-assembly methods for lipid nanoparticle preparation, which for instance requires low energy consumption and is suitable for industrial applications, make lipid nanoparticles a potential and commercially viable drug delivery platform [18-20]. The aim of this study was to coat ACV loaded NLCs with chitosan in order to obtain sustained drug release, increase the corneal residence time and enhance the corneal permeability of ACV.

## 2. MATERIALS AND METHOD (FOR RESEARCH ARTICLES ONLY)

### 2.1. Materials

ACV powder (Jai Radhe Sales, India), Lauroglycol® 90, Compritol® 888 ATO, low molecular weight chitosan (LCH) and Tween® 40 (Sigma, Germany) were used in the preparation of NLCs. Sodium fluorescein (Sigma, Germany), gastric mucin (Hangzhou Dayangchem Co. Limited, China), CoverWell™ perfusion chambers (20 mm×2.5 mm) were purchased from Sigma-Aldrich (New Zealand) and a fluorescence microscope (Axioplan2, Zeiss, Germany) was used for the measurement of precorneal residence time. Zovirax ophthalmic antiviral ointment (GlaxoSmithKline, New Zealand) was used as a control in the *in vivo* release study. Petroleum jelly (Multichem, New Zealand) was used to dilute the Zovirax ointment. Size 26G ½ (0.45 x 13mm) and 21G 1 ½ TW (0.8mm x 38mm) needles (BD PrecisionGlide™ Needles, Singapore) were used to collect ocular fluid samples. Guanosine (Sigma, Germany) was used as high performance liquid chromatography (HPLC) internal standard. Phosphate buffer solution (PBS) and all other chemical used were of analytical grade.

### 2.2. HPLC method of detection:

HPLC was used to analyse ACV concentrations based on a previously published method [21]. An Agilent 1200 series (Agilent Technologies, Germany) equipped with a quaternary pump, a vacuum solvent microdegasser, an autosampler with 100-well tray and an online diode array detector were employed [22]. The output signal was monitored and processed using ChemStation software (Agilent Technologies, Germany). The mobile phase comprising of acetonitrile and 0.05 M ammonium acetate with a pH of 5.4 using acetic acid (3:97, v/v) was pumped through a cosmosil packed column (5C18-AR, 4.6×250 mm, Nacalai Tesque, Japan) at a rate of 1 mL/min at 25 ºC with a detection wavelength of 254 nm. Calibration curves were linear over the concentration range of the study ($r^2$=0.999).

### 2.3. Preparation of NLCs

NLCs were prepared according to the previously published hot microemulsion technique [2]. Briefly, ACV (7% w/w) was dispersed in the liquid lipid (Lauroglycol® 90) now before adding the solid lipid (Compritol® 888 ATO) (58% w/w). The solid lipid was then melted at 80 ºC which was 10 ºC above its melting point. The aqueous phase containing the surfactant (Tween® 40) was heated to the same temperature. A primary o/w emulsion was formed by homogenisation (3 min at 20,500 rpm using an Ultra-Turrax T-10, IKA, Germany) of the lipid melt in the aqueous phase. The hot emulsion was then cooled in an ice bath and homogenisation was continued for 7 min at 20,500 rpm using an Ultra-Turrax T-10 until NLCs were formed. Particles were separated by ultracentrifugation at 30,000 rpm for 30 min and washed with cold water to remove excess surfactants. The purified particles were dispersed in 5 mL of cold Milli Q water and were deep-frozen overnight. Subsequently NLCs were lyophilised at -20 ºC at below 0.133 mBar for 24 hrs to obtain a dry powder (Labconco-7806020, USA). NLCs were coated by dispersing them in different concentrations of chitosan solutions (0.1, 0.25, 0.5 and 1% w/v of LCH) at a 1:5 (w/v) ratio and stirring at 600 rpm for 20 min at ambient temperature.

The fluorescein containing NLCs were prepared by replacing the drug with sodium fluorescein and the whole procedure was carried out in a dark room to avoid photobleaching. The concentration of fluorescein in NLCs was 0.01 mg $mL^{-1}$.

### 2.4. Characterisation of NLCs

The average particle size and zeta potential of nanoparticles were measured immediately after preparation by the light scattering technique (Malvern Zetasizer, ZEN 3600, Malvern Instruments, UK). The entrapment efficiency (EE%) was determined in triplicate using the total amount of drug added and the amount of drug in nanoparticles according to the following formula:

EE%= (Amount of drug in nanoparticles)/(Total amount of drug) ×100

The amount of drug in NLCs was measured after extracting the drug from the particles by phase separation using PBS: octanol (1:3 v/v) where the NLCs were initially dissolved in octanol prior to the addition of PBS [23, 24]. The amount of drug in a known volume of PBS was then determined by HPLC.

