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Design and Development of Solid Lipid Nanoparticles for Ocular Delivery of Acyclovir

Ali Seyfoddin

Abstract

Background and aim: Ocular infections caused by Herpes keratitis remain the leading cause of infectious blindness in the developed world and there is a need for the development of a novel drug delivery system to improve treatment options. The complex structure of the eye poses several challenges which ocular drug delivery methods seek to address. The aim of this thesis was to improve the ocular bioavailability of acyclovir (ACV) by incorporating it into solid lipid nanoparticles (SLNs) and nano-structured lipid carriers (NLCs).

Methods:
SLNs and NLCs were prepared by a hot microemulsion technique. Nanoparticles were characterised by laser diffraction size measurements, zeta potential, scanning electron microscopy (SEM), x-ray diffraction (XRD), Fourier transform infrared (FTIR) and differential scanning calorimetry (DSC). The drug was quantified by high performance liquid chromatography (HPLC) for the measurement of entrapment efficiency, drug loading, in vitro and ex vivo drug release studies. ACV-β-cyclodextrin (ACV-βCD) inclusion complexes were prepared by lyophilisation. Nanoparticles were coated with the biodegradable, cationic and bioadhesive polymer, chitosan and were characterised for cytotoxicity, cellular uptake, antiviral efficiency and stability. Finally, the chosen chitosan coated NLCs formulation was subjected to an in vivo animal ocular pharmacokinetic study.

Results: The prepared nanoparticles were spherical and within the size range suitable for ocular drug delivery. The incorporation of liquid oils in the structure of SLNs resulted in the formation of NLCs with higher entrapment efficiency (91.64%) compared to that of SLNs (11.14%). The complexation of ACV with βCD led to the formation of inclusion complexes with higher solubility. Incorporation of complexes in NLCs resulted in a sustained drug release profile, increased stability and increased ex vivo corneal permeability. Coating
nanoparticles with chitosan inverted the zeta potential from \(-25.5 \pm 1.65\) to \(+28.1 \pm 0.72\), increased stability, prolonged corneal residence time, sustained the drug release and increased \textit{ex vivo} corneal permeation of ACV. Finally, the chitosan coated formulation was selected for \textit{in vivo} animal studies and showed a 4.5 times increase in ocular bioavailability in comparison with the marketed ophthalmic ACV ointment.

**Conclusion:**

The findings of this thesis demonstrated the suitability of lipid based novel drug delivery systems for ocular administration and their capability to increase the ocular bioavailability and therefore antiviral efficacy of ACV.
This thesis is dedicated to my parents, Hamidreza Seyfoddin and Zahra Bozorgian and to my wife Carol Greene without whose support and kindness completing this degree was not achievable. Their support, encouragement and constant love have sustained me throughout my life. This thesis is also dedicated to my younger brother Vahid.
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Publications Arising from this Thesis


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Other publications


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List of Abbreviations

ACV: Acyclovir
AUC: Area under the curve
βCD: β-Cyclodextrin
Da: Dalton
DSC: Differential scanning calorimetry
EE%: Percentage entrapment efficiency
FTIR: Fourier transform infrared
HPLC: High performance liquid chromatography
HSV: Herpes simplex virus
LCH: Low molecular weight chitosan
LDCs: Lipid drug conjugates
mL: Mililiter
μL: Microliter
μm: Micrometer
NLCs: Nanostructured lipid carriers
nm: Nanometer
NMR: Nuclear magnetic resonance
P_app: Apparent permeability coefficient
PBS: Phosphate buffered saline
PDI: Poly dispersity index
PEG: Polyethylene glycol
pH: Negative log hydrogen ion concentration
RPE: Retinal pigment epithelium
rpm: Rotations per minute
®: Registered trade mark

SLNs: Solid lipid nanoparticles

v/v: Volume per volume

w/o: Water in oil

w/v: Weight per volume

WAXD: Wide angle X-ray diffraction
"In literature as in love, we are astonished at what is chosen by others."

— Andre Maurois
1.1. Herpes simplex virus ocular infection

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 type 2 (HSV-2) infections are common types of viral infections seen in patients of every age and ethnicity which can affect almost any organ of the body at any time of the year (Nahmias 1975; Liesegang 2001). However, HSV-1 prevalence is affected by geographical location, socioeconomic status and age (Liesegang 2001). HSV-1 is often acquired in early childhood as an oral infection while HSV-2 is acquired as genital infection in adults (Nesburn, Burke et al. 1994). Ocular infection is usually caused by HSV-1 (Nahmias 1975). “The virus replicates in the skin or mucosal epithelium, infects sensory nerves and is transported to the involved sensory ganglia” (Nesburn, Burke et al. 1994). HSV can remain in the body for a long period of time and recurrences of type 1 and 2 HSV infections are common (Nahmias 1975; Nesburn, Burke et al. 1994). HSV infections should be considered as potential cause of ophthalmia neonatorum and may also play a significant role in complicated cases of xerophthalmia (Whitcher, Srinivasan et al. 2001).

Table 1-1 Types of herpes virus (Miyagawa and Yamanishi 1999; Liesegang 2001).

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<thead>
<tr>
<th>Types of HSV</th>
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<td>HSV type 1</td>
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<td>2</td>
<td>HSV type 2</td>
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<td>3</td>
<td>Varicella-zoster virus</td>
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<td>4</td>
<td>Cytomegalovirus</td>
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<td>5</td>
<td>Epstein-Barr virus</td>
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Ocular infections can be primary or recurrent with lid, conjunctival, corneal, or intraocular infections and retinitis as some of most common ocular complications (Liesegang 2001). 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 (Nesburn, Burke et al. 1994). Once reactivated, HSV-1 can infect the posterior of the eye via axonal transport resulting in corneal disease and stromal scarring; these can be detected by culturing tear films (Newell, Martin et al. 1989; Nesburn, Burke et al. 1994). Recurrent infections can induce scarring as a result of an immune-mediated inflammatory process which usually causes blindness (Smith, McDonald et al. 1980; Nesburn 1983; Newell, Martin et al. 1989). Ocular complications arising from HSV-1 can be classified into blepharitis, conjunctivitis, epithelial keratitis, stromal keratitis, iridocyclitis and retinitis based on the inflammed tissue (Liesegang 2001). However, the most common manifestations associated with HSV ocular infections are acute primary blepharokeratoconjunctivitis and chronic recurrent keratitis (Figure 1-1) (Darougar, Hunter et al. 1978). Typically, superficial ocular infection does not scar, but deeper infections can cause lesions characterised by dendritic keratitis (Nahmias 1975). The infection can be clinically diagnosed with high accuracy, however, conjunctivitis may require laboratory aid as the early stages of acute viral conjunctivitis are difficult to differentiate from bacterial or allergic conjunctivitis (Nahmias 1975; Wishart, James et al. 1984). Morphological examination of infected cells with characteristic multinucleated giant cells and inter-nuclear inclusions, has been the most common method of diagnosis (Nahmias 1975). Epidemiological investigations have revealed that due to its recurrent nature, HSV still remains the most common infective cause of corneal blindness in most developed countries (Darougar, Hunter et al. 1978; Shuster, Kaufman et al. 1981; Wishart, James et al. 1984; Darougar, Wishart et al. 1985; Shimomura, Ohashi et al. 1987; Liesegang, Melton et al. 1989; Wilhelmus, Beck et al. 1998; Liesegang 2001).
1.2. Acyclovir

Acyclovir (ACV), also known as acycloguanosine, is an effective and selective agent against HSV and varicella-zoster virus with very low toxicity (Schaeffer, Beauchamp et al. 1978; Jones, Fison et al. 1979; Davidson, Kaufman et al. 1981; Dorsky and Crumpacker 1987; Miwa, Kurosaki et al. 2005). ACV was the first selective antiviral agent released for clinical use in 1982 which requires a viral enzyme for its activation, therefore, it exerts no substantial toxic effects on uninfected cells (Hung, Patterson et al. 1984; Dorsky and Crumpacker 1987). As once anticipated, ACV is now the prototype of new antiviral drugs which are potent inhibitors of viral polymerase enzymes after activation by viral thymidine kinase (Dorsky and Crumpacker 1987).

1.2.1. Chemistry and mechanism of action

ACV is chemically 9-[2-hydroxyethoxymethyl] guanine (Figure 1-2) which is a synthetic acyclic analogue of 2´-deoxiguanosine (Elion, Furman et al. 1977; Dorsky and Crumpacker 1987).
A systematic process aimed to prepare synthetic acyclic nucleoside analogues led to the discovery of ACV which was found to be active against HSV (Elion, Furman et al. 1977; Schaeffer, Beauchamp et al. 1978; Dorsky and Crumpacker 1987).

DNA elongation occurs via the 3’-hydroxyl group of guanosine. The acyclic ACV acts as a chain terminator of DNA synthesis as it lacks the essential 3’-hydroxyl group (Dorsky and Crumpacker 1987). The mechanism of antiviral selectivity of ACV is through activation by phosphorylation through viral thymidine kinase which has an affinity for ACV 200-fold higher than cellular thymidine kinase. As a result, ACV is $3 \times 10^6$ times more phosphorylated by the viral enzyme (Fyfe, Keller et al. 1978; Davidson, Kaufman et al. 1981; Keller, Fyfe et al. 1981; Dorsky and Crumpacker 1987). ACV triphosphate acts as a suicide inactivator which binds tightly to viral HSV DNA polymerase through a self-limited reaction in the form of a complex which is difficult to dissociate (Dorsky and Crumpacker 1987). ACV triphosphate has 30 to 50-fold greater affinity for the viral enzyme compared with the natural substrate deoxyguanosine triphosphate which makes it highly selective (Figure 1-3) (Dorsky and Crumpacker 1987).
1.2.2. Clinical indications

ACV is used in a range of clinical conditions and is available via the topical, oral and intravenous routes of administration (Dorsky and Crumpacker 1987). It was initially used topically to treat mucocutaneous and primary genital infections when the drug was released in 1982 (Jones, Fison et al. 1979; Hung, Patterson et al. 1984; Dorsky and Crumpacker 1987). Subsequently, intravenous ACV was used to treat mucosal and cutaneous HSV-1 and HSV-2 infections in immunocompromised patients and to treat severe initial genital herpes infections (Dorsky and Crumpacker 1987). A combination of topical and oral administration has been used in the treatment of genital herpes infections where five daily doses are usually required to attain the minimal effective concentration. ACV is also administered intravenously to treat acute retinal necrosis (Carney, Peyman et al. 1986).
Topical use of ACV has been a major route in the treatment of herpetic keratitis (Shiota, Inoue et al. 1979; Laibson, Pavan-Langston et al. 1982; McGill and Tormey 1982; Shiota 1982; Collum, Akhtar et al. 1985; de Vrueh, Smith et al. 1998). Results of a randomized double-controlled clinical trial showed that topical ACV is as effective as the existing drug vidarabine in the treatment of epithelial herpetic keratitis, although neither of them could prevent the stromal changes beneath the epithelial infection (Laibson, Pavan-Langston et al. 1982). It was suggested that a systemically available and safe antiviral drug may be required to heal stromal changes (Laibson, Pavan-Langston et al. 1982). Although, oral ACV has shown to be effective in preventing stromal changes, the development of a novel ocular drug delivery system would expand the clinical indications of ACV. ACV is particularly useful in the treatment of ulcerative herpes keratitis and results of an early clinical trial in Japan have shown that 96.8% of patients had excellent or good responses to ACV treatment (Shiota 1982).

It is also evident that ACV is effective against both herpes simplex and herpes zoster ocular infections and can be used in the treatment of herpes zoster ophthalmicus to reduce the severity and incidents of postherpetic pain and to protect against long term ocular complications (McGill and Tormey 1982; Harding and Porter 1991). It should be pointed out that the severity of HSV infections has increased over the past decade as a result of increased numbers of immunocompromised patients produced by aggressive chemotherapy, expanded organ transplantation and a greater occurrence of HIV infections (de Jalón, Blanco-Príeto et al. 2003).

1.2.3. Dosage form and dosing

ACV is a potent antiviral agent and the dose for 50% inhibition (ED₅₀) is only 0.1 μM (Elion, Furman et al. 1977). However, due to its poor bioavailability frequent administration of large
doses is often necessary to attain the minimum effective concentration. Five times daily administration is 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 applied on the affected area for up to six times a day for one week.

1.2.4. Toxicity and side effects

Due to its high viral selectivity, ACV administered as various dosage forms and via different routes of administration has shown a good safety profile and it is generally well tolerated in humans in a variety of disease states, population types and age groups (Keeney, Kirk et al. 1982; Dorsky and Crumpacker 1987). Inhibition of HSV transformed and non-transformed mouse cells have shown that ACV has higher affinity for viral thymidine kinase and DNA polymerase which makes the drug safe and selective against viral cells (Davidson, Kaufman et al. 1981).

Local irritation and phlebitis have been documented in clinical trials after high dose intravenous injections which is probably due to the basic pH (9-11) of the ACV solution (Keeney, Kirk et al. 1982; Sylvester, Ogden et al. 1986; Dorsky and Crumpacker 1987). Topical administration may cause burning when applied to mucosal surfaces although some of this maybe due to the irritancy of the polyethylene glycol base (Corey, Nahmias et al. 1982; Dorsky and Crumpacker 1987). High intravenous and oral administration can adversely affect renal function (Dorsky and Crumpacker 1987). Thanks to its high affinity for the viral enzyme, there is no significant evidence of teratogenicity or mutagenicity associated with ACV (Clive, Turner et al. 1983; Moore, Szczech et al. 1983; Tucker, Krasny et al. 1983; Tucker, Macklin et al. 1983).
1.2.5. Viral resistance to ACV

Viral resistance to ACV develops by mutation of the HSV thymidine kinase and DNA polymerase (Dorsky and Crumpacker 1987). The most common mechanism is through decreased thymidine kinase production resulting in less virulent progenies with reduced ability to establish latency (Balfour 1983). Other mechanisms, such as decreased DNA polymerase sensitivity to ACV, result in progenies as virulent as wild-type parents and altered substrate specificity for thymidine kinase possibly results in reduction in neurovirulence (Balfour 1983). This is a distinguishing property as in the case of other antiviral agent’s resistant isolates that had increased pathogenicity, whereas resistant HSV isolates have reduced virulence (Bacon, Levin et al. 2003). This possibly explains why resistance to ACV is so rare.

Like all antiviral drugs, resistance to ACV is increasing (Morfin and Thouvenot 2003), but only few resistant viral isolates have been recognized in immunocompromised patients including thymidine kinase-negative and DNA-polymerase mutants (Dorsky and Crumpacker 1987; Bacon, Levin et al. 2003; Miwa, Kurosaki et al. 2005). Although ACV has been used extensively in the past two decades, the prevalence of antiviral resistance in immunocompetent and immunocompromised patients has not significantly increased (Bacon, Levin et al. 2003). It should be noted that ACV is only active against the replicating virus and does not eliminate the latent herpes simplex genome; therefore, recurrences should not be mistaken for viral resistance unless proven (Balfour 1983). The use of ACV in herpes keratitis has caused resistance in only very rare cases merely due to difficulty in maintaining an adequate concentration of the drug at the site of ocular infection which increases the opportunity for the virus to develop drug resistance (Bacon, Levin et al. 2003).
1.2.6. Physiochemical properties and solubility of ACV

ACV is available as a free flowing white crystalline powder with a molecular weight of 225 Da. According to the British Pharmacopeia, ACV is only slightly or sparingly soluble in water, sparingly soluble and soluble in slightly acidic or basic solutions. Its aqueous solubility is reported to be between 1.25-1.3 mg mL$^{-1}$ or 5.6 mM with an octanol to water partition coefficient of 0.018 (Miranda, Krasny et al. 1982; Tucker, Macklin et al. 1983; Dorsky and Crumpacker 1987). Its poor aqueous and lipid solubility are the main factors contributing to its poor oral bioavailability (de Vrueh, Smith et al. 1998) and contribute equally to the poor ocular bioavailability of the drug. It is evident that by increasing the solubility of the drug, or by using more soluble derivatives, one can increase the corneal penetration of ACV (Dias, Nashed et al. 2002).

1.3. Ocular drug delivery

With the advancement of therapeutic agents in recent years ocular drug delivery has become extremely important to treat a variety of ocular conditions (Lang 1995; Ghate and Edelhauser 2006). Moreover, more sophisticated delivery systems such as solid lipid nanoparticles and liposomes have been developed to optimise conventional solution based simple eye drops (Meisner and Mezei 1995; Law, Huang et al. 2000; Cortesi, Argnani et al. 2006; Vega, Gamisans et al. 2008; Araújo, Gonzalez et al. 2009; Basaran, Demirel et al. 2009; Wadhwa, Paliwal et al. 2009; Seyfoddin, Shaw et al. 2010; Shen, Sun et al. 2010; Abdelkader, Ismail et al. 2011). Ocular drug delivery is focused on delivering the drug to either the anterior or posterior chambers. The two routes are entirely different and the focus here is on topical ophthalmic drug delivery. Topically applied formulations are absorbed through the corneal route as well as conjunctival and scleral transfer (Ghate and Edelhauser 2006).
1.3.1. Challenges and obstacles

Drugs have to encounter several obstacles before reaching their target site where they exert their pharmacological effect. The main obstacle is the ability to cross the tissue epithelium while maintaining stability. Novel drug delivery carriers, such as solid lipid nanoparticles (SLNs), can help to alleviate these problems. SLNs are important because it was found that one of the criteria for a particle to enter ocular mucosa, apart from its lipophilicity, is that it should be of submicron size (Calvo, Thomas et al. 1994; Alonso and Alonso 2004). For an ocular drug delivery system to be successful it should have small particle size (less than 10 μm) (Zimmer and Kreuter 1995) with a narrow size range, it should be non-irritant, adequately bioavailable, compatible with ocular tissues and not cause any blurred vision (Bourlais, Acar et al. 1998; Sahoo, Dilnawaz et al. 2008).

Ocular drug delivery is a challenge for the pharmaceutical scientists because of the complex nature and structure of the eye (Figure 1-4). Barriers such as the epithelial, aqueous-vitreous, blood-aqueous and blood-retinal barriers limit the entry of drugs via different routes to the eye. Usually deep drug penetration into the posterior chamber is necessary to treat glaucoma or uveitis and fight viral infections proliferated within the eye (Seal, Bron et al. 1998).

Most drugs are formulated as simple eye drops and ointments for ophthalmic use. Eye drops account for more than 90% of ocular preparations (Bourlais, Acar et al. 1998). Although eye drops are cost effective, patient compatible and simple to formulate, a major fraction of the drug applied topically is washed away with the tears or removed by other mechanisms. Due to this removal, several applications in a day are required to achieve a therapeutic effect. Ocular defence mechanisms limit the drug residence time on the corneal surface and reduce its absorption. It was estimated that using a conventional ophthalmic dosage form only about 5% of the drug enters the eye intact (Gaudana, Jwala et al. 2009).
In many cases, posterior eye conditions are treated by intravenous or intravitreal administration of high doses of a drug with high therapeutic index, as in the case of antibiotics (Urtti 2006). The patient’s exposure to high concentrations of drugs is risky because certain disease processes require chronic medication or continuous supply of the drug over a period of time to achieve therapeutic objectives (Moshfeghi and Peyman 2005). Drugs administered orally, intravenously, or through extravascular junctions barely reach the retina and the posterior chamber, thus delivering very low concentrations to the sites of action (Moshfeghi and Peyman 2005). Systemic administration of drugs to treat eye conditions such as macular disease can only be optimised if the medicament is lipophilic because the hydrophilic agents remain behind the blood retinal barrier unless it is interrupted (Moshfeghi and Peyman 2005). Periocular and intravitreal administration routes have provided advantages over conventional eye drops and ointments but there are still many drawbacks associated. The delivered drug via these routes is cleared rapidly from the site of action and repeated administration of high doses is often necessary (Moshfeghi and Peyman 2005).

Moreover, the intravitreal route of administration is considered invasive which may cause endophthalmitis, cataracts, vitreous haemorrhages and retinal detachment, especially if repeated exposure is necessary. Due to these reasons, the intravitreal route is used less frequently unless the expected therapeutic outcomes outweigh the risks (Yasukawa, Kimura et al. 2001; Urtti 2006). Sustained drug delivery devices including Ocusert®, Vitrasert® and Retisert® offer several therapeutic improvements but their use is somewhat limited due to a surgical procedure required to implant them. Therefore, it is increasingly desirable to develop novel delivery systems to achieve optimised treatment.
1.3.2. Ocular barriers to drug delivery

1.3.3. Surface removal

Lachrymal secretions wash away the topically applied drug continuously and the excess of the lachrymal fluid flows down the nasolachrymal duct swiftly (Urtti and Salminen 1993; Urtti 2006). Due to the presence of an extensive network of capillaries in the conjunctival sac and the nasal cavity, most of the drugs applied topically are absorbed into the systemic circulation, thereby reducing the ocular bioavailability to only 5-10% (Urtti and Salminen 1993; Gaudana, Jwala et al. 2009). Systemic absorption from the ocular surface can cause side effects, especially if the patient has various medication needs. For instance, timolol and other intraocular pressure reducing agents can cause cardiac and vascular complications since the venous drainage of the face goes straight to the heart (Attama, Reichl et al. 2009). Other pre-corneal factors limiting ocular drug absorption are drainage of the instilled solution, tear production (induced lachrymation), drug metabolism and normal tear turn-over (Mainardes, Urban et al. 2005).

The human cul-de-sac can usually accommodate about 30 μL of fluid, whereas the instilled volume from eye droppers is about 50 μL (Mainardes, Urban et al. 2005). Therefore, large volumes of the medicament are lost through spillage from the cul-de-sac. Nasolachrymal drainage further limits the ocular absorption. These limiting factors undermine therapeutic efficiency and reduce the pre-corneal half-life of drugs to about 1-3 mins (Zimmer and Kreuter 1995).
Chapter 1 Introduction and literature review

The Cornea:
1. Epithelium (barrier to hydrophilic drugs)
2. Stroma (barrier to lipophilic drugs)
3. Endothelium (barrier to hydrophilic and lipophilic drugs)

Ciliary bodies and Iris pump out the drugs penetrated through the cornea to the systemic circulation

Absorption of drugs through systemic administration is limited due to the presence of Blood-retinal barrier and Blood-aqueous barrier.

70-100% of drugs administered locally will be lost due to:
- Nasolacrimal drainage and absorption to the systemic circulation
- Spillage due to limited capacity of human cul-de-sac
- Metabolism by enzymes

Aqueous humour

Figure 1-4 The structure of the eye and barriers for drug absorption.

1.3.4. Blood-aqueous barrier

This barrier is situated in the anterior segment of the eye and is composed of endothelial cells in the uvea (Urtti 2006). It limits the entry of hydrophilic drugs from the systemic circulation into the aqueous humour. This barrier can be disrupted by inflammation which temporarily results in enhanced drug permeation (Urtti 2006). Together with the blood-retinal barrier they make up the blood-ocular barrier. The epithelium of the iris and the ciliary bodies pump anionic drugs out from the aqueous humour to the systemic circulation (Seal, Bron et al. 1998).
1.3.5. **Blood-retinal barrier**

Situated in the posterior chamber, this barrier limits the entry of drugs from the systemic circulation to the retina. It is composed of the retinal pigment epithelium (RPE) and the tight walls of the retinal capillaries (Hornof, Toropainen et al. 2005; Urtti 2006). Although drugs can reach the choroidal extravascular space easily through the leaky and extensive vasculature of the choroid, their retinal access is denied by RPE and retinal endothelia (Urtti 2006). The blood-retinal barrier, along with the blood-aqueous barrier protect the eyes from the entry of xenobiotics and harmful substances (Urtti 2006). This physiological defence mechanism limits drug delivery to the retina and vitreous humour via the choroid through the systemic circulation.

1.4. **Application of nanotechnology in ocular drug delivery and the concept of modified eye drops**

Nanotechnology is changing the perception of drug administration using conventional dosage forms with the potential to revolutionize the way we develop new therapies, as well as to optimize existing ones. In pharmaceutical science, the term nanoparticle refers to a particulate drug delivery system where the size is in the nanometre range (1-1000 nm). Nanoparticles are being investigated extensively in order to develop drug delivery systems capable of allowing penetration through physiological barriers. They are either in the form of a matrix-dispersion (nanosphere) or a membrane-reservoir type (nanocapsule) where drugs can be dissolved, entrapped, encapsulated and dispersed within the particles or adsorbed on the particle surface (Bourlais, Acar et al. 1998) (Figure 1-5).
A wide range of chemical and physiological materials have been used to prepare SLNs including polymers, lipids, phospholipids and metals. These multifunctional drug carriers are expected to accommodate large drug loads, help target them to the site of action and promote sustained/controlled drug delivery while maintaining a minimum size of 30-300 nm (Debbage 2009). Submicron sized particles have a very high surface to volume ratio. Therefore, according to the Noyes Whitney and Kelvin equations, they have increased dissolution rates enabling them to enhance the absorption of poorly soluble drugs such as cyclosporine, paclitaxel or amphotericin B (Wissing, Kayser et al. 2004). On the contrary, there are a few disadvantages associated with nanoparticles such as difficulty of production, stability during their storage, aggregation and complexity of administration (Wissing, Kayser et al. 2004). Another drawback is the faster release rates associated with nanoparticles when compared to microspheres (Janoria, Hariharan et al. 2007). Physicochemical properties such as particle size, surface net charge, shape, solubility, degree of ionisation and lipophilicity influence drug ocular absorption and determine the route of administration (Mainardes, Urban et al. 2005). These factors can be tailored using novel nanoparticulate drug delivery systems to enhance the ocular bioavailability of drugs.

In recent years, scientists have been interested in incorporating drugs and other therapeutics into nanoparticulate carriers administered as modified eye drops which are cost effective and
therapeutically efficient (Nagarwal, Kant et al. 2009). The modified eye drops provide better penetration, extended ocular surface residence time, minimized drainage owing to mucoadhesive properties, simple administration and patient compatibility (Nagarwal, Kant et al. 2009). Moreover, colloidal and particulate drug delivery systems can also be utilised for subconjunctivital, periocular and intraocular injections (Janoria, Hariharan et al. 2007).

Many nano-structured systems such as solid lipid nanoparticles, niosomes, nanocapsules, nanospheres, dendrimers, nanosuspensions, liposomes and nanoemulsions have been employed in ocular drug delivery to alleviated problems associated with poorly soluble drugs increasing their bioavailability while decreasing their administered dose and toxicity (Wissing, Kayser et al. 2004). For many serious eye conditions (e.g. chronic cytomegalovirus rhinitis (CMV)) a constant and prolonged controlled drug release is necessary to achieve therapeutic goals. The ultimate goal for nanotechnology based delivery systems including SLNs is to provide controlled release of drugs to reduce frequent administrations associated with conventional delivery systems. Further, nanomedicines have several advantages such as the possibility of formulation as modified eye drops which are easily self-administered by the patient, elimination of the need for repeated administration and offering protection against metabolic enzymes present on the ocular surfaces by constructing a protective barrier (Kaur, Garg et al. 2004; Araújo, Gonzalez et al. 2009; Hironaka, Inokuchi et al. 2009). Due to their submicron size range they have the added advantage of not impairing vision (Araújo, Gonzalez et al. 2009). Hironaka et al. (2009) designed a submicron sized liposomal drug delivery system capable of delivering hydrophilic drugs to the posterior segment of the eye (Hironaka, Inokuchi et al. 2009). The liposomes were formulated in the form of modified eye drops and the authors suggested that this system could replace currently used invasive delivery routes in the treatment of major ocular conditions such as age related macular degeneration, diabetic macular oedema and endophthalmitis. Others have reported that ACV
containing liposomes are more effective than simple eye drop preparations due to better penetration and absorption through the cornea (Law, Huang et al. 2000). Nanoparticles such as positively charged liposomes and SLNs are expected to increase corneal absorption of drugs by increasing drug residence time through ionic interactions. Cortesi et al. (2006) developed cationic liposomes to administer certain peptides with anti-herpetic activity to the eye (Cortesi, Argnani et al. 2006). Moreover, it is widely believed that nanoparticles owe their therapeutic efficiency to their bioadhesive nature (Bourlais, Acar et al. 1998).

1.5. Solid lipid nanoparticles (SLNs)

Lipids have been used as an excipient or drug delivery vehicle for accommodating lipophilic drugs and elevating their poor physiological water solubility (Chen 2008). Water-in-oil emulsions and microemulsions have been extensively investigated to solubilise hydrophilic drugs for ocular drug delivery. Although microemulsions have been known to scientists since 1928, research for potential use in ocular delivery has only begun in the past decade (Vandamme 2002). Despite their simple production, easy sterilization and modest stability, the use of microemulsions in ocular drug delivery is limited by the choice of ingredients possessing good ocular tolerability (Vandamme 2002). Oil-in-water emulsions and microemulsions require a high surfactant concentration to ensure formulation stability. This limits their ocular drug delivery applicability as surfactants are usually not well tolerated.