### 2.5. Ex vivo measurement of precorneal residence time

A system based on the *ex vivo* method for evaluation of precorneal residence of topical ophthalmic formulations was used with some modifications [25]. It consisted of a perfusion chamber, two precision pumps to pump in/out the perfusion solution and a fluorescence microscope. Rat eyes were used as the biomembrane in this study. The cornea was removed immediately after euthanizing the animal and placed on a glass slide. The mucin solution (5 µL, 4% w/v) was added onto the surface of the cornea followed by 5 µL

of fluorescein containing NLCs. Subsequently, the perfusion chamber was attached to the cover slip and the pumps were turned on to allow the perfusion medium (PBS, pH 7.4) to flow over the corneal surface at a rate of 4 µl $mL^{-1}$. Fluorescent images of the cornea were taken at 30 sec intervals for 60 min and the fluorescein intensity was measured with ImageJ software (National Institutes of Health, USA).

### 2.6. In vitro drug release

*In vitro* release studies were carried out using Franz-type diffusion chambers (Logan Instruments, USA) at 34 ± 1 ºC under stirring at 100 rpm. A cellulose dialysis membrane with a molecular weight cut-off of 12,400 Dalton was used as diffusional barrier. Membranes were soaked in PBS overnight and washed with water before being mounted onto the diffusion cells. The receptor compartment was filled with freshly prepared PBS of pH 7.4. Samples were taken from the receptor chamber at predetermined time intervals and replaced with an equal volume of fresh buffer to maintain sink conditions. The amount of drug diffused was determined using HPLC [2].

### 2.7. Ex vivo drug permeation through the cornea

Experiments to determine drug permeation through the cornea were conducted using Franz-type diffusion chambers under the same condition as described above, except that bovine corneas were used instead of the cellulose membrane. Bovine eyes were collected from Auckland Meat Processors Limited (Auckland, New Zealand) immediately after slaughtering of the animal and corneas were excised carefully to avoid any damage. Corneas were then placed between the donor and receptor compartments.

The apparent permeability coefficient was calculated using the following equation [26]:

$$P_{app} = \Delta Q/\Delta t*(3600)*A*C_0$$

where $\Delta Q/\Delta t$ is the permeation rate (µg $h^{-1}$) obtained from the slope of the linear line of corneal penetration of ACV versus time, 3600 is the unit conversion from hrs to sec, $A$ is the sample surface area (1.7 $cm^2$) and $C_0$ is the initial concentration of ACV in the carrier system (µg $mL^{-1}$).

### 2.8. MTT cell proliferation assay

A MTT stock solution (5 mg $mL^{-1}$) was prepared in RPMI-1640 without phenol red, filtered through a 0.2 µm filter and stored at 2-8 °C. A MTT working solution was prepared by a 1:10 dilution of the 5 mg $mL^{-1}$ stock. Thereafter, cultured Vero cells were washed with warm RPMI-1640 without phenol red before incubating with MTT working solution at 37 °C for 2 hrs. The converted dye was then solubilized with 1 mL of acidic isopropanol (0.04 M HCl in absolute isopropanol), transferred into a 1.5 mL Eppendorf tube and centrifuged at 13,000 rpm for 2 min. The supernatant was transferred into a new Eppendorf tube. Absorbance of the converted dye was measured at 570 nm with background subtraction at 650 nm.

### 2.9. Determination of the antiviral efficacy of ACV loaded NLCs

Monkey kidney cells (CV-1) were used for the antiviral efficiency assay. Cell cultures were grown and maintained in DMEM supplemented with 10% v/v FBS and 1% w/v penicillin-streptomycin. HSV-1 was a hospital isolate amplified in human fetal fibroblasts that stained positive for a HSV-1 specific antibody. The virus titre was determined by a plaque reduction assay expressed as plaque forming units (pfu $mL^{-1}$). Cells were infected by allowing them to absorb the virus for 2 hrs at 37 ºC. The non-absorbed virus in solution was then removed and cells were washed with culture medium. A plaque assay was then performed to measure the number of infectious virus particles. Plaques were produced in a monolayer of CV-1 where a single virus infects a cell and the progeny produced infects the neighbouring cells.

The antiviral efficacy of NLCs containing ACV was measured by a plaque reduction assay of HSV-1 replication in Vero cells. Concentrations of released drug necessary to reduce plaque formation by 50% ($EC_{50}$) was measured and compared to an ACV solution. Briefly, cells were plated in six-well plates and incubated at 37 °C. When cells reached confluence, the medium was aspirated and a quantity of virus sufficient to yield 20 to 30 plaques per well was added. After removal of the inoculum (1 hr), cells were incubated in growth medium containing the NLCs at a range of concentrations. When plaques were formed, monolayers were stained with 1% w/v crystal violet in 20% v/v methanol and plaques were counted using a stereomicroscope. The $EC_{50}$ was calculated using Prism software by comparing drug-treated with untreated cultures.

### 2.10. Cellular uptake of fluorescein-loaded NLCs

Primary human corneal epithelial cells (HCEC) were plated in a 24-well plate as a monolayer on round coverslips at a density of $4\times10^4$ cells/well at 37 ºC in a 5% $CO_2$ and 95% air atmosphere and incubated for 24 hrs to attach. Dulbecco modified Eagle's medium (DMEM), supplemented with 15% v/v heat inactivated fetal bovine serum (FBS), 10 ng $mL^{-1}$ human epidermal growth factor (EGF), 5 µg $mL^{-1}$ insulin, 5 µg $mL^{-1}$ human transferrin, 0.4 µg $mL^{-1}$ hydrocortisone, 2 mM L-glutamine and 100 U $mL^{-1}$ penicillin–100 µg $mL^{-1}$ streptomycin was used as the culture medium. When the cells were at least 90% confluent, they were treated with fluorescein-loaded NLC dispersions in a supplement free culture medium. The concentration of lipid and fluorescein in the samples was maintained at 4.0 and 0.01 mg $mL^{-1}$ respectively. The control was incubated with the fluorescein solution (0.01 mg $mL^{-1}$) in a supplement-free culture medium.

At different time points (0, 2, 4 and 12 hrs) cells were washed three times with PBS (pH 7.4), cover slips were mounted on a glass slide and observed under a fluorescence microscope. Each treatment was performed in triplicate and at least three independent fields of each slide were photographed using a confocal laser scanning microscope (FV1000, Olympus, Germany).

### 2.11. Cellular uptake of ACV-loaded NLCs

HCEC cells were grown as described above with 1 mL DMEM in each well before inoculation with 10 µL of the following test solutions: chitosan coated NLCs, uncoated NLCs and ACV suspension all containing 0.3% w/v of ACV. At specific time points (0.5, 1, 2 and 4 hrs), the medium was removed and cells were washed with PBS. A 200 µL volume of trypsin was added to each well and the contents were centrifuged at 12,000 rpm for 10 min. Sedimented cells were re-suspended in 150 µL of PBS and freeze-thawed thrice to lyse the cell membranes. A volume of 100 µL of the cell suspension was diluted with 100 µL of PBS, vortexed for 5 min and centrifuged at 12,000 rpm for 10 min. The resultant solution was subjected to HPLC to quantify the amount of ACV taken up by the cells. The protein content of the cells was measured using a Coomassie Blue staining protein assay kit. The uptake of the drug was calculated using the following equation:

$$\text{Drug uptake } (\mu g\ mg^{-1} \text{ protein}) = C/M$$

Where C is the intracellular concentration of ACV and M is the unit weight (mg) of cellular protein. All experiments were carried out in triplicate.

### 2.12. In vivo evaluation of ACV loaded NLCs

New Zealand albino rabbits (3-4 kg) were used in accordance with the University of Auckland guidelines for the use of laboratory animals in research and were checked for any signs of ocular damage. Rabbits were confined in standard rabbit cages in a light and temperature controlled room and were free to move with no restriction of food, water or eye movements. Animals were divided into five groups: the first group received the commercially available 3% w/v ACV ophthalmic ointment, the second group received a 0.3% w/v ACV ophthalmic ointment diluted in petroleum jelly, the third group received 0.3% w/v ACV loaded NLCs in the form of an ocular suspension and the fourth and fifth group received a 3% w/v and 0.3% w/v ACV suspension in buffer respectively. Therefore, 36 animals were used in each study group from which six animals were humanely killed at each time point by injection of an overdose of sodium pentoparbitone (300 mg $mL^{-1}$) into the marginal ear vein.

The formulation (20 µL) was instilled in the cul-de-sac of the eye by pulling the lower lid forward. After instillation, the lower lid was lifted back and forth to allow mixing of the formulations with the lacrimal fluid [21]. At specific time intervals (0.5, 1, 2, 4, 6 and 24 hrs) six animals were euthanized and aqueous humour samples were obtained by a syringe using a 26G ½ needle. An appropriate amount of the internal standard, guanosine, was added to each vial and 150 µL of collected aqueous humour samples were treated with 150 µL of 2% w/v zinc sulphate. The mixture was vortexed for 1 min and centrifuged at 6,000 rpm for 20 min [9, 27, 28]. The clear supernatant was filtered through a 0.22 µm filter and 50 µL were injected into the HPLC for analysis with guanosine used as HPLC internal standard.

### 2.12. Statistical analysis

Student t-test was used to analyse data obtained from two populations at the 0.05 level of significance while one-way analysis of variance (ANOVA) was used for analysis of more than two populations by GraphPad software.

## 3. RESULTS AND DISCUSSIONS

### 3.1. Effects of chitosan coating on NLC characteristics

Chitosan is expected to cover the surface of the NLCs in a saturable process with the negatively charged lipid Compritol providing sufficient negative charge to attract the positively charged chitosan molecules. The finished product can be in the form of a freshly formed suspension for immediate application or a freeze-dried redispersible powder providing longer shelf life [29, 30].

### 3.2. Freeze-dried formulation

Table 1 lists the effect of increasing chitosan concentrations on particle size and zeta potential of freeze dried NLCs, with coating resulting in a significant increase in the particle size ($P<0.05$). It is apparent that particles coated with the lowest concentration of chitosan (0.1% w/v) had the largest size compared to uncoated particles. This may be due to a very low zeta potential (3.86 ± 0.73) leading to particle-particle attraction and aggregation. Generally, a value close to ±30 mV is considered optimal to stabilize a nanosuspension where particle-particle repulsion forces inhibit aggregation of the nanoparticles [31]. Increasing the concentration of chitosan resulted in an increase in the zeta potential and a decrease in particle size, although NLCs coated with 0.25 and 0.5% w/v chitosan solution remained in the micro size range. Coating of freeze-dried NLCs resulted in agglomeration and the formation of microparticles similar to what was reported by Dharmala [32]. Therefore, to obtain chitosan coated particles in the nano-size range freshly prepared nano-suspensions were used.