SLNs were first patented by Müller and Lucks in 1996. Since then they have become a widely used, stable, nontoxic and reliable particulate drug delivery vehicle. SLNs are structured as a solid lipid core in the nanometre range accommodating the drug stabilised by a layer of surfactants (Sawant and Dodiya 2008). They have several advantages over other colloidal carriers such as the possibility of controlling drug release, drug targeting, long term stability, good drug loading (whether hydrophilic or lipophilic), absence of biotoxicity due to
the use of physiological lipids, possibility of sterilization by autoclaving and easy large scale production (Mehnert and Mäder 2001). In addition, due to their nano size range, SLNs can be an effective ocular drug delivery system by enhancing corneal absorption, improving ocular bioavailability, prolonging the ocular retention time and providing a sustained drug release profile (Figure 1-6) (Kaur, Kanwar et al. 2002). A wide range of drugs can be loaded into solid lipid nanoparticles, the only disadvantage being the burst effect associated with hydrophilic drugs (Kaur, Rana et al. 2008). The burst effect is often associated with drug adsorption on the surface of the nanoparticles which results in the release of a major fraction of the dose in a short period of time. SLNs control and stop the degradation process of sensitive lipophilic materials and drugs, due to the fact that the mobility of the reactive agents is hindered in the solid state when compared to the liquid state (Helgason, Awad et al. 2009). Also, the mechanism of drug loading and location of microphase surfaces can affect the position of the active substances. The presence of drugs on the surface of particles promotes their chemical degradation, whereas drug orientation towards the centre inhibits the degradation process (Helgason, Awad et al. 2009). These types of physiochemical phenomena can be well controlled through the careful design of solid lipid nanoparticles. They offer other advantages such as biodegradability, safety, low cost, simple production and, importantly, free dispersibility in aqueous media enabling them to be formulated as modified eye drops (Sawant and Dodiya 2008). SLNs are mainly built from triglycerides in a specific orientation consisting of a polar core with polar heads facing towards the aqueous phase (Wadhwa, Paliwal et al. 2009). Many different lipids (triglycerides, hard fat types, partial glycerides, steroids and waxes) and all classes of emulsifiers (ionic and non-ionic) have been used to prepare SLNs (Mehnert and Mäder 2001).
In comparison to an aqueous eye drop, an ocular solid lipid nanoparticular system can have extended residence time on the ocular surface and in the conjunctival sac (Gaudana, Jwala et al. 2009) leading to sustained release of the drug revealed by in vivo studies (Cavalli, Gasco et al. 2002). However, their efficiency for ocular drug delivery has not been studied extensively (Velpandian and Velpandian 2009) and further investigation is necessary. Drug particles engulfed in SLNs should effectively cross the epithelium due to the SLNs lipophilic properties. Moreover, the epithelium is slightly negatively charged, hence cationic solid lipid nanoparticles can increase the corneal residence time of the drug and increase its absorption levels.
1.5.1. Modifications of SLNs and emergence of NLCs and lipid drug conjugates (LDCs)

Apart from their initial success, SLNs are associated with a number of drawbacks including: limited drug loading capacity, possibility of drug expulsion during phase modifications and high water content of aqueous dispersions of SLNs (70-90%). The introduction of nanostructured lipid carriers (NLCs) aims to address these shortcomings (Mehnert and Mäder 2001; Müller, Radtke et al. 2002; Sawant and Dodiya 2008). NLCs are produced by the controlled addition of a spatially incompatible liquid lipid to the solid lipid component in order to accommodate a larger quantity of the drug and achieve a better release profile (Müller, Radtke et al. 2002; Sawant and Dodiya 2008). This can be achieved by increasing the space between the fatty acid chains of the glycerides, allowing more drug to be accommodated and formation of imperfect lipid crystals avoiding drug expulsion during storage (Müller, Radtke et al. 2002). Although NLCs contain up to 30% of liquid lipids, the final product is in the solid state with no crystalline structure.

1.5.1.1. Imperfect NLCs

Those systems in which a liquid lipid (e.g. glycerides) is added to the solid lipid can be regarded as imperfect NLCs. The liquid lipids induce a larger distance between the fatty acid chains of the main solid lipid core which causes imperfections in the crystalline structure of the lipid where a very large amount of the drug can be incorporated (Sawant and Dodiya 2008). For example Jenning et al. (2000) incorporated a medium length chain triglyceride oil in a matrix of a solid long chain glyceride achieving higher payloads and controlled release properties (Jenning, Mäder et al. 2000).
1.5.1.2. Multiple type NLCs

In another approach, liquid lipids are mixed in excess with solid lipids resulting in nanocompartments of liquid lipid formed within the solid core which is already dispersed in the aqueous medium (Müller, Radtke et al. 2002). The nanocompartments of liquid lipids are dispersed uniformly in the matrix of solid lipids, which are protected from degradation and potentiate prolonged release behaviour of the system (Müller, Radtke et al. 2002). This multiple system is beneficial for drugs that have higher solubility in liquid oils (Sawant and Dodiya 2008). Excess of liquid lipids used in such cases inhibits drug expulsion especially if a high concentration of drug is used in the preparation step (Müller, Radtke et al. 2002). For example; NLCs of Compritol 888 ATO can be prepared with incorporation of incompatible liquid lipids such as α-tocopherol or Miglyol 812 (Jenning, Mäder et al. 2000; Souto, Mehnert et al. 2006). These NLCs present a better drug protection and entrapment efficiency when compared to normal SLNs. Moreover, the result of DSC and X-ray diffraction studies have revealed that NLCs are in a less crystalline state than SLNs of Compritol (Jenning, Mäder et al. 2000; Souto, Mehnert et al. 2006).

1.5.1.3. Structureless NLCs

Another approach is to mix and melt together carefully selected liquid and solid lipids which, upon cooling, solidify but do not crystallize. These are regarded as structureless SLNs and can avoid drug expulsion caused by crystallization (Sawant and Dodiya 2008).

1.5.1.4. Lipid drug conjugates (LDCs)

Alternatively, lipid drug conjugates are developed by converting a hydrophilic drug to a lipophilic drug conjugate or a prodrug by addition of an ester or amide group (Sawant and Dodiya 2008). These are either used alone or combined with other solid lipids and are
expected to enhance biological transport and targeting of hydrophilic drugs (Sawant and Dodiya 2008).

1.5.2. Fabrication of SLNs

The preparation of SLNs is based on solidified emulsion technologies. It is comparatively easy to incorporate lipophilic drugs into SLNs; however, hydrophilic drugs, especially proteins and peptides, tend to partition in the aqueous phase during the preparation process. This problem can be solved by using surfactants as emulsion stabilisers (Almeida and Souto 2007).

SLNs can be prepared by several methods which were classified by Mehnert and Mader (2001) into four categories; namely high shear homogenisation and ultrasound (Hou, Xie et al. 2003), high pressure (hot and cold homogenization) (zur Mühlen, Schwarz et al. 1998; Mehnert and Mäder 2001; Olbrich, Bakowsky et al. 2001; Radomska-Soukharev 2007; Helgason, Awad et al. 2009), solvent emulsification/evaporation and microemulsion based (Heydenreich, Westmeier et al. 2003; Vighi, Ruozi et al. 2007; Kuo and Chen 2009). In addition to the above methods, SLNs composed of glyceryl monostearate (GMS) can be successfully prepared by a solvent emulsification-diffusion technique (Trotta, Debernardi et al. 2003), or by a double emulsion technique (Lv, Yu et al. 2009). More recently, Hu et al. (2002) used a novel solvent diffusion method in an aqueous system to prepare SLNs with clobetasol propionate as the loading drug (Hu, Yuan et al. 2002). Yun et al. (2009) presented a continuous method of SLNs preparation by liquid flow focusing and gas displacement method in microchannels, whereas Zhang et al. (2008) prepared SLNs in a microchannel system with cross shaped junctions. A number of these methods will be discussed in more detail in the following subsections:
Chapter 1 Introduction and literature review

1.5.2.1. High pressure homogenisation

High pressure homogenisation has been used extensively to produce SLNs and nanoemulsions for parenteral use (Mehnert and Mäder 2001). High pressure homogenisers are widely available in pharmaceutical production plants and laboratories and scaling up the method only represents a few problems. During the process of hot homogenization the dispersion is passed through a narrow gap by applying a high pressure (Mehnert and Mäder 2001). Passage through a short distance combined with the applied pressure produces high shear stress, breaking down the accelerated particles to submicron size. Lipid concentrations between 5-10% are easily handled in this technique, although higher concentrations reflect no limitation (Mehnert and Mäder 2001). A preparatory step is involved in both hot and cold homogenization where the drug has to be dissolved or dispersed in the lipid melt. This step eliminates the need to use an organic solvent which might cause toxicity concerns if residues remain.

Hot homogenisation

In hot homogenisation the mixture of drug inside the lipid melt is first emulsified with a solution of proposed surfactant(s) using a high speed stirrer. The primary emulsion affects the quality of the final particles and should ideally produce particles in or below micrometre range (Mehnert and Mäder 2001). The primary emulsion is then subjected to high pressure homogenization at temperatures above the melting point of the lipid. The homogenization can be repeated a few times to achieve the desired sized particles taking into account that extra cycles can actually yield bigger sized particles due to increased kinetic energy causing agglomeration (Mehnert and Mäder 2001). Finally, the hot oil-in-water emulsion is cooled to room temperature to produce a super-cooled melt to yield SLNs (Mehnert and Mäder 2001). One of the drawbacks of this method is the elevated temperatures required to melt the lipid which might further increase while applying high shear stress. This can affect the chemical
stability of thermo-labile drugs and therapeutics such as antibiotics, peptides and proteins which are widely used in ophthalmic procedures. However, if the drug is thermo-stable, the high shear and temperature employed in this technique can help producing smaller sized particles.

**Cold homogenisation**

In cold homogenization excessive heating is avoided by solidifying the lipid-drug mixture in liquid nitrogen and grinding it to a powder with particle sizes below 100 nm before dispersing it in a cold aqueous solution containing the surfactant(s). However, the preparatory step which involves melting of the lipid and dispersing/dissolving the drug is still necessary. Therefore, in cold homogenization, a suspension is introduced to high shear homogenization rather than an emulsion as in the case of hot homogenization (Mehnert and Mäder 2001). Although cold homogenization can control the complex crystallization behaviour of lipids during super-cooling and drug distribution through the aqueous phase during homogenization, it usually yields particles with higher average particle sizes compared to hot homogenisation (Mehnert and Mäder 2001).

**1.5.2.2. Water-in-oil-in-water (w/o/w) double emulsion**

The double w/o/w emulsion technique is a relatively new method which has been utilized in recent years to prepare nanoemulsions and SLNs (Lv, Yu et al. 2009). The method, presented in Figure 1.7, first involves dissolving a drug in an aqueous solvent (Lv, Yu et al. 2009). The aqueous solution is then emulsified in an oil phase containing the lipid(s) dissolved in an organic solvent to form a primary w/o emulsion. The primary w/o emulsion is then dispersed into an aqueous solution containing the surfactant. The w/o/w double emulsion system is mechanically agitated to allow complete evaporation of the organic solvent until SLNs are formed. Heating is not required during the process which makes it suitable for thermo-labile
substances and SLNs of good quality can be prepared by adjusting the sonication intensity. The only disadvantages associated with this method are the use of organic solvents and the possibility of the presence of metal impurities from the sonicator (Mehnert and Mäder 2001).

\[ \text{Drug} + \text{Fatty material} + \text{Organic solvent} \xrightarrow{\text{Ultrasonication}} \text{I. (Inner aqueous phase)} \]

\[ \text{II. (Oil phase)} \]

\[ \text{III. (Primary W/O emulsion)} \]

\[ \text{Surfactant} + \text{Water} \xrightarrow{\text{Ultrasonication and homogenisation}} \text{IV. (Outer aqueous phase)} \]

\[ \text{V. (W/O/W emulsion)} \]

Figure 1-7 Schematic representation of the double emulsion technique.

1.5.2.3. Solvent emulsification-evaporation

Sjöström and Bergenståhl (1992) developed a method for preparing lecithin stabilised nanoparticles containing cholesteryl acetate. In this method the lipid component is dissolved in a water immiscible organic solvent and the drug is either dissolved or dispersed in the lipid solution. For preparation of cationic SLNs the main lipid core (e.g. Precirol® ATO 5) is
dissolved in an organic solvent (e.g. dichloromethane) and the cationic lipid (e.g. DODAB) can be dispersed in the aqueous phase containing the surfactant (del Pozo-Rodríguez, Delgado et al. 2008). This organic phase is then emulsified in an aqueous solution containing a biocompatible cosurfactant such as bile salts (e.g. sodium glycocholate and phosphatidylcholine). If used in combination, the ratio of different cosurfactants is a critical factor which decides the final particle size. Emulsification can be achieved using a high speed stirrer Ultra-Turrax followed by high pressure homogenization. The system is mechanically stirred at room temperature until the solvent has completely evaporated.

The quality of the primary emulsion directly affects the final SLNs average size. The smaller the droplets in the primary emulsions, the smaller will be the final SLNs (Sjöström and Bergenståhl 1992). Hence, it is important to optimise all parameters which influence the emulsification process such as the lipid and emulsifier concentration and type. If optimized, this method can produce particles with an average size below 100 nm by precipitation into an o/w emulsion (Sjöström and Bergenståhl 1992).

1.5.2.4. Solvent emulsification-diffusion

This method is based on the water miscibility property of certain organic solvents such as butyl lactate or benzyl alcohol used as the oil phase (Hu, Hong et al. 2004; Trotta, Cavalli et al. 2005). A primary oil-in-water emulsion is prepared containing the drug and the lipid phase solution in a water-miscible solvent. This emulsion is then transferred into water. The hypothesis is that upon introduction to an aqueous phase, the water-miscible solvent will diffuse. Hence, the lipophilic material dissolved in the solvent will solidify due to the diffusion of solvent from droplets to the continuous phase (Trotta, Cavalli et al. 2005).

Trotta et al. (2005) used this method to encapsulate insulin into glyceride monostearate solid lipid micro- and nanoparticles using isobutyric acid as a water-miscible solvent (Trotta,
Cavalli et al. 2005). The entrapment efficiency was high (80%); however, the burst effect which released 20% of the drug suggested the presence of insulin on the surface of the particles rather than within. This method is reliable for the entrapment of lipophilic and hydrophilic drugs if optimized.

1.5.2.5. Oil-in-water (o/w) microemulsion

The oil-in-water microemulsion technique is an easy and suitable method for the preparation of SLNs. It does not use organic solvents; however, a pre-heating step is required which may be disadvantageous to thermolabile substances. Briefly, the oil components are melted at about 10°C above their melting point and the drug to be loaded inside SLNs is mixed with the lipid melt. The lipid melt is then dispersed and homogenised in a hot aqueous phase containing the surfactant heated to the same temperature as the lipid melt. The hot o/w microemulsion is then cooled rapidly while maintaining the mechanical stirring until SLNs are formed.

The theory is that oil droplets are present in the hot o/w microemulsion. Upon a sudden decrease in the temperature, the nanoparticles are expected to crystallize rapidly, forming SLNs (Marengo, Cavalli et al. 2000). Marengo et al. (2000) developed an apparatus for SLNs preparation which works by quenching of the warm o/w microemulsion into cold water. The warm o/w emulsion can be either cooled using an ice bath (Vighi, Ruozi et al. 2007) or it can be placed into cold water directly (Cavalli R. 1996; Marengo, Cavalli et al. 2000).

In another study Cavalli et al. (1996) developed an instrument with the capacity to handle 1 mL of oil-in-water microemulsion at a time in which a glass syringe thermostated with a bronze jacket, injects the hot emulsion into cold water. Factors such as the temperature and the delivery rate of the hot emulsion into cold water are very important in deciding the final nanoparticle size and composition (Marengo, Cavalli et al. 2000).
1.5.2.6. Preparation of SLNs in co-flowing microchannels

Preparation of SLNs using microchannels is investigated to avoid critical procedures associated with earlier production methods such as high speeds, high pressures, high temperatures and the use of toxic organic solvents (Yun, Zhang et al. 2009). Microchannels offer several advantages such as efficient mass transfer, stable flow field and uniform concentration distribution which results in continuous production of SLNs with a narrow size range (Yun, Zhang et al. 2009).