**Table 1** Physiochemical properties of freeze-dried NLCs coated with different LCH concentrations (n=3, mean ± SD).

| Chitosan (% w/v) | Zeta potential (mV) | Particle size (nm) |
|---|---|---|
| 0 | -32.16 ± 3.39 | 801.33 ± 84.05 |
| 0.1 | 3.86 ± 0.73 | 4690.00 ± 86.86 |
| 0.25 | 30.26 ± 1.40 | 4439.00 ± 252.93 |
| 0.5 | 45.96 ± 1.70 | 3084.00 ± 725.53 |

### 3.3. Freshly prepared formulation

Table 2 lists the physiochemical properties of freshly prepared coated and uncoated NLCs. Plain NLCs were 323.33 ± 14.6 nm in diameter with the diameter increasing as a result of surface coating with chitosan. At relatively low concentrations of chitosan (0.1% w/v), the particle size increased significantly ($P<0.05$) as the induced positive charges were not sufficient to stabilize the NLC suspension [33]. In other words, due to the low surface charge, particle-particle attraction forces were strong and resulted in aggregation of the nanoparticles.

However, as the concentration of chitosan increased, the positive surface charges also increased resulting in particle-particle repulsion. This led to the formation of a stable suspension in which the nanoparticles did not aggregate and therefore the mean particle size was increased only slightly compared to uncoated NLCs. The zeta potential of the NLCs was converted from -25.5 ± 1.65 to +28.1 ± 0.72 mV confirming successful coating. Statistical analysis showed that the change in particle size was significant for all samples ($P<0.05$).

Table 2 also lists the ACV entrapment efficiency of the coated formulations. When compared to the uncoated particles, the slight decrease in the EE% was insignificant after coating ($P>0.05$). This means that the coating did not interfere with the encapsulated drug, although some of the drug adsorbed onto the surface of the nanoparticles might have been lost in the aqueous solution of chitosan leading to the slight decrease in EE% [33].

**Table 2** Physicochemical properties of fresh NLCs coated with different chitosan concentrations (n=3, mean ± SD).

| Chitosan (% w/v) | Particle size (nm) | Zeta potential (mV) | Entrapment efficiency (%) |
|---|---|---|---|
| 0 | 323.33 ± 14.60 | -25.50 ± 1.65 | 90.54 ± 6.74 |
| 0.1 | 529.90 ± 86.86 | 5.03 ± 1.01 | 85.63 ± 5.14 |
| 0.25 | 444.73 ± 21.16 | 17.56 ± 0.85 | 86.32 ± 4.51 |
| 0.5 | 457.30 ± 44.38 | 28.10 ± 0.72 | 84.76 ± 1.23 |
| 1 | 467.46 ± 56.25 | 28.20 ± 0.57 | 87.94 ± 1.93 |

### 3.4. Corneal perfusion study and ex vivo corneal residence time

Figure 1 shows the fluorescent images obtained at different time points when various NLCs loaded with fluorescein were applied onto the surface of the rat corneas illustrating a typical fluorescein intensity decay profile for each formulation. One of the advantages of NLCs is that they are capable of entrapping both hydrophilic and lipophilic molecules. This allows the hydrophilic sodium fluorescein to be entrapped within the NLCs internal spaces. Images were taken from the center of the rat excised cornea placed in the perfusion chamber. The decrease in fluorescence intensity over time predicts the precorneal residence of the formulations [25].

A 0.01 mg $mL^{-1}$ solution of fluorescein sodium used as control was completely flushed away from the cornea after only 15 min with the image being completely dark at 30 min. NLCs, however, retained the dye on the surface of the cornea for an extended period of time. As the concentration of chitosan increased from 0.1 to 0.5% w/v the corneal residence time also increased significantly ($P<0.05$) due to interactions of the positively charged particles with the negative charges on mucin and epithelial cells. NLCs coated with 0.5% w/v chitosan solution retained the fluorescent dye even after 45 min of exposure as shown in Figure 1.

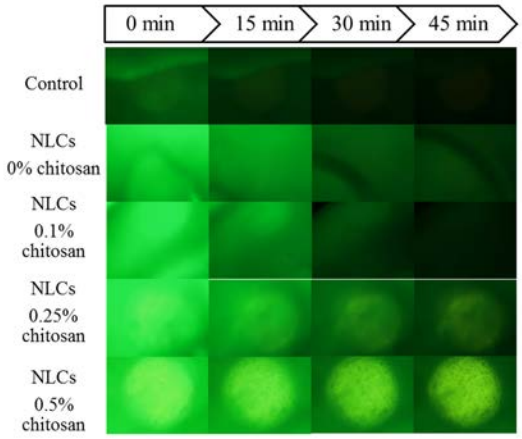


**Figure 1** Precorneal residence of the fluorescein loaded formulations over time.

Figure 2 shows the fluorescein decay as a function of time. To quantitatively analyze the data obtained from the corneal perfusion study, the fluorescence intensity of each concurrent image taken at 30 sec intervals was integrated using ImageJ software (National Institutes of Health, USA). It is evident from the obtained data that coating of NLCs with an increasing chitosan concentration resulted in increased bioadhesion and extended corneal residence time. The bioadhesive nature of chitosan, its cationic charge and increased viscosity of the system hereby contributed to the increased surface adhesion of the nanoparticles. Other studies have also shown that the addition of a bioadhesive coating can significantly increase the precorneal retention time of NLCs [34]. In a study by Shen [34], a dramatic increase in periocular retention time of NLCs coated with a thiolated non-ionic surfactant, cysteine-polyethylene glycol stearate (Cys-PEG-SA) was demonstrated for up to 6 hrs.