The basic principle behind this method is solvent displacement. As described by Zhang et al. (2008), the apparatus consists of a co-flowing microchannel system with inner and outer capillaries. The lipid is dissolved in a water-miscible solvent which is injected into the inner capillary. The aqueous phase containing the surfactant is injected into the outer capillary simultaneously. Displacement of the solvent from the lipid phase to the aqueous phase causes local supersaturation and solidification of lipids resulting in the formation of SLNs. This instrument also employs a digital inversion microscope equipped with a video camera for capturing images of flow patterns in the microchannels (Zhang, Shen et al. 2008).

Particles with different size and morphology can be prepared by altering parameters such as the velocities of the liquids flow to adjust focused flow patterns (Yun, Zhang et al. 2009). The small diameter of the microchannels is often blocked by SLNs hindering continuous production, however, this can be avoided by gas-liquid slug flow (Yun, Zhang et al. 2009).
1.5.3. Post preparation procedures

1.5.3.1. Purification and separation of SLNs

Purification is an important step in SLN preparation to avoid toxicity associated with extra surfactants. Therefore, methods like ultrafiltration, ultracentrifugation and dialysis have been employed (Heydenreich, Westmeier et al. 2003; Trotta, Cavalli et al. 2005).

1.5.3.2. Sterilization

Sterilization is a required step for all ocular preparations unless prepared aseptically. Ophthalmic products usually have a short shelf life and must be used within one month of opening due to contamination risks. In many cases, a sterilisation technique has to be carefully chosen to ensure formulation sterility without degradation and aggregation of the solid lipids to avoid toxicity and instability (Mehnert and Mäder 2001). Commonly used techniques for sterilization include autoclaving, filtration, γ-radiation and aseptic production.

One of the advantages of SLNs over other colloidal systems is that they can be sterilized by autoclaving, a commonly used, straightforward and reliable technique. A study on trilaurin SLNs loaded with azidothymidine palmitate showed that SLNs dispersions maintained good stability after autoclaving at 121°C for 20 mins (Heiati, Tawashi et al. 1998). However, it should be noted that heating can induce physical instability and particle aggregation. An increase in the average particle size is usually observed after sterilization by heating as SLNs melt and recrystallize in a controlled manner. Therefore, certain structural features assigned to SLNs by controlling the production parameters will be lost by autoclaving, the severity of which is defined by the composition of the SLNs (Müller, Mäder et al. 2000).

It is important to note, however, that heat or radiation induced degradation is not always associated with increased particles size. In some cases, breakdown of lipids results in
decreased particles size (Mehnert and Mäder 2001). The degradation of lipids during sterilization is also an important issue which can cause potential toxicity. A filtration technique as well as an aseptic preparation strategy can be also employed for SLNs sterilization similar to the sterilization of parenteral emulsions for nutrition (Müller, Radtke et al. 2002).

1.5.3.3. Freeze drying

Freeze-drying, also known as lyophilization, is a process that allows the stabilization of biomaterials so that they can be stored. It involves the removal of water by employing a process known as sublimation, wherein a solid is converted to the vapour state without first passing through the liquid phase. Lyophilisation is utilized as a critical technique to convert the lipid dispersion to a solid state to extend the stability and to avoid particle aggregation (Mehnert and Mäder 2001).

Lyophilisation is the main method used for drying vaccines, pharmaceuticals and other delicate, heat sensitive materials. With respect to SLNs, the freeze-drying process improves their long-term chemical and physical stability. This means that degradation reactions such as hydrolysis are prevented and the initial particle size is preserved (Vighi, Ruozi et al. 2007). The freezing procedure also affects the crystal structure and properties of the lyophilizate (Vighi, Ruozi et al. 2007). Cooling may be done either rapidly or slowly. Rapid cooling can be performed by dipping the vial containing the preparation into liquid nitrogen or by adding the SLNs dispersion drop wise to liquid nitrogen (Mehnert and Mäder 2001). This results in the formation of small heterogeneous crystals, whereas slow cooling causes large crystals to form as is done by placing the vials in a freeze drier having a shelf temperature of -25 °C for 24 hrs (Mehnert and Mäder 2001). Each cooling type is associated with certain advantages and disadvantages. While rapid cooling decreases freezing out effects, it causes slower
sublimation. Therefore, freeze-drying must be done in a sample-specific manner by optimizing the lyophilization process (Mehnert and Mäder 2001).

The process of freeze-drying may have some unfavourable effects on the SLNs themselves; therefore, it may sometimes be necessary to use certain protective materials known as cryoprotectants. These additives help to decrease particle aggregation and improve redispersion of the dry product (Vighi, Ruozi et al. 2007). Commonly used cryoprotective agents are sorbitol, mannose, trehalose, glucose and polyvinylpyrrolidone (PVP). In a study by Zimmermann et al. (2000), trehalose was found to be the most effective while PVP was found to be least effective (Zimmermann, Müller et al. 2000). As with the type of freezing process used, cryoprotectants need to be chosen specifically according to the lipid excipients used for SLNs preparation. However, the concentrations of the cryoprotectants need to be optimized. As an example, a study by Schwarz and Mehnert (1997) showed that trehalose proved to be the most effective for Compritol-SLNs and Dynasan-SLNs when used at high concentrations. Although the solid state of the SLNs are chemically and physically more stable, attention must be given to the effects of the lyophilization process and the process of redispersion on the overall stability of the SLNs once it is reconstituted. The negative effects of these processes can be lessened by employing the optimum freezing velocity and by selecting the best redispersion method i.e. either sonication or manual shaking (Schwarz and Mehnert 1997).

1.5.3.4. Spray drying

Spray drying is used as an alternative method to increase SLNs stability (Freitas and Müller 1998). It involves the conversion of a solution or a suspension into a dry product. Although spray drying is a widely used process in the pharmaceutical industry and is cheaper than lyophilisation, it has rarely been used for SLNs formulations (Mehnert and Mäder 2001).
Freitas and Müller (1998) described a process for carrying out spray drying of SLNs wherein a product suitable for i.v. administration was obtained. They briefly summarized the steps into the following: (1) atomization of the feed into the spray, (2) spray-air contact, (3) drying of the spray and (4) separation of the dried product from the drying gas. Several factors such as spray drying parameters, chemical nature of the lipid and type of redispersion medium need to be taken into consideration in order to obtain a product which has optimum particle size and redispersability (Freitas and Müller 1998).

1.5.4. Limitations of SLNs

1.5.4.1. Drug expulsion during storage

Crystalline behaviour of lipids in the nanoscale is very complex. In SLNs, the solidification temperature is usually lowered compared to bulk lipids (Bunjes and Koch 2005) and less ordered lipid modifications are often formed (Freitas and Müller 1999; Müller, Radtke et al. 2002). However, drug expulsion during storage is one of the most encountered issues associated with the lipid crystal transformation to a more stable $\beta$-modification with more perfect crystalline lattice leaving behind a reduced space for drug accommodation (Figure 1-8) (Müller, Radtke et al. 2002).
SLNs after production
(Uniform dispersion in an imperfect crystalline lattice)

Drug expulsion due to the lipid crystals modifications and formation of ordered crystalline lattice

Figure 1-8 Drug expulsion during storage (Modified from (Müller, Radtke et al. 2002)).

1.5.4.2. Drug enriched shell and burst release

The burst effect is a common problem associated with SLNs release profile. It can be explained by the room temperature phase separation effect (Figure 1-9) (Müller, Radtke et al. 2002). It is proposed that when the hot o/w emulsion containing the drug is cooled to solidify, lipids crystallize to form SLNs with a drug-free core and a drug enriched shell (Müller, Radtke et al. 2002). This soft deformable drug enriched surface shell was detected in a study by atomic force microscopy (AFM) and small angle X-ray scattering (SAXs) measured as a 15-18 nm thick layer (zur Mühlen, Schwarz et al. 1998). To achieve a prolonged release profile of SLNs, lower temperatures and surfactant concentrations during production are advised as optimal conditions (Mehnert and Mäder 2001; Müller, Radtke et al. 2002).
1.5.6. Characterisation and quality assessment of SLNs

Many factors apart from the preparation methods influence the final quality of SLNs. The quality, composition, properties and quantities of lipids and surfactants used can greatly influence the quality of the final product (Mehnert and Mäder 2001).

The formulation stability can be evaluated via particle size, zeta potential and entrapment efficiency studies (Lv, Yu et al. 2009). Characterisation of structure and quality is a very important step in determining stability and release kinetics of SLNs. A number of methods have been employed to measure SLNs particle size and morphology, amongst them photon correlation spectroscopy (PCS) (Mehnert and Mäder 2001; Olbrich, Bakowsky et al. 2001; Hu, Yuan et al. 2002; Kuo and Chen 2009; Lv, Yu et al. 2009), atomic force microscopy (AFM) (Olbrich, Bakowsky et al. 2001; Vighi, Ruozi et al. 2007), transmission electron microscopy (TEM) (Hou, Xie et al. 2003; Trotta, Debernardi et al. 2003; Lv, Yu et al. 2009) and field-flow-fractionation (FFF) being the most popular. Zeta potential measurement is equally important in order to predict the stability of colloidal dispersions. It is known that particles of the same charges tend to repel each other and hence avoid aggregation (Mehnert...
and Mäder 2001). The measurement of crystallinity and lipid modification is important because these parameters influence drug incorporation, stability and release rates. Methods like differential scanning calorimetry (DSC) and X-ray scattering are often used to examine the status of lipids. In addition, nuclear magnetic resonance (NMR) and electron spin resonance (ESR) methods are used to assess the presence of other colloidal structures such as microparticles in the formulation (Mehnert and Mäder 2001).

1.5.6.1. Particle size

One of the most important characteristic features of SLNs is their nanometre scale. Particle size measurements can be regarded as the easiest way to estimate SLNs stability in dispersion over a period of time. Methods widely used to measure particle size are photon correlation microscopy (PCS) and laser diffraction (LD) (Ronald and Hong-Shian).

PCS does not measure the size directly but it determines the fluctuation of the intensity of the scattered light as a function of particle movement (Müller, Mäder et al. 2000). It is a sensitive method for particles in the nanometre range but is inefficient for particles larger than a few micrometres. LD, on the other hand, can measure particles ranging in size from a few nanometres to sub-millimetres with the measurement based on the dependency of the diffraction angle on the particle radius where smaller particles cause more scattering at high angles compared to larger particles (Müller, Mäder et al. 2000).

The data obtained from PCS and LD are not always absolute as some SLNs preparation contain particles of irregular shape (not necessarily spherical) which beside being in the nanometre range, are wrongly measured as microparticles. In such a case, other methods which provide direct information on particle shape such as TEM, SEM and AFM are useful (Müller, Mäder et al. 2000).
Many factors such as the nature of the lipid matrix, concentration of lipids in the formulation, type of surfactant, surfactant concentration and the viscosity of the lipid and aqueous phase influenced the SLNs particle size.

1.5.6.2. Zeta potential

The electrokinetic potential of colloidal systems referred to as zeta potential determines the stability of colloidal dispersions. The zeta potential indicates the extent of particle-particle repulsion forces necessary to avoid agglomeration and aggregation. Higher zeta potential values, whether positive or negative, generally indicate higher dispersion stability. The zeta potential of an emulsion is determined by the chemical nature of the surfactant, with a zeta potential of 30 mV considered to be sufficient to ensure physical stability of the emulsion (Li, Lin et al. 2008). Apart from the stability of the dispersion, surface charges are important in molecular mechanisms of drug absorption. In ocular drug delivery, this is explained by the fact that the corneal epithelial cells are negatively charged. Therefore, to increase drug residence time and penetration, cationic SLNs can be used.

1.5.6.3. Morphology

The following methods can be used to determine particle size, size distribution and morphology of SLNs.

**Transmission electron microscopy (TEM)**

TEM evaluates particle morphology by examining the electrons that are transmitted through the specimen. An image is produced by interpreting the interaction of the electrons passed through the specimen which is visualised by an imaging device or detected by a special sensor. Nanoparticles can be visualised using TEM after freeze fracturing and freeze substitution (Sawant and Dodiya 2008).
Scanning electron microscopy (SEM)
This method offers excellent resolution and an easy sample preparation procedure for SLNs morphological examination. SEM measures electrons transmitted from the particle surfaces to evaluate their morphology (Sawant and Dodiya 2008).

Atomic force microscopy (AFM)
AFM produces a three dimensional image of the nanoparticles. It is a very sensitive device and spatial resolution of up to 0.01 nm can be achieved by measuring the force acting between the probing tip and the particle surface (Sawant and Dodiya 2008).

1.5.6.4. Crystalline properties

Differential scanning calorimetry (DSC)
Differential scanning calorimetry can be described as a thermal analysis technique used in the investigation of melting, crystallization, solid-to-solid transition temperatures of lipids and the determination of the solid fat content of the excipient (Jannin, Musakhanian et al. 2008). It enables the measurement of temperature change and heat flow which occurs when a material undergoes phase transition. Basically it involves the comparison of the heat required to raise the temperature of a sample and a reference wherein both the sample and the reference are maintained at the same temperature throughout a given experiment. Such data allows the quantitative and qualitative assessment of physical and chemical properties of the material by measuring changes in either enthalpy or heat capacity. The thermal events that can be detected by this method may be endothermic phenomena such as melting or exothermic phenomena such as crystallization (Coleman and Craig 1996).

DSC has various applications and is successfully used in the pharmaceutical industry as a method of drug analysis and characterization of new delivery systems (Gill, Sauerbrunn et al. 1993). With specific reference to SLNs, DSC allows the study of their melting and
recrystallisation behaviour. This is important as the crystallinity of a lipid matrix has an effect on the functional properties such as drug incorporation and release rates of the SLNs derived from it (Gill, Sauerbrunn et al. 1993).

Attama et al. (2007) described the characterization of SLNs prepared with a mixture of theobroma oil and goat fat as the main lipids, phospholipon 90G as the heterolipid and polysama orbate 80 as the mobile surfactant. Methods of characterization used were time-resolved particle size analysis, zeta potential and osmotic pressure measurements, differential scanning calorimetry (DSC), transmission electron microscopy (TEM) and isothermal heat conduction microcalorimetry (IMC) (Attama, Schicke et al. 2007).

**Wide angle x-ray diffraction (WAXD)**

This is a well-established method used to investigate the crystalline nature of the formulated SLNs it can qualitatively determine the crystalline ingredients of a dispersion according to their individual diffraction patterns (Attama, Schicke et al. 2007; Bunjes and Unruh 2007; Attama, Reichl et al. 2008). If a crystalline structure is identified, information about polymorphic modifications can be obtained (Bunjes and Unruh 2007). For example, Jenning et al. (2000) confirmed the presence of the β’-modification of Compritol using X-ray diffraction (Jenning, Mäder et al. 2000). It is based on the fact that the X-rays reflected as the crystalline lipids appear well above the amorphous background of non-crystalline lipids (Attama and Müller-Goymann 2007).

**1.5.6.5. Drug encapsulation and loading efficiency**

Determination of encapsulation efficiency is based on the separation of lipids from the aqueous phase of the dispersion achieved by either ultrafiltration, ultracentrifugation, gel filtration using a sephadex column or dialysis (Sawant and Dodiya 2008). In ultrafiltration the amount of drug loading can be calculated indirectly after centrifugation in a membrane.
concentrator (Attama, Reichl et al. 2008). Sometimes ultracentrifugation for a period of time is sufficient to separate SLNs from the aqueous phase (Kuo and Chen 2009). The amount of drug present in the aqueous continuous phase can be determined using a sensitive method like HPLC or UV spectroscopy. The loading capacity can then be calculated considering the initial drug concentration. Attama et al. (2008) repeated these measurements thrice with two weekly intervals to allow the complete crystallisation of the lipids in order to determine if the crystalline lattice modifications cause drug expulsion. The entrapment efficiency and drug loading can be calculated using these formulas (Lv, Yu et al. 2009):

\[
\text{Entrapment efficiency} = \left( \frac{W_{t_{\text{initial drug}}} - W_{t_{\text{free drug}}}}{W_{t_{\text{initial drug}}}} \right) \times 100\% \\
\text{Drug loading} = \left( \frac{W_{t_{\text{initial drug}}} - W_{t_{\text{free drug}}}}{W_{t_{\text{lipid}}}} \right) \times 100\%
\]

Ultrafiltration and microdialysis are regarded as the most accurate methods to measure entrapment efficiency of encapsulated systems (Liu, Zhang et al. 2009). Although ultracentrifugation is the fastest and easiest method to use, the results obtained are not reliable (Liu, Zhang et al. 2009). Basaran et al. (2009) used a different method to determine the drug contents in SLNs. They melted an accurately weighed amount of SLNs at about 80°C in tightly closed glass tubes containing ethanol. Cooling of the obtained hot dispersion to -18°C in a deep freezer resulted in the precipitation of the solid matrix which left behind a transparent liquid mass containing the drug after ultracentrifugation. The amount of drug present was then determined.

1.5.6.6. Drug release studies

Drugs incorporated into SLNs are usually released by diffusion through the lipid matrix and/or biodegradation and surface erosion of the lipid matrix (Sawant and Dodiya 2008). This should allow a sustained and controlled release of drug from the colloidal system. The extent
of the drug incorporation within the SLNs can be revealed by its release profile. An immediate burst effect can release a major portion of the drug in a short period of time. This can be due to surface adsorption of active rather than encapsulation or dispersion in the SLNs. Many factors such as the concentration of lipids, drug solubility inside the lipid core, drug interaction with the lipids, production temperatures, surfactants used and particle size can influence and promote surface adsorption (Sawant and Dodiya 2008). The aqueous solubility of most drugs is enhanced at higher temperatures. This causes a change in the partition coefficient of the drug and its position in the SLNs. Therefore, higher production temperatures promote drug localization on the surface of SLNs causing a burst effect (Sawant and Dodiya 2008).

*In vitro* release studies can be performed in a modified Franz diffusion cell (Franz 1975). A diffusion barrier like siliconised Spectrapore® MWCO 6000-8000 can be used to mimic physiological conditions (Attama, Reichl et al. 2008). Alternatively, fresh cornea obtained from white male New Zealand rabbits can be used as the diffusion barrier mounted on a modified Franz-type cell (Li, Nie et al. 2008). Specific amount of SLNs are placed on the donor compartment and apparatus openings are sealed to prevent evaporation (Attama, Reichl et al. 2008). A suitable buffer like phosphate buffer pH 7.4 is used as the receptor medium which is magnetically stirred continuously. At specific time intervals aliquots of samples containing the released drug are taken from the receptor compartment and are quantified using a suitable method of determination like HPLC or spectroscopy. Sink conditions are usually maintained by replacing the volume of aliquots taken by similar volume of the buffer to resemble constant clearance of the drug from their physiological site of action.

Animal studies are usually necessary to investigate the ocular bioavailability and release profile of drugs incorporated into different drug delivery vehicles. One of the most extensively used animals for ocular studies is the rabbit (Hornof, Toropainen et al. 2005),
although larger animals are also employed in some studies (Basaran, Demirel et al. 2009). The rabbit’s eye has obvious morphological and physiological differences with the human eye. The most important difference is the infrequent blinking rates which can potentially affect pre-ocular retention of the medicament (Hornof, Toropainen et al. 2005). Moreover, ethical and economic issues associated with the use of animals in clinical studies have further limited this approach. Alternatively, release studies can be performed on a cell culture developed to mimic the eye physiology and its barriers. Many different well defined cell culture models of the ocular barriers are available in which parameters and conditions can be easily tailored according to the needs of a particular study (Hornof, Toropainen et al. 2005). Ocular cell cultures avoid inter-species variabilities and can be used to study the release profile, mechanisms of cellular transport, metabolism, protein expression and toxicity of SLNs and other ocular preparations (Hornof, Toropainen et al. 2005).

Attama et al. (2008) studied the *in vivo* drug release of diclofenac sodium from SLNs by preparing a human cornea construct (HCC) using a method described in previous literature (Reichl, Bednarz et al. 2004; Reichl, Döhring et al. 2005). The use of HCC can resolve the problems associated with the use of rabbits cornea such as differences in physiological structure, enzymes presents, transporters, efflux proteins, surface proteins and mucins (Attama, Reichl et al. 2008).

The cornea of the eye is a multilayer barrier consisting of three layers namely: the epithelium, the stroma and the endothelium. This multilayered tissue can be engineered and created step by step in a Transwell cell culture insert using SV-40 immortalised human endothelial and epithelial cells and native fibroblasts (stromal cells) (Reichl, Bednarz et al. 2004). As a result, the HCC prepared by this method will have a cellular structure resembling the real cornea with seven to nine layers of flattened epithelial cells, microvilli and microplicae present (Reichl, Bednarz et al. 2004). The method is promising since the permeability characteristics
of HCC also closely resemble the permeation behaviour of excised porcine cornea (Reichl, Bednarz et al. 2004). The HCC can be then mounted on a modified Franz diffusion cell and the amount of drug permeated to the receptor medium can be obtained following a similar procedure as described before.

1.5.4. Toxicity of lipid based nanoparticles

Lipid based nanoparticles are produced from long or medium chain triglycerides usually mixed with mono- and di-glycerides combined with individual or mixed ionic and/or non-ionic surfactants. They contain a wide range of compositions while having complicated characters and functionalities therefore, the toxicity of lipids as an excipient as well as in formulations needs thorough investigations (Chen 2008). Nanoparticles can induce cell toxicity by either one or a combination of the following mechanisms: adherence to the cell membrane due to their nanometre scale or ionic charge, degradation, release of cytotoxic degradation products and cellular internalization followed by degradation and toxic effects inside the cells (Lherm, Müller et al. 1992; Pardeike, Hommoss et al. 2009). SLNs are constructed from physiological lipids which are enzymatically biodegradable (Pardeike, Hommoss et al. 2009). Most lipids and their degradation products are considered non-toxic to cells of the human body. However, due to their nano size range, their biotoxicity is an important issue as the human body reacts very differently to nanoparticles when compared to larger particles of the same material (Pardeike, Hommoss et al. 2009). In vitro assays can be performed to evaluate topical and ocular toxicity and irritation caused by SLNs (Küchler 2008; Pardeike, Hommoss et al. 2009). In a study, Küchler et al. (2008) found no ocular irritation potential associated with SLNs from the results of a HET-CAM test based on the EU classification system R38 (Küchler 2008). Considering the results obtained by many studies, SLNs are actually very well tolerated at the cellular level (Pardeike, Hommoss et al.
2009). All surfactants are potential irritants, hence the biotoxicity of them remains an independent issue (Pouton and Porter 2008). The choice of surfactant used is also an important issue as water insoluble surfactants penetrate and fluidize biological membranes, whereas water soluble surfactants can solubilise the membrane components (Pouton and Porter 2008). It should be noted that cationic surfactants are more toxic than anionic and non-ionic surfactants (Pouton and Porter 2008) due to their ionic interaction with negatively charged cellular membranes. In the following sections a summary of both in vivo and in vitro tests are provided:

1.5.4.1. In vivo tests

Draize test

First developed in 1944 by Draize et al., this was one of the earliest attempts to assess irritancy of substances applied topically to the skin and mucous membranes (Draize, Woodard et al. 1944). This assay, which involves the use of rabbits, has received a large interest from the research community and hundreds of journal articles have been published based on the Draize’s principle of ocular opacity. The test involves instillation of 0.1 mL of the test substance into the conjunctival sac of at least six rabbits. Readings are obtained at 1, 24 and 48 hrs post treatment and scored based on a scoring table for the assessment of severity of ocular lesions. This traditional assay has long been recognised by governments and regulatory authorities, but the demand for reducing the number of animals used in research has driven scientists towards developing more efficient in vitro assays. Apart from the use of animals, the Draize test has attracted sound scientific criticism. The variability of the test results are of great concern and are deemed to be due to the small group size and the inability of the current scoring system to account for the complexities of the total in vivo response (York and Steiling 1998).
Low volume eye test (LVET)

LVET is a refinement of the Draize test in which only 0.01 mL is instilled (Bruner, Parker et al. 1992). The eyes are examined using a slit-lamp at 0.5, 1, 3 and 12 hrs intervals and the irritancy is measured based on a scoring scale (Bottari, Giannaccini et al. 1978; Cavalli, Gasco et al. 2002). Alternatively, the frequency of rabbit blinking within 5 mins after administration to the lower cul-de-sac is measured against the frequency of blinking occurred, with the reference physiological solution (Li, Nie et al. 2008). It is thought that the low irritation level and brief exposure time in this test are less stressful for the animals (Bruner, Parker et al. 1992). Moreover statistical analysis has shown that this three-animal model essentially provides the same eye irritation classification as the six-animal model (Bruner, Parker et al. 1992).

1.5.4.2. In vitro tests

There have been many attempts to develop in vitro ocular irritancy assays to replace the need for the use of animals (Bruner, Kain et al. 1991; Vinardell and Mitjans 2008). For an in vitro test to be scientifically meaningful, the end point of the assay must correlate in a predictable manner with the in vivo biological response and the in vitro assay should have a biological basis linking it to the processes involved in ocular injury (Bruner, Kain et al. 1991). Below is a discussion of several in vitro tests that have been reported and utilised in ocular formulation development over the last two decades (Bruner, Kain et al. 1991; Sina, Galer et al. 1995; Vinardell and Mitjans 2008).

Silicon microphysiometer

Silicon microphysiometer is a light addressable potentiometric sensor based device which can be used to measure the metabolic rate of cell cultures indirectly (Bruner, Kain et al. 1991; Bruner, Miller et al. 1991; Catroux, Rougier et al. 1993; Harbell, Osborne et al. 1999). Using
this device, the metabolic rate of the cell population can be measured for each dose and the dose inducing a 50% reduction in the metabolic rate (MRD50) is then used to estimate the ocular irritation potential (Harbell, Osborne et al. 1999).

Neutral red assay
Neutral red, which is 3-amino-7-dimethylamino-2-methylphenazine hydrochloride, can be used to provide a quantitative measure of the number of viable cells in culture (Rougier, Cottin et al. 1994; Vian, Vincent et al. 1995; Courtellemont, Hébert et al. 1999; Repetto, del Peso et al. 2008). According to a protocol published in Nature Protocols: “Cells are seeded in 96-well tissue culture plates and are treated for the appropriate period. The plates are then incubated for 2 hrs with a medium containing neutral red. The cells are subsequently washed, the dye is extracted in each well and the absorbance is read using a spectrophotometer” (Repetto, del Peso et al. 2008).

Total protein assay
This assay is usually carried out immediately after a neutral red assay using the same cells. The total protein remaining in each cell well is determined using a bovine albumin standard (Bruner, Kain et al. 1991; Bruner, Miller et al. 1991; Vian, Vincent et al. 1995). Dose response curves are then obtained and the concentration of the test material that causes a 50% reduction in total protein is used to determine ocular toxicity (Bruner, Miller et al. 1991).

MTT assay
The colorimetric based MTT, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, assay can also be used in determining the viability of cells. This assay is a fast and sensitive alternative to assess epithelial toxicity. It can be performed on primary corneal epithelial cells or any other specific cell line (Repetto, del Peso et al. 2008). The principle of the colorimetric determination is based on the conversion of the yellow tetrazolium salt MTT
to a purple formazan dye by mitochondrial succinate dehydrogenase of viable cells which indicates their metabolic activity (Vian, Vincent et al. 1995; Repetto, del Peso et al. 2008).

**Bovine corneal opacity-permeability assay (BCOP)**

The BCOP assay is an alternative to the Draize test and is the closest to an *in vivo* test without the need for live animals (Vinardell and Mitjans 2008). It is an *ex vivo* assay based on the measurement of corneal swelling, opacity and fluorescein permeability. A careful investigation of corneal opacity and fluorescein staining is sufficient to determine the corneal irritating potential of a given material (Prinsen and Koëter 1993). The BCOP assay, combined with histopathological evaluations and a cell culture method, is a reliable strategy to determine the corneal irritation potential of a formulation. The cornea is generally a transparent and lustrous tissue thus opacity would signify toxicity. Due to the structure of the corneal epithelium and the presence of tight junctions, a healthy cornea is practically impermeable to fluorescein dye. Any changes in the opacity and permeability of the cornea may therefore be associated with irritation and toxicity of a topically applied material.

**Histopathological examination following BCOP**

The corneal opacity and permeability endpoints associated with *in vitro* tests are often indicators of epithelial and stromal changes associated with ocular toxicity (Harbell 2006). To further investigate the toxicity at a cellular level, histological evaluation can be used to assess cellular toxicity as a result of the chemicals reacting with nucleic acids, mitochondrial proteins or other cellular targets, which often do not lead to immediate loss of cellular integrity or protein precipitation (Harbell 2006). Histopathological evaluation is performed on the three major layers of the cornea to provide a direct measure of the depth of injury, as well as better indication of the types of lesions produced, to rule out ocular lesions and to help comparing the depth of injury between control and test materials (Harbell 1999).
et al. (2006) presented a study where a series of chemicals were used to illustrate the type of lesions that can be induced by membrane lyses, protein denaturation, saponification and reactive/oxidative damage. The cornea is avascular, transparent and disk-like anterior section of the eye. The central thickness of the human cornea is about 500 µm (Hogan and Weddell 1971). The non-keratinizing stratified squamous epithelial layer with a basal cell layer produces five to six superficial layers with a total thickness of about 50 µm (Figure 5-8). The corneal epithelium undergoes mitosis and regenerates every 6 to 24 hrs whereas Bowman’s membrane is acellular and once destroyed cannot regenerate. This explains why mild corneal surface injuries are reversible. The stroma represents 90% of the corneal thickness and consists of collagen fibrils which are parallel to each other in a special arrangement to maintain transparency. Unlike epithelial cells, endothelial cells do not undergo mitosis. They regulate the amount of water in the cornea and prevent corneal opacification.