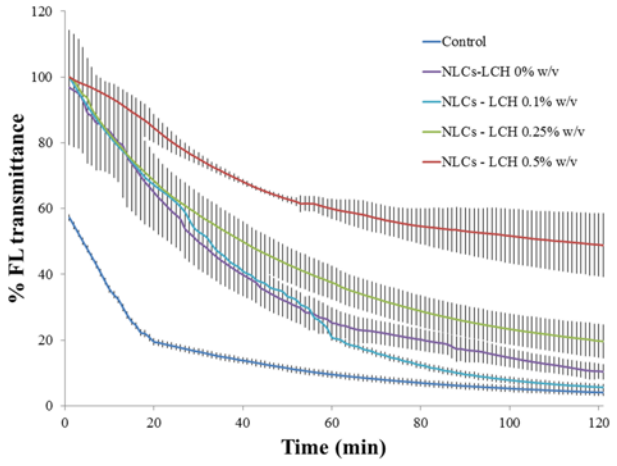


**Figure 2** Precorneal residence time of fluorescein loaded formulations as a function of time measured by ImageJ software.

### 3.5. In vitro drug release

The *in vitro* drug release profiles of different formulations are shown in Figure 3. The ACV suspension in PBS pH 7.4 freely passed through the artificial membrane proving that

drug diffusion through the artificial barrier was not the rate limiting factor. The freshly prepared NLCs had a slower drug release profile compared to the freeze-dried particles. However, after coating NLCs with chitosan, there was a 25% reduction in the drug release rate. The chitosan coat is insoluble at physiological pH and retains its integrity and therefore restrains drug release [17]. Although water insoluble, the chitosan coating layer absorbs water and swells which provides hydrated channels for slow release of the drug over time [35]. Figure 3 reveals that increasing the concentration of chitosan did not influence the release parameters significantly (P<0.05).

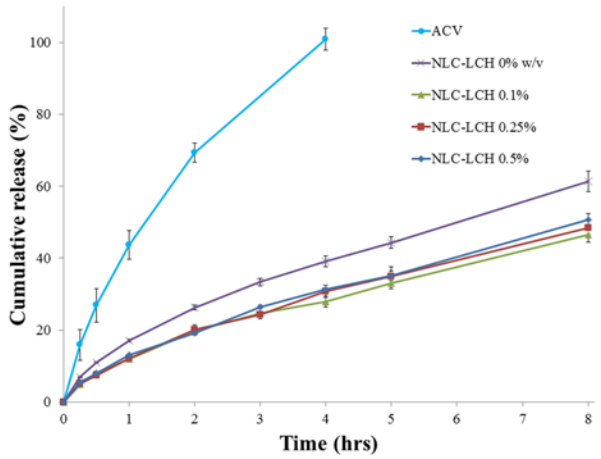


**Figure 3** *In vitro* release profiles of ACV suspension, uncoated NLCs and NLCs coated with 0.1 , 0.25 and 0.5 % w/v chitosan (n=3, mean ± SD).

### 3.6. Ex vivo corneal penetration studies

Bovine eyes were used in *ex vivo* corneal permeation studies because they are easily obtained and the diameter allows a proper fit over Franz cells [36, 37]. Figure 4 displays the *ex vivo* ACV corneal penetration profiles for the different formulations.

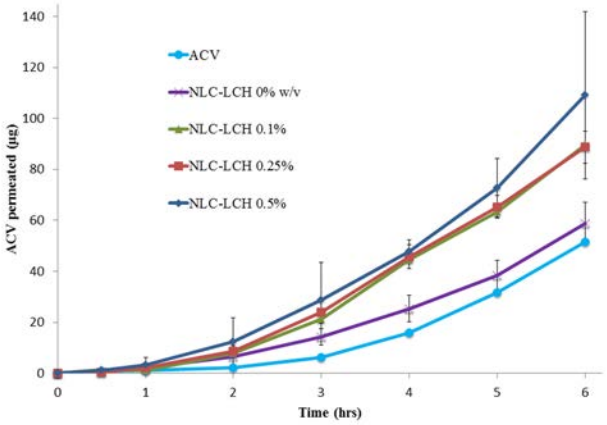


**Figure 4** Corneal penetration profile of the ACV suspension, uncoated NLCs and NLCs coated with 0.1, 0.25 and 0.5% w/v chitosan (n=3, mean ± SD).