**Hen’s egg test–chorioallantoic membrane (HET-CAM)**

The HET-CAM is designed to measure the conjunctivital toxicity of a test substance which is based on the assumption that the chorioallantoic membrane of embryonated hen’s eggs is similar to the vascularised mucosal tissues of the eye (Vinardell and Macián 1994; Gilleron, Coecke et al. 1996; Barile 2010).

**1.5.7. Materials used in the preparation of SLNs**

In general, the type of lipids, surfactants and drugs used in the preparation of SLNs have their own influence on the final quality of the product. For instance, release of a lipophilic compound from a lipid carrier depends upon partitioning between the aqueous medium surrounding the carrier, the surfactant and the lipid exploited (Vyas, Rai et al. 2008). Many factors contribute to the drug disposition in either the lipid or the surfactant phase which need thorough investigation and characterisation.
1.5.7.1. Lipids

With respect to lipids, crystalline modification, structure, concentration and hydrophobicity characteristics are important factors in SLNs formation (Vyas, Rai et al. 2008). Supercooling of SLNs is also an important feature to be ensured after melt homogenisation, otherwise the final product would appear as a nanoemulsion rather than a colloidally dispersed SLNs (Bunjes, Westesen et al. 1996). Moreover, the solid matrix is expected to remain in the solid state at body temperatures so that controlled and sustained drug release properties can be achieved (Bunjes, Westesen et al. 1996). It is well known that lipids which form highly ordered crystalline lattice are unfavourable as drug expulsion is greater. Therefore, it is desirable to employ lipids with complex structures containing fatty acid chains of different lengths which are able to accommodate the drug efficiently. However, it has to be noted that lipids with long fatty acid side chains usually form larger particles. The pharmaceutical industry encourages scientists to use a combination of long chain and short chain fatty acids to achieve better quality, stability and higher drug loading capacities. One important advantage of SLNs over other nano-carriers, apart from its physiological acceptability, is their stability and longer shelf life. Radomska-Soukarev (2007) studied the chemical stability of several lipids after SLNs production over a period of two years and found that the lipids were sufficiently stable. It was observed that the initial preparation method did not affect the chemical stability of the lipids and that triglycerides produced much more stable SLNs formulations than mono- and diglycerides (Radomska-Soukharev 2007). A list of lipids used in the preparation of SLNs for ocular drug delivery is given in Table 1-2.
Table 1-2 Lipids used in the production of SLNs for ocular drug and gene delivery.

<table>
<thead>
<tr>
<th>Lipids used in preparing SLNs and NLCs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solid lipids:</strong></td>
<td></td>
</tr>
<tr>
<td>Compritol® 888 ATO</td>
<td>(Li, Nie et al. 2008; Basaran, Demirel et al. 2009)</td>
</tr>
<tr>
<td>Precirol® ATO 5</td>
<td>(del Pozo-Rodríguez, Delgado et al. 2008)</td>
</tr>
<tr>
<td>Phospholipon 90G®</td>
<td>(Attama, Reichl et al. 2008; Attama, Reichl et al. 2009)</td>
</tr>
<tr>
<td>Gelucire 44/14</td>
<td>(Li, Nie et al. 2008)</td>
</tr>
<tr>
<td>Stearylamine</td>
<td>(Li, Nie et al. 2008)</td>
</tr>
<tr>
<td>Tripalmitin (Dynasan® 116)</td>
<td>(Basaran, Demirel et al. 2009)</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>(Cavalli, Gasco et al. 2002)</td>
</tr>
<tr>
<td><strong>Cationic lipids:</strong></td>
<td></td>
</tr>
<tr>
<td>DOTAP</td>
<td>(del Pozo-Rodríguez, Delgado et al. 2008)</td>
</tr>
<tr>
<td>Octadecylamine</td>
<td>(Basaran, Demirel et al. 2009)</td>
</tr>
<tr>
<td><strong>Liquid lipids:</strong></td>
<td></td>
</tr>
<tr>
<td>Miglyol 812</td>
<td>(Li, Nie et al. 2008)</td>
</tr>
</tbody>
</table>

Triglycerides

Triglycerides are either short (<5 carbons), medium (6-12 carbons), or long chain (>12 carbons) and may be synthetically hydrogenated to reduce oxidative degeneration (Hauss 2007). The longer chain triglycerides have higher melting points, believed to be more stable and expected to produce better SLNs (Radomska-Soukharev 2007). Many triglycerides such as tricarpin (Domb 1995), trilaurin (Domb 1995; Westesen and Bunjes 1995; Bunjes, Westesen et al. 1996; Heiati, Tawashi et al. 1997; Schwarz and Mehnert 1997; Heiati, Tawashi et al. 1998; zur Mühlen, Schwarz et al. 1998), trimyristin (Westesen and Bunjes 1995; Bunjes, Westesen et al. 1996; Westesen, Bunjes et al. 1997), tripalmitin (Westesen and Bunjes 1995; Bunjes, Westesen et al. 1996; Westesen and Siekmann 1997), tristearin (Domb 1995; Westesen and Bunjes 1995; Bunjes, Westesen et al. 1996; Westesen, Bunjes et al. 1997) and hydrogenated coco-glycerides (Almeida, Runge et al. 1997) have been used in the preparation of SLNs for several purposes (Mehnert and Mäder 2001). These widely used triglycerides are considered...
as suitable solid lipid core materials for the production of particles because their melting point is well above the body temperature to allow controlled drug release and sufficiently low to assure solidification after melt homogenisation (Bunjes, Westesen et al. 1996). However, it has been shown that melt homogenisation with tripalmitin, tristearin and trimyristin produces a crystalline solid lipid matrix, whereas trilaurin dispersions remained in the form of an emulsion even after exposure to refrigerated temperatures (Bunjes, Westesen et al. 1996). This problem can be rectified by addition of longer chain triglycerides to speed up nucleation and crystallization of trilaurin or other resembling glycerides (Bunjes, Westesen et al. 1996). During the course of production and storage, glycerides may exist under various polymorphic forms such as hexagonal (α), orthorhombic (β’) and triclinic (β) which can be confirmed by X-ray diffraction (Jannin, Musakhanian et al. 2008). Polymorphic changes depend on the thermal history of the lipid (Jannin, Musakhanian et al. 2008) and need to be investigated from SLNs as the transition to a more stable crystalline form potentiates drug expulsion. It is suggested that the use of a mixture of triglycerides produce crystals with a poorly ordered matrix (Bunjes, Westesen et al. 1996) which can accommodate more quantities of the drug while avoiding drug expulsion during storage. The properties of colloidally dispersed glycerides, especially the melting, crystallization and the kinetics of polymorphic transitions, differ greatly from those of their bulk material (Bunjes, Westesen et al. 1996). Therefore, the suitability of these lipids for the SLNs formulation should not be evaluated from their bulk properties and the dispersion properties should be carefully studied to optimize the production parameters which decide the quality of the final product.
Chapter 1 Introduction and literature review

![Chemical structure of triglycerides](image)

**Figure 1-10 Chemical structure of triglycerides (adopted from (Seniha Güner, Yagci, & Tuncer Erciyes, 2006)).**

**Compritol 888 ATO**

The solid lipid glycerol behenate (Compritol®888 ATO) is used very often to prepare SLNs. It is composed of a mixture of mono-, di- and triacylglycerols with a very small amount of α-form which disappears under thermal stress owing to its thermodynamic instability (Souto, Mehnert et al. 2006).

Compritol usually crystallizes in its βʹ modification which is very sensitive to elevated temperatures (Souto, Mehnert et al. 2006). It has been shown that Compritol has higher loading capacities compared to stearic acid, monostearin and tristearin (Vyas, Rai et al. 2008) and reportedly can be used to avoid re-crystallisation problems associated with other solid lipid used (zur Mühlen, Schwarz et al. 1998).

![Diagram of Compritol 888 ATO](image)

**Figure 1-11 Compritol 888 ATO consists of mixture of monobehenate (A), dibehenate (B) and tribehenate (C) of glycerol (Adopted from (Brubach, et al., 2007)).**
**Gelucires**

Gelucires are semisolid lipids mostly used in the preparation of NLCs for ocular drug delivery (Li, Nie et al. 2008). They are saturated polyglycolized glycerides which consist of a mixture of mono-, di- and tri-glycerides and di-fatty acid esters of polyethylene glycol (Li, Nie et al. 2008). It is reported in the literature that they can enhance transdermal absorption of certain drugs (Nilüfer, Aysegül et al. 2003). This finding was applied to the corneal permeation issue and some promising results were obtained (Li, Nie et al. 2008).

**Cationic lipids**

Cationic lipids are often used alone (e.g. stearic acid) or in combination (e.g. octadecylamine and N-[1-(2, 3-Dioleoyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTAP)) to induce a positive charge on SLNs. Cationic SLNs obtained using these cationic lipids have shown better corneal penetration and gene transfection properties (del Pozo-Rodríguez, Delgado et al. 2008; Basaran, Demirel et al. 2009). The enhanced drug penetration through cornea is probably due to the ionic interaction of cationic particles with negatively charged epithelial cells (Law, Huang et al. 2000; Cortesi, Argnani et al. 2006). These ionic interactions promote prolonged corneal residence time of the formulation and possibly corneal morphological changes resulting in enhanced drug penetration and absorption. The same ionic interactions are known to be responsible for binding of cationic SLNs used as non-viral gene delivery vectors to the targeted negatively charged cell surfaces promoting enhanced transfection and cellular trafficking (del Pozo-Rodríguez, Delgado et al. 2008).
1.5.7.2. Emulsifiers

Several emulsifiers have been used to prepare SLNs including polysorbates (e.g. Tween-80), polyoxyls (e.g. Cremophor® EL), sodium lauryl sulphate, bile salts (e.g. cholic acid), Poloxomer® 188, Brij® 78 and saponins with HLB values ranging from 6 to 18 (Mehnert and Mäder 2001; Hauss 2007). Many of these agents are also used as corneal epithelium penetration enhancers (Kaur, Kanwar et al. 2002). Studies have shown that higher concentrations of emulsifiers produce smaller sized particles while low concentrations produce relatively large particles (Radomska-Soukharev 2007; Helgason, Awad et al. 2009).

During the emulsification process, creation of smaller sized particles produces a large increase in surface area of the system which requires excessive amounts of surfactants to coat the newly formed surfaces in order to stop the uncovered surfaces from colliding and agglomerating (Vyas, Rai et al. 2008). Usually, a mixture of surfactants yields smaller sized particles and the system shows a lower polidispersity index and higher lipid stability (Radomska-Soukharev 2007). It has to be noted that the type and concentration of the surfactant can affect the chemical stability of physiological lipids used to prepare SLNs (Radomska-Soukharev 2007). In ocular drug delivery the use of cationic surfactants is favoured in order to induce a positive charge to the formulated SLNs. This allows longer residence time on the corneal epithelium and better penetration to the posterior chamber of the eye. Stearylamine has been used in the preparation of cationic SLNs for ocular (Li, Nie et al. 2008) and non-ocular drug delivery.
In a study, Helgason et al. (2009) used Tween 20 to investigate the effect of surfactant concentration and surface coverage on the stability and crystalline structure of SLNs. They suggested that “simple addition of surfactant to the emulsion prior to lipid crystallization helps prevent gel formation”. This means that the surfactant molecules cover the newly formed surfaces inhibiting their agglomeration and increasing the SLNs emulsion stability. However, at low excess surfactant concentrations there may not be sufficient surfactant molecules to cover the entire surface, hence, optimal surface coverage may not be achieved (Helgason, Awad et al. 2009). It was shown that by adding a higher concentration of a surfactant during the cooling process and crystallization of the lipid, one can control the overall stability of the particles as well as particle shape changes could be controlled, thereby avoiding agglomeration and transition from oval or spherical to needle or disc shaped particles.

Table 1-3 Emulsifiers used in the production of SLNs for ocular drug delivery.

<table>
<thead>
<tr>
<th>Emulsifiers used in preparing SLNs and NLCs for ocular drug delivery</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor® EL</td>
<td>(Cavalli, Gasco et al. 2002; Li, Nie et al. 2008)</td>
</tr>
<tr>
<td>Transcutol® P</td>
<td>(Li, Nie et al. 2008)</td>
</tr>
<tr>
<td>Stearylamine</td>
<td>(Li, Nie et al. 2008)</td>
</tr>
<tr>
<td>Polysorbate 80 (Tween®80)</td>
<td>(Attama, Reichl et al. 2008; del Pozo-Rodríguez, Delgado et al. 2008; Attama, Reichl et al. 2009; Basaran, Demirel et al. 2009)</td>
</tr>
<tr>
<td>Soya phosphatidylcholine (Epikuron 200)</td>
<td>(Cavalli, Gasco et al. 2002)</td>
</tr>
<tr>
<td>Sodium taurocholate</td>
<td>(Cavalli, Gasco et al. 2002)</td>
</tr>
</tbody>
</table>

1.5.4.3. Drug molecule

One of the advantages of SLNs over other particular carrier systems is that both hydrophilic and lipophilic drugs can be incorporated. SLNs can extend drug shelf life and half-life by protecting them from the external environment. SLNs allow controlled/sustained release of
drugs, thereby reducing the required administered dose to achieve therapeutic objectives. This in turn reduces the patients’ risk of exposure to toxic doses of drugs. SLNs are also believed to increase bioavailability of poorly penetrating drugs. Drugs can be incorporated into SLNs through deposition between fatty acid chains, between lipid layers or in imperfections (Vyas, Rai et al. 2008).

1.6. Thesis question, aims and objective

This thesis attempts to answer the research question of whether a lipid nanoparticle based novel drug delivery system can enhance the ocular bioavailability of ACV in comparison with a commercially available ACV ophthalmic ointment.

The aim of this thesis is to improve ocular bioavailability of ACV by designing and developing a lipid based nanoparticulate ocular drug delivery system to encapsulate and deliver the antiviral drug. ACV is a compound of medium polarity which is poorly soluble in water and oil. SLNs were primarily chosen based on their lipophilic properties which could aid drug penetration through biological membranes. The choice of SLNs was also based on the advantages they offer. The following objectives were pursued to complete this thesis:

1. to design and develop a lipid based nanoparticulate system for ocular delivery of ACV (Chapter 2),
2. to modify the physiochemical properties of the drug by β-Cyclodextrin (βCD) complexation to increase solubility, enhance entrapment efficiency into SLNs and modulate the nanoparticle drug release profile (Chapter 3),
3. to enhance and optimise the developed system for in vivo application by coating nanoparticles with chitosan which is a cationic, natural and bioadhesive polymer (Chapter 4),
4. to undertake relevant antiviral efficacy, toxicity assay and cell uptake studies to assess the efficacy and suitability of the formulation for ocular application (Chapter 5) and,

5. to perform an *in vivo* animal ocular pharmacokinetic study to determine whether or not the nanoparticulate system is more efficient than the commercially available ophthalmic ACV ointment (Chapter 5).
Chapter 2. Formulation and Characterisation of Acyclovir Containing
Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Chapter Two

“Many things difficult to design prove easy to perform. “

— Samuel Johnson
2.1. Introduction

Acyclovir (ACV) is an antiviral drug used in the treatment of HSV ocular disease. It is a compound of medium polarity which is practically insoluble in water with very limited lipophilicity (de Vrueh, Smith et al. 1998; Ghera, Perret et al. 2009). For this reason it has poor ocular bioavailability and cannot be formulated as conventional eye drops. Currently, an ACV ointment is the only dosage form available but, is known to be efficient only against superficial and mild herpes keratitis (Richards, Carmine et al. 1983). The patient is generally required to apply the ointment 5-6 times daily for a period of 1-2 weeks. Not only is the ointment difficult to administer resulting in patient incompliance, but it may also delay the healing of infective lesions. Due to side effects associated with the ointment therapy, its use is not approved in the United States (Anand, Hill et al. 2003; Foster, Azar et al. 2005; Zaal and Opstelten 2005). Systemic administration of ACV requires large doses to attain the minimum effective concentration at the site of action resulting in an increased risk of systemic side effects and therapeutic complications.

Several trials have attempted to enhance the ocular bioavailability of ACV with liposomes and microparticles with enhanced antiviral efficiency demonstrated (Law, Huang et al. 2000; de Jalón, Blanco-Príeto et al. 2003). However, no liposomal ACV formulation has made its way to the market mainly due to toxicity and instability issues associated with liposomes. Other approaches such as the presence of permeation enhancers including chitosan, ethylenediaminetetraacetic acid (EDTA), inclusion complexes with cyclodextrines, cross-linked malonylchitosan microspheres and mucoadhesive microspheres have been used to enhance the bioavailability of ACV (Majumdar, Hippalgaonkar et al. 2008; Stulzer, Lacerda et al. 2008; Ghera, Perret et al. 2009; Tao, Lu et al. 2009).
Lipid based carriers have the ability to improve the corneal penetration of drugs. Solid lipid nanoparticles (SLNs) offer an attractive choice as ocular drug delivery carriers due to their stability and non-toxicity as they are prepared from physiological and biocompatible lipids (Mehnert and Mäder 2001). Due to their nano size range, SLNs can be effective ocular drug delivery systems by enhancing corneal absorption, improving ocular bioavailability, prolonging the ocular retention time and providing a controlled drug release profile (Kaur, Kanwar et al. 2002; Seyfoddin, Shaw et al. 2010). They offer other advantages such as drug targeting, long term stability, good drug loading, accommodation of hydrophilic/lipophilic drugs, possibility of sterilization by autoclaving and easy large scale production (Mehnert and Mäder 2001; Seyfoddin, Shaw et al. 2010). It has been documented that SLNs are capable of enhancing corneal penetration due to their lipophilicity which increases their uptake by ocular tissues and allows more drugs to penetrate through the barriers (Cavalli, Gasco et al. 2002; Kaur, Rana et al. 2008). Using cationic lipids in SLNs can further enhance drug penetration into the cornea through ionic interactions with negatively charged corneal epithelial cells.

The main concern about incorporating ACV into SLNs is its solubility profile which might hinder its entrapment inside a vesicular carrier. It has been shown that by controlling the formulation parameters such as lipid and surfactant concentrations and types, the entrapment efficiency and quality of the final product can be optimized (Almeida, Runge et al. 1997; Attama and Müller-Goymann 2007). Incorporation of liquid lipids into the structure of SLNs results in the formation of nano-structured lipid carriers (NLCs) which often show better entrapment capacity and a sustained release profile (Hu, Jiang et al. 2005; Li, Nie et al. 2008; Shen, Sun et al. 2010).

The general aim of this study was to improve the ocular delivery and antiviral activity of ACV by incorporating it into SLNs and NLCs. Therefore, the effects of formulation parameters on the quality of the resultant nanoparticles were studied and the physiochemical
properties such as the drug entrapment efficiency, loading, particle size, zeta potential, melting properties and *in vitro* drug release profiles were investigated. Bovine cornea was used as a barrier in corneal drug permeation studies to mimic a corneal barrier.

### 2.2. Materials and methods

#### 2.2.1. Materials

ACV (batch 116471554, Jai Radhe Sales, India) was the drug, stearic acid (Scharlau chemie S.A., EU), Compritol® 888 ATO and Cithrol GMS were used as solid lipids and Capryol® 90 Lauroglycol® 90 (Gattefosse, France) as liquid lipids. The following surfactants were used: Tween® 40, Tween® 80 (Sigma, Germany), Poloxamer® 188 (BASF corporation, USA) and Brij® 78 (Sigma, Germany). Phosphate buffer saline (PBS) and all other chemicals used were of analytical grade.

#### 2.2.2. HPLC method of detection

High performance liquid chromatography (HPLC) was used to analyse ACV concentrations in *in vitro* and corneal penetration studies based on a method described in other reports (Law, Huang et al. 2000). 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 was employed. The output signal was monitored and processed using ChemStation software (Agilent Technologies, Waldbronn, Germany). The mobile phase comprising of acetonitrile and 0.05 M ammonium acetate with the pH adjusted to 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 °C with a detection wavelength of 254 nm. Calibration curves were linear over the concentration range of the study ($r^2=0.999$). The HPLC method was validated for linearity, accuracy and precision.
2.2.3. Fabrication of SLNs and NLCs

A modified version of the hot oil-in-water (o/w) microemulsion technique (Heydenreich, Westmeier et al. 2003; Vighi, Ruozi et al. 2007) was used to prepare the nanoparticles. The lipid was melted at 10 °C above its melting point and the drug was dispersed in the lipid melt. The melting points of lipids used were as follows: Compritol 72.09 °C, stearic acid 69.8 °C and Cithrol GMS 70 °C. The aqueous phase containing the surfactant was heated to the same temperature. A primary o/w emulsion was formed by homogenisation (3 min at 20,500 rpm using 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 the stirring was continued for 7 min or until the formation of SLNs. 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 SLNs were lyophilised at -20°C at below 0.133 mBar for 24 hrs to obtain a dry powder (freeze dryer system: Labconco-7806020, USA).

NLCs were prepared using the same methodology, but ACV was dispersed in the liquid lipid before the addition of the solid lipid. The formulation parameters investigated for the preparation of SLNs and NLCs are shown in Table 2-1.
Table 2-1 Sample composition and experimental parameters investigated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compritol (mg)</th>
<th>Stearic acid (mg)</th>
<th>Cithrol (GMS) (mg)</th>
<th>ACV (mg)</th>
<th>Capryol-90 (mg)</th>
<th>Lauroglycol-90 (mg)</th>
<th>Surfactant type and concentration (% v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tween-40, Tween-80, Brij-78, Poloxamer-188</td>
</tr>
<tr>
<td>S(1)</td>
<td>120</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>S(2)</td>
<td>200</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>S(3)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>2</td>
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</tr>
<tr>
<td>S(4)</td>
<td>600</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>S(5)</td>
<td>-</td>
<td>40</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>S(6)</td>
<td>-</td>
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<td>40</td>
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<tr>
<td>S(7)</td>
<td>400</td>
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<td>5</td>
<td>-</td>
</tr>
<tr>
<td>S(8)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>7.5</td>
<td>-</td>
</tr>
<tr>
<td>S(9)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>S(10)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>S(11)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>S(12)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>N(1)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>120</td>
<td>2</td>
<td>-</td>
<td>-</td>
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<tr>
<td>N(2)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>200</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>N(3)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>120</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N(4)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>200</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N(5)</td>
<td>400</td>
<td>-</td>
<td>40</td>
<td>100</td>
<td>100</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2.4. Particle size and zeta potential

The average particle size and zeta potential of nanoparticles were measured immediately after preparation by light scattering (Malvern Zetasizer, ZEN 3600, Malvern Instruments, UK). A volume of 0.1 mL of the nanoparticle suspension was diluted in 5 mL of Milli-Q water. The applied voltage and the number of runs were determined automatically by the software with all experiment performed in triplicate at 25 °C.

2.2.5. Scanning electron microscopy (SEM)

SEM was used to examine the morphology of wet and freeze-dried samples. Samples were coated in a Polaron SC 7640 sputter coater and analysed by a Philips XL30S FEG with a SiLi
(Lithium drifted) Super Ultra-Thin Window EDS detector. SEM images of the bovine cornea after *ex vivo* release studies were taken to examine any change in the corneal epithelial and endothelial morphology. For this, corneas were washed with PBS, frozen overnight and freeze-dried before being coated as described above.

### 2.2.6. Differential scanning calorimetry (DSC)

A differential scanning calorimeter (DSC Q1000 V9.9 Build 303, Universal V4.5A TA Instruments, USA) was used to evaluate the physical state of particles. Accurately weighed 5 mg of lyophilised samples were placed in aluminium pans which were hermetically sealed, while empty pans were used as a reference. The thermal behaviour of samples was determined in the range of 10-280 °C at a heating rate of 10 °C min⁻¹. The crystallinity index of each sample was calculated using the below formula by assuming the melting enthalpy of Compritol as 100% (Shen, Sun et al. 2010):

\[
\text{Crystallinity Index (CI\%) = \left( \frac{\text{Enthalpy}_{SLNs\ or\ NLCs}}{\text{Enthalpy}_{Bulk\ lipid} + \text{Concentration}_{Lipid\ phase}} \right) \times 100}
\]

### 2.2.7. Entrapment efficiency (EE%) 

A method based on the extraction of drug from SLNs was developed to determine the percentage of entrapment efficiency. An accurately weighed amount of nanoparticles (10 mg) was dissolved in 1-octanol (1 mL) and was shaken vigorously for a min. A volume of 5 mL of phosphate buffer solution (PBS, pH 6.8) was added and the mixture was shaken again to allow drug partitioning. The mixture was left to stand for 2 hrs to allow phase separation. The aqueous phase containing the drug was collected and the amount of the drug was quantified using HPLC.
Percentage entrapment efficiency (EE%) and drug loading (DL%) were determined using the total amount of drug added and the amount of drug in nanoparticles according to the following formulae:

\[
EE\% = \frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of drug}} \times 100
\]

\[
DL\% = \frac{\text{Amount of drug in nanoparticles}}{\text{Total amount of drug} + \text{Total amount of lipid}} \times 100
\]

2.2.8. *In vitro* drug release studies

The *in vitro* release studies were carried out using Franz-type diffusion chambers (Logan Instruments, USA) at 34 ± 1 °C under stirring at 100 rpm. Cellulose dialysis membrane with a molecular weight cut-off of 12,400 Dalton was used to mimic a diffusional barrier. Membranes were soaked in PBS overnight and washed with water before being mounted on to the diffusion cells. An appropriate quantity of nanoparticles and freeze-dried complexes containing 3 mg of ACV were dispersed in 2 mL of PBS pH 7.4 and were added into the donor chamber. ACV suspension in PBS was used as control (1.5 mg mL\(^{-1}\)). The receptor compartment was filled with freshly prepared PBS pH 7.4. Samples of 0.5 mL were taken from the receptor chamber at predetermined time intervals and replaced with an equal volume of fresh buffer. The amount of drug diffused was determined using HPLC. All experiments were performed in triplicate.

The cumulative amount of drug released (Qn) was plotted as a function of time and was calculated based on the following equation:

\[
Qn = Cn \times V0 + \sum_{i=1}^{n-1} Ci \times Vi
\]
where \( C_n \) was the drug concentration in the receptor medium at each sampling time point, \( C_i \) was the drug concentration of the sample, \( V_0 \) and \( V_i \) were the volumes of the dissolution medium and the sample respectively (Li, Jiang et al. 2010).

### 2.2.9. 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 membranes. Bovine eyes were collected from Auckland Meat Processors Limited (Auckland, New Zealand) immediately after slaughtering of the animal. The eyes were transported in PBS to the laboratory and corneas were excised carefully to avoid any damage. The corneas were placed between the donor and receptor compartments. As above ACV suspension in PBS was used as the control. Accurately weighed freeze dried samples of SLNs and NLCs containing 3 mg of ACV were dispersed in 2 mL of PBS pH 7.4 and were placed into the donor compartments. Samples were analysed by HPLC and all experiments were repeated in triplicates.

The apparent permeability coefficient was calculated using the following equation (Schoenwald and Huang 2006):

\[
P_{\text{app}} = \frac{\Delta Q}{\Delta t} (3600) A C_0
\]

where \( \Delta Q/\Delta t \) is the permeation rate (\( \mu \text{g} \text{ h}^{-1} \)) obtained from the slope of the linear line of corneal penetration of ACV vs. 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 (\( \mu \text{g} \text{ mL}^{-1} \)).
2.2.10. Determination of corneal hydration levels

The wet weight, $W_a$, of each corneal sample was determined after the permeation study. The cornea was carefully excised off the scleral ring, rinsed with methanol and excess fluid was removed using a filter paper (Saettone, Chetoni et al. 1996; Monti, Chetoni et al. 2002; Liu, Zhang et al. 2011). The dried weight, $W_b$, was obtained after drying the corneas in an oven at 60 °C for 16 hrs. The percentage corneal hydration level (HL%), was determined for both untreated corneas and those treated with the formulations using the following formula:

$$HL(\%) = (1 - \frac{W_b}{W_a}) \times 100$$

2.2.11. Statistical analysis

All data obtained are shown as mean ± SD. All experiments were repeated at least thrice and experimental data were analysed using a $t$-test. If $p < 0.05$, the difference was considered significant.

2.3. Results and discussion

2.3.1. HPLC validation

The peak for ACV was eluted at about 7.9 min post injection. There was no interfering peak around the eluting time of the drug and the peak appeared sharp.
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Figure 2-1 HPLC chromatogram of ACV in PBS (1 mg mL\(^{-1}\)).

**Linearity**

A calibration curve was plotted and the plot was subjected to regression studies. The concentration of ACV examined ranged from 10-1000 ng mL\(^{-1}\) (Figure 2-2) and 1.25-25 µg mL\(^{-1}\) (Figure 2-3). The data obtained confirmed that the plots are linear. The equation of the line for the 10-1000 ng mL\(^{-1}\) concentration range was \(y = 0.1461 \times \) with \(r^2 = 0.9998\). The equation of the line for 1.25-25 µg mL\(^{-1}\) concentration range was \(y = 145.53 \times \) with \(r^2 = 0.9998\).

Figure 2-2 Calibration curve of ACV (10-1000 ng mL\(^{-1}\), n=3, mean ± SD).
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Figure 2-3 Calibration curve of ACV (1.25-25 µg mL\(^{-1}\), n=3 mean ± SD).

**Precision**

Intra- and inter-day precision studies were performed. The results indicated that RSD (%) values for intra-day studies ranged from 0.23 to 3.68 and for inter-day precision from 1.21 to 3.62 for the concentration range of 10-1000 ng mL\(^{-1}\) (Table 2-2). For the concentration range of 1.25-25 µg mL\(^{-1}\), RSD (%) values for intra-day studies ranged from 0.47 to 2.93 and for inter-day precision from 2.24 to 4.26 (Table 2-3).

Table 2-2 Precision studies for the concentration range of 10-1000 ng mL\(^{-1}\).