The ACV control suspension had the slowest penetration rate but inclusion of ACV into NLCs slightly improved its corneal penetration due to their lipophilic nature [2]. There was a slight improvement in $P_{app}$ of ACV from 8.78 ± 0.01 to 9.12 ± 1.16 which was insignificant (P>0.05). Coating of NLCs with chitosan and increasing its concentration also resulted in an insignificant increase in the drug permeation through excised bovine corneas (P>0.05). However, NLCs coated with 0.5% w/v chitosan had the maximum penetration through the cornea ($P_{app}$=14.74 ± 5.03) and there was more than a 1.5 fold increase in permeability when compared to the ACV suspension although the difference was statistically insignificant (P>0.05). In addition to its bioadhesive nature, chitosan is also a cell membrane penetration enhancer [38], which has been attributed to the modulation of the tight junction barriers between corneal epithelial cells [39] and also intracellular routes [17, 40]. In a recent study, NLCs coated with chitosan oligosaccharides also improved corneal penetration of a model drug, flurbiprofen, by 2.4 folds compared to uncoated NLCs supposedly due to opening of corneal epithelial tight junctions by chitosan [41]. There is no established data for the exact mechanism of chitosan mediated increased corneal epithelial permeation although it has been illustrated in Caco-2 cells that chitosan causes a reversible, time and dose dependent decrease in trans-epithelial electrical resistance by affecting paracellular and intracellular pathways of epithelial cells in a reversible manner without causing cellular toxicity [40].

### 3.7. MTT cell proliferation assay

The MTT cell proliferation assay on Vero cells showed that the cytoxicity increased with an increase in the concentration of NLCs used. The $LD_{50}$ of ACV loaded NLCs was 1086 µg $mL^{-1}$ which closely resembles values reported for other lipid based nanocarriers [42]. The $LD_{50}$ of the empty NLCs was 1015 µg mL-1 which was not significantly different to that of the drug loaded NLCs (P>0.05). This shows that the drug itself does not impose any toxic effects on the cells. The $LD_{50}$ of the chitosan coated NLCs was 1047 µg $mL^{-1}$ which was not significantly different to the values obtained for the uncoated nanoparticles (P>0.05).

### 3.8. Antiviral efficacy of NLCs

The antiviral efficacy of the NLCs was determined after 24 and 48 hrs of treatment on a monolayer of Vero cells infected with the purified HSV-1 viral stock. The ACV containing NLCs exhibited a concentration dependent plaque reduction profile. Figure 5 shows the dose response curve of the ACV suspension and the ACV containing NLCs after 24 and 48 hrs of treatment respectively. The effective concentration to achieve 50% inhibition of viral replication ($IC_{50}$) was calculated by plotting the Log concentration versus plaque reduction graphs. Encapsulation of ACV in NLCs resulted in a 3.5-fold reduction in the required dose to achieve 50% reduction in viral plaques. The $IC_{50}$ of ACV in suspension versus ACV in NLCs was 0.018 ± 0.010 versus 0.063 ± 0.030 after 24 hrs and 0.017 ±0.020 versus 0.061 ± 0.010 after 48 hrs of treatment. Based on these results, the antiviral efficacy of ACV was increased when formulated as NLCs.

NLCs are thought to enhance the antiviral efficiency of ACV by enhancing drug penetration into infected cells as well as

prolonging the exposure of the infected cells to the antiviral agent through their sustained release characteristics. Moreover, the membrane perturbation caused by the virus makes the infected cells a natural target for particulate drug delivery systems [43, 44]. The enhanced antiviral property of ACV in NLCs could be due to two different mechanisms: 1) The sustained drug release properties of NLCs which would have increased the exposure time of the infected cells to the drug resulting in a more efficient treatment and 2) the cell internalizing properties of NLCs as the drug in solution would not be taken up by cells in sufficient amounts to eradicate the virus within the cells.

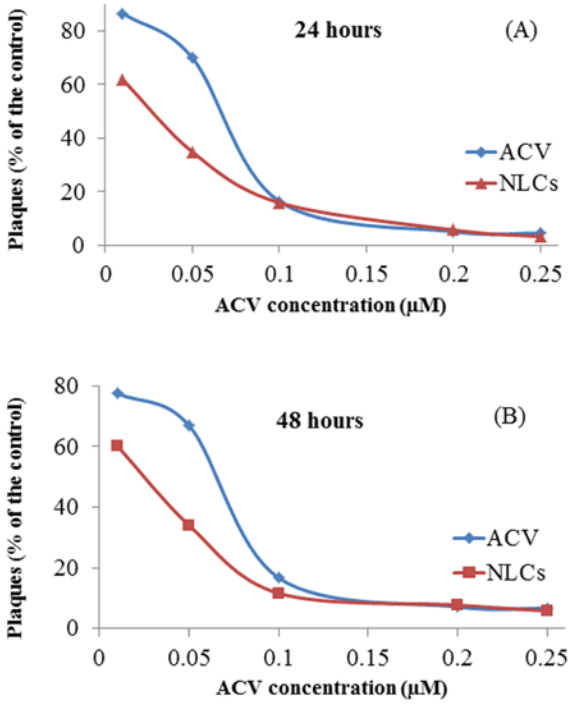


**Figure 5** Antiviral efficacy of ACV in solution (ACV) and ACV encapsulated NLCs (NP) after (A) 24 hrs and (B) 48 hrs treatment.

### 3.9. Cellular uptake of NLCs

Figure 6 shows the fluorescein uptake into corneal epithelial cells after being treated with the control fluorescein solution, fluorescein loaded NLCs and fluorescein loaded chitosan coated NLCs.

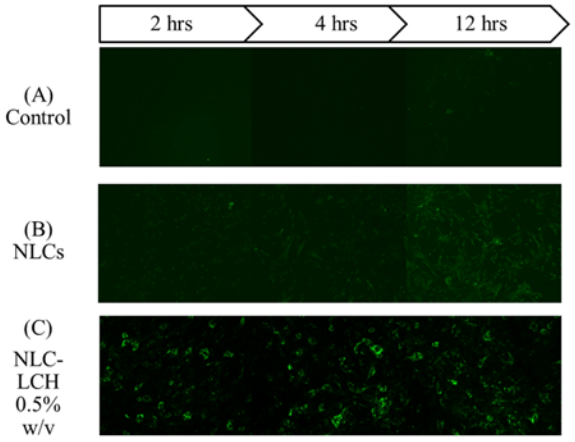


**Figure 6** Fluorescein uptake of (A) control fluorescein solution, (B) NLCs and (C) NLCs coated with 0.5% w/v chitosan after 2, 4 and 12 hrs.

It is evident that cells were confluent and remained impermeable to fluorescein even after 12 hrs of incubation. When cells were treated with NLCs the particles seemed to adhere to the exterior surface of the cells without internalization (Figure 6B). The fluorescein intensity on the cells increased with longer incubation times as NLCs are expected to form a film on the surface of the corneal epithelial cells. A closer look at the fluorescein images displayed in Figure 6C revealed that they differ considerably from the images obtained after treating the cells with the control and plain NLCs. Decisively, addition of chitosan to the formulations allowed the nanoparticles to be internalized with fluorescein present in the cytoplasm of the epithelial cells which seemed to intensify over time. This was expected as chitosan is known for its corneal penetration enhancing properties [38, 45-47].

Figure 7 shows the intracellular ACV concentrations after exposure to the ACV suspension and ACV loaded NLCs (coated and uncoated). The cellular uptake of ACV in chitosan coated NLCs was higher than that of uncoated NLCs and ACV in suspension. The chitosan coated system had the highest drug cellular uptake due to the cell penetration enhancing properties of chitosan. It should be noted that the results obtained here are only a relative measure of ACV cell uptake since the Coomassie Brilliant Blue test used to quantify the total amount of cellular proteins is only semi-quantitative.

**Figure 7** Cellular uptake concentration profile of ACV in solution (ACV), uncoated NLCs (NLCs) and NLCs coated with 0.5% chitosan (NLCs-LCH).

| Formulation | $C_{max}$ (µg mL$^{-1}$ ) | $T_{max}$ (min) | AUC (min µg mL$^{-1}$ ) | Relative AUC |
|---|---|---|---|---|
| NLC-LCH | 1.56 ± 0.43 | 60 | 275.9 ± 70.49 | 4.17 |
| 3% w/v Ointment | 1.80 ± 0.96 | 60 | 252.7 ± 84.42 | 3.82 |
| 0.3% w/v Ointment | 0.52 ± 0.10 | 60 | 66.07 ± 66.06 | 1.00 |
| 3% w/v ACV suspension | 0.42 ± 0.23 | 30 | 44.46 ± 32.10 | 0.67 |
| 0.3% w/v Eye drop | 0.34 ± 0.11 | 30 | 39.78 ± 29.62 | 0.60 |

**Table 3** Aqueous humour pharmacokinetic parameters of the formulations.

Therefore, to establish a control formulation of similar concentration, Zovirax™ ointment was diluted with petroleum jelly to obtain a 0.3% w/v ACV ointment. Figure 8 shows the ACV concentrations in the aqueous humour with the corresponding pharmacokinetic data reported in Table 3. The relative AUC for ACV in NLCs increased 4.17 times when compared to the ointment and 6.93 times when compared to the eye drop. The $C_{max}$ for ACV in NLCs increased by 3 and 4.5 times respectively when compared to the ointment and eye drop controls.

Both ophthalmic ointment and NLCs reached the $C_{max}$ 1 hr after topical administration ($T_{max}$=60 min); however, the eye drop control reached $C_{max}$ 30 min post administration. This is due to the immediate availability of the drug from solution for absorption and penetration across the cornea [50].

### 3.10. In vivo corneal permeation

The only marketed ophthalmic preparation of ACV is in the form of a 3% w/v ointment (Zovirax™) which is due to the low solubility of the drug in aqueous medium. Novel drug delivery systems promise to increase the ocular bioavailability of drugs and by doing so would decrease the required loading dose [48, 49]. The chitosan coated NLCs system under investigation contained 0.3% w/v of ACV which is ten times lower than the marketed ophthalmic ointment.