<table>
<thead>
<tr>
<th>Actual concentration (ng mL(^{-1}))</th>
<th>Measured concentration ± SD; RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Repeatability (n=6)</td>
</tr>
<tr>
<td>10</td>
<td>10.72 ± 0.39; 3.68</td>
</tr>
<tr>
<td>500</td>
<td>497.60 ± 1.18; 0.23</td>
</tr>
<tr>
<td>1000</td>
<td>995.89 ± 4.48; 0.45</td>
</tr>
</tbody>
</table>
Table 2-3 Precision studies for the concentration range of 1.25-25 µg mL⁻¹.

<table>
<thead>
<tr>
<th>Actual concentration (ng mL⁻¹)</th>
<th>Measured concentration ± SD; RSD (%)</th>
<th>Repeatability (n=6)</th>
<th>Intermediate precision (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>1.21 ± 0.03; 2.93</td>
<td>1.21 ± 0.05; 4.26</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.07 ± 0.04; 0.47</td>
<td>10.07 ± 0.25; 2.64</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>24.86 ± 0.27; 1.10</td>
<td>24.335 ± 0.54; 2.24</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2. Characterisation of the developed SLNs

Hot microemulsion is a suitable method for the fabrication of ACV loaded SLNs. ACV has high thermal stability and when the drug was kept at 70 °C for 15 days, 98.14% of the drug was retained (Sinha, Trehan et al. 2007). Ultracentrifugation was used for the separation and purification of SLNs. Although it is an easier and cheaper technique compared to dialysis and ultrafiltration, it is compromised in terms of the preparation yield. Only when large amount of lipids (600 mg) were used the preparation yield reached 19.25%, otherwise it remained between 3-6% w/w.

Table 2-4 Effect of drug to lipid ratio on the characteristics of ACV loaded SLNs.

<table>
<thead>
<tr>
<th>Drug : lipid ratio</th>
<th>EE%</th>
<th>DL%</th>
<th>(Z_{\text{average}}) (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 3</td>
<td>4.10 ± 0.15</td>
<td>2.05 ± 0.07</td>
<td>185.43 ± 2.75</td>
<td>-34.23 ± 0.50</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>1: 5</td>
<td>8.34 ± 0.80</td>
<td>2.78 ± 0.26</td>
<td>283.16 ± 1.42</td>
<td>-30.3 ± 1.22</td>
<td>0.49 ± 0.12</td>
</tr>
<tr>
<td>1: 10</td>
<td>11.14± 0.67</td>
<td>2.02 ± 0.01</td>
<td>465.86 ± 7.15</td>
<td>-35.13 ± 0.32</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>1: 15</td>
<td>34.39± 1.55</td>
<td>4.29 ± 0.19</td>
<td>766.66± 11.11</td>
<td>-30.73 ± 0.66</td>
<td>0.75 ± 0.12</td>
</tr>
</tbody>
</table>

EE%, entrapment efficiency; DL%, drug loading; \(Z_{\text{average}}\), average particle size; PDI, polydispersity index. Results are presented as mean ± SD (n=3).
Table 2-4 shows the effect of drug to lipid ratio on the physicochemical properties of SLNs. As can be seen, an increase in the mass of Compritol resulted in an increase in EE% of SLNs. This could be due to the fact that more core material is available to accommodate drug molecules under a given surfactant concentration which was in accordance with previous studies (Lv, Yu et al. 2009).

As shown, an increase in the concentration of lipid significantly increased the particle size of SLNs measured after preparation. A linear relationship exists between the mean diameter of particles and mass of Compritol ($R^2 = 0.98$). The same relationship has previously been reported for glyceryl monostearate (GMS) SLNs prepared by the solvent emulsification-diffusion technique (Trotta, Debernardi et al. 2003). Lower lipid concentrations would result in more surfactant available on the newly formed surfaces which leads to the formation of smaller sized particles during the formation of the hot microemulsion. In other terms, increasing the lipid content results in poor emulsifying efficiency and therefore an increase in particle agglomeration (Subedi, Kang et al. 2009). In addition, the viscosity of the molten lipid phase increases by increasing the lipid concentrations. PDI was also increased by increasing lipid concentration probably due to a reduction in homogenisation efficiency attributed to changes in the viscosity of the lipid melt.

From the zeta potential data obtained immediately after preparation, it can be seen that increasing the mass of Compritol has little or no effect on the surface charges. The reliability of this observation was checked by 95% confidence intervals (CI). The average zeta potential of particles was $-32.59 \pm 2.38$ mV (mean + CI). The CI value contains the “value of no effect” ranged between -2 and +3 which means that the risk reduction is not statistically significant (Attia 2005). The zeta potential of all formulations ranged from -30 to -34 mV indicating good physical stability. In addition, the steric hindrance effect due to the presence
of non-ionic surfactants, Tween 40, was an additional factor contributed to the stability of the colloidal dispersion (Liu, Hu et al. 2007; Fang, Fang et al. 2008).

Formulations prepared by constituting 400 mg of several individual lipids, namely Compritol, stearic acid and Cithrol GMS, showed varying entrapment efficiencies (Table 2-5). Cithrol GMS constituted nanoparticles showed the highest entrapment with smaller particle size followed by stearic acid and Compritol nanoparticles. Stearic acid has a required HLB value of 15 which exactly matches that of the surfactant used (Tween-40, HLB=15.6). This is probably the reason for the higher EE% and the smaller particle size of stearic acid SLNs compared to Compritol SLNs (HLB=2). As the HLB value of the surfactant increases, the surface free energy of the particles reduces leading to a smaller particle size, an increased surface area and ultimately increased drug entrapment efficiency especially if drug loading is a surface phenomenon.

### Table 2-5 Effect of lipid type on the characteristics of ACV loaded SLNs.

<table>
<thead>
<tr>
<th>Lipid type</th>
<th>EE%</th>
<th>Z\textsubscript{average} (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic acid</td>
<td>18.51 ± 3.72</td>
<td>318.93 ± 7.18</td>
<td>-32.2 ± 0.34</td>
<td>0.63 ± 0.01</td>
</tr>
<tr>
<td>Cithrol GMS</td>
<td>26.05 ± 0.18</td>
<td>312.16 ± 0.58</td>
<td>-22.43 ± 1.88</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>Compritol</td>
<td>11.14 ± 0.67</td>
<td>465.86 ± 7.15</td>
<td>-35.13 ± 0.32</td>
<td>0.53 ± 0.05</td>
</tr>
</tbody>
</table>

EE%, entrapment efficiency; Z\textsubscript{average}, average particle size; PDI, polydispersity index. Results are presented as mean ± SD (n=3).

Interestingly, Cithrol GMS which had the highest entrapment efficacy has the smallest particle size after preparation followed by stearic acid and Compritol. Even after freeze drying the particle size remained in the nanometre range (769.5 ± 101.97 nm), whereas that of stearic acid and Compritol was in the micrometre range (1333.66 ± 42.52 and 6220.33 ± 1617.92 nm respectively). Glyceryl monostearates are highly lipophilic compounds (HLB=3-5) which are practically non-emulsifying in the absence of an auxiliary surfactant. Cithrol GMS is a more hydrophilic derivative of GMS which is classified as a self-emulsifying agent (Abdalla and Mäder 2007). This self-emulsifying property when combined with the
emulsifying effect of Tween-40 results in higher EE% and smaller particle size. It was observed repeatedly that stearic acid and Cithrol GMS SLNs had a very low yield (less than 2%), formed a thick solidified layer on the top of the tube after ultracentrifugation with large agglomerates in the final product. Therefore, they were omitted from further studies.

On the other hand, Compritol 888 ATO is a non-toxic, inert, chemically very stable (peroxide value lower than 6 meq O$_2$kg$^{-1}$) and highly compatible pharmaceutical ingredient which is suitable for the development of controlled release systems (Swart, Hundt et al. 1994). Compritol 888 ATO is a mixture of glycerol tribehenate, glycerol dibehenate and glycerol monobehenate and it possesses amphiphilic properties due to the presence of partial acylglycerols (Souto, Mehnert et al. 2006).

Table 2-6 shows the effect of concentration and type of surfactant on the quality of SLNs. Surface coverage by surfactants can have a major effect on the formation and stability of SLNs. Any concentration below 1% w/v is known to result in gelling of SLNs, hence the minimum concentration used in these formulations was 2% w/v (Helgason, Awad et al. 2009). An optimum concentration of surfactant is required to cover the newly formed surfaces during homogenisation. It has been reported that addition of the surfactant after homogenisation will not significantly affect the particle size but results in the formation of more stable SLNs (Helgason, Awad et al. 2009). Our observations, however, showed that smaller sized particles with lower PDI were formed by adding higher concentrations of the surfactant after homogenisation, which was in agreement with previous studies (Westesen and Siekmann 1997; Radomska-Soukharev 2007; Helgason, Awad et al. 2009). On the other hand, incorporation of increasing concentration (2-5% w/v) of surfactant resulted in an increase in EE%. It has been reported that the prerequisite to obtain a sufficiently high loading capacity is good solubility of the drug in the lipid melt. Surfactants have been widely used as wetting and solubilising agents. They reduce the solid/liquid interfacial tension and
permit the liquid to wet the solid more effectively. Therefore, the observed increase in EE% could be explained by the increase of wetting of ACV and its solubility in the molten lipid by the addition of the surfactant.

Table 2-6 Effect of surfactant concentration and type on the quality of SLNs.

<table>
<thead>
<tr>
<th>Type of surfactant</th>
<th>Concentration (% w/v)</th>
<th>EE%</th>
<th>$Z_{\text{average}}$ (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 40</td>
<td>2</td>
<td>11.14 ± 0.67</td>
<td>465.86 ± 7.15</td>
<td>-35.13 ± 0.32</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Tween 40</td>
<td>5</td>
<td>15.50 ± 1.98</td>
<td>241.74 ± 35.55</td>
<td>-30.13 ± 0.37</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>Tween 40</td>
<td>7.5</td>
<td>10.00 ± 0.13</td>
<td>201.85 ± 16.36</td>
<td>-28.1 ± 0.43</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2</td>
<td>13.32 ± 1.39</td>
<td>362.76 ± 20.00</td>
<td>-31.93 ± 0.41</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>Brij 78</td>
<td>2</td>
<td>5.07 ± 3.92</td>
<td>701.30 ± 12.17</td>
<td>-28.36 ± 0.37</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>Poloxamer 188</td>
<td>2</td>
<td>0.85 ± 0.29</td>
<td>679.63 ± 90.82</td>
<td>-26.50 ± 1.41</td>
<td>0.50 ± 0.09</td>
</tr>
</tbody>
</table>

EE%, entrapment efficiency; DL%, drug loading; $Z_{\text{average}}$, average particle size; PDI, polydispersity index.
Results are presented as mean ± SD (n=3).

The EE% of the formulation containing 2% w/v Tween-80 did not increase significantly (P>0.05) in comparison to the formulation containing 2% w/v Tween 40 (13.32 ± 1.4 vs 11.14 ± 0.67% respectively) and formulations containing Brij 78 and Poloxamer 188 showed even lower EE%. Tween 80 and Tween 40 are both from the polysorbate families which are similar in their properties. Although possessing a high zeta potential value (>35 mV) is essential in developing a highly dispersed suspension of nanoparticles, steric hindrance property of polysorbates further helps maintaining SLNs/NLCs stability by keeping the particles apart (Liu, Hu et al. 2007; Fang, Fang et al. 2008). To the contrary, Brij-78 and Poloxamer-188 are both non-ionic surfactants; their chemical structure and their effect on hydrophilie-lipophile balance (HLB) could be the reason for reduced EE% values and increased average particle size (Lv, Yu et al. 2009).

2.3.3. Characterisation of the developed NLCs

NLCs are structural modifications of SLNs developed to address problems such as drug expulsion during storage, low drug loading and poor stability (Müller, Mäder et al. 2000;
Chapter 2 Formulation and characterisation of ACV containing SLNs and NLCs

Seyfoddin, Shaw et al. 2010). Addition of liquid lipids, namely lauroglycol-90 and capryol-90, to the SLNs resulted in the formation of NLCs. The effects of the type, concentration and combination of liquid lipids on the quality of NLCs were investigated (Table 2-7).

Table 2-7 The effect of the liquid lipid concentration and type on the quality of NLCs.

<table>
<thead>
<tr>
<th>Liquid lipid</th>
<th>Concentration (%) w/v</th>
<th>EE%</th>
<th>Z_{average} (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>11.14 ± 0.67</td>
<td>465.86 ± 7.15</td>
<td>-35.13 ± 0.32</td>
<td>0.530 ± 0.05</td>
</tr>
<tr>
<td>Capryol-90</td>
<td>30</td>
<td>25.51 ± 1.93</td>
<td>777.56 ± 5.05</td>
<td>-27.85 ± 0.07</td>
<td>0.265 ± 0.02</td>
</tr>
<tr>
<td>Capryol-90</td>
<td>50</td>
<td>37.85 ± 0.84</td>
<td>472.36 ± 10.63</td>
<td>-30.10 ± 0.56</td>
<td>0.700 ± 0.06</td>
</tr>
<tr>
<td>Lauroglycol-90</td>
<td>30</td>
<td>56.88 ± 0.66</td>
<td>656.2 ± 5.21</td>
<td>-25.70 ± 0.10</td>
<td>0.752 ± 0.10</td>
</tr>
<tr>
<td>Lauroglycol-90</td>
<td>50</td>
<td>91.64 ± 5.85</td>
<td>319.76 ± 9.55</td>
<td>-28.13 ± 0.64</td>
<td>0.509 ± 0.04</td>
</tr>
<tr>
<td>Capryol-90:Lauroglycol-90</td>
<td>25:25</td>
<td>33.38 ± 1.51</td>
<td>638.06 ± 12.52</td>
<td>-27.4 ± 0.26</td>
<td>0.42 ± 0.02</td>
</tr>
</tbody>
</table>

It was noticed that NLCs can be formulated even after addition of a large quantity of the liquid lipid (up to 50% of the weight of the solid lipid) without gelling. The resultant nanoparticles were separated more efficiently and the preparation yield was increased. Table 2-7 shows that the addition of capryol-90 and lauroglucol-90 increased EE% from 11.14 to 25.51 and 56.88% respectively. However, when the concentration of liquid lipid was increased to 50% of the weight of the solid lipid, the EE% was increased further. In comparison to SLNs, NLCs are known to have less lipophilic entities and the drug could be more soluble in the liquid lipid phase which can result in increased EE% and drug loading (Fang, Fang et al. 2008; Shen, Sun et al. 2010).

Another possible explanation for the increase in the entrapment efficiency is the lipid forms a highly crystalline state with a perfect lattice which could lead to drug expulsion. Any imperfections (lattice defects) in the lipid structure could offer space to accommodate more drugs. Thus, incorporation of a liquid into solid lipid could have led to crystal order
disturbances and thus imperfections in the crystal lattice which may have created space to accommodate ACV molecules and increased the drug loading (Helgason, Awad et al. 2009). It has been reported that NLCs generally have smaller particle sizes in comparison to similarly constituted SLNs (Fang, Fang et al. 2008). However, our results showed that addition of liquid