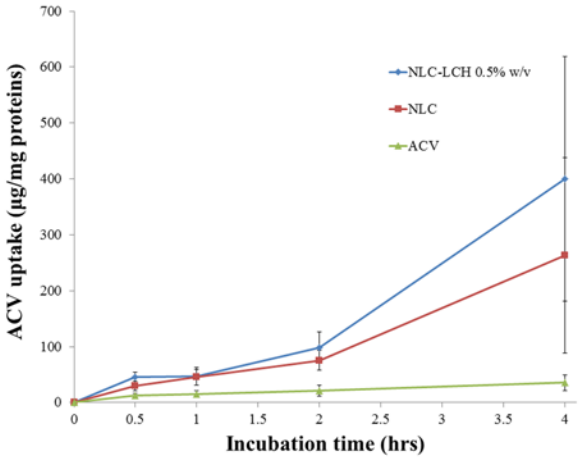


**Figure 8** Drug concentration profile of NLCs coated with 0.5% w/v chitosan (NLCs-LCH 0.5%w/v), a 0.3% w/v ACV eye drop and a 0.3% w/v ophthalmic ointment in the aqueous humour.

Figure 9 shows the aqueous humour ACV profiles for the chitosan coated NLCs when compared to the 3% w/v ophthalmic ointment and the 3% w/v ACV suspension. Although the commercial ointment had ten times more ACV loaded, the AUC for both NLCs and ointment remained similar with the difference being insignificant (P>0.05) (275.9 ± 70.49 and 252.7 ± 84.42 respectively). This

illustrates the low efficacy of the ophthalmic ointment for this particular treatment [51]. By increasing the concentration of the ACV control suspension from 0.3 to 3% w/v, the ocular bioavailability of ACV did not increase significantly (P>0.05) and the time to reach the maximum concentration ($T_{max}$) remained the same. This illustrates that the drug reached super saturated solubility levels and addition of an excess of ACV in the form of a suspension offered no obvious benefit. The $T_{max}$ for the NLCs was very similar to what has been reported for ACV loaded liposomes [21]. This shows that the positively charged chitosan coated NLCs had a controlled and slow penetration through the cornea [21]. Also, NLCs had much higher aqueous humour concentrations when compare to the ointment and eye drop formulations of the matching concentration confirming that the NLCs coated with chitosan enhanced the penetration of ACV through the cornea.

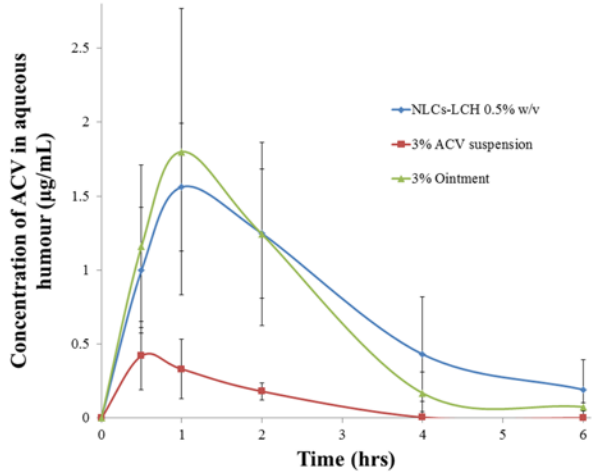


**Figure 9** ACV concentration profiles in aqueous humour after application NLCs coated with 0.5% w/v chitosan (NLCs-LCH 0.5% w/v), 3% w/v ACV aqueous suspension and 3% w/v ACV ophthalmic ointment.

Most nano- and microencapsulated systems are capable of enhancing the aqueous humour permeation profile of ACV when compared to the control solution of matching concentration [21, 27, 28, 51]. Only one study included the commercial ophthalmic ointment as a control [51]. It is vital to maintain a therapeutic concentration of ACV throughout the ocular tissues to eradicate any sign of a deeper ocular infection, to inhibit viral latency development and to avoid vision loss associated with secondary herpes infections [52]. This study has shown that NLCs can deliver increased amounts of drug in cornea, aqueous humour and vitreous humour over an extended period of time in comparison to the commercial ointment.

## CONCLUSION

A novel lipid based nanoparticulate drug delivery system (NLCs) coated with a bioadhesive and biodegradable polymer, chitosan, was designed and developed for ocular delivery of the antiviral drug ACV. In summary, the developed ocular drug delivery system was able to:

- enhance the antiviral efficacy of the drug,
- illustrate no toxicity and thus suitability for ocular drug delivery,
- show increased drug cellular uptake,
- demonstrate a controlled drug release profile *in vitro*,
- increase the *ex vivo* penetration of the drug through the cornea,
- exhibit a prolonged precorneal residence time and
- achieve higher ocular drug bioavailability in comparison to the commercially available ophthalmic ointment.

It is therefore reasonable to state that pharmaceutical nanotechnology has the potential to optimize topical ocular drug delivery of poorly soluble drugs. The commercial potential and ease of the scale up manufacturing process support the recognized pharmaceutical advantage of lipid nanoparticles over conventional ophthalmic dosage forms. This nanoparticulate formulation is a versatile delivery system capable of encapsulating both hydrophilic and lipophilic drugs. Thus, it could serve as a delivery platform for many other drugs including anti-inflammatory agents, antibacterials, gene therapeutics and glaucoma medications.

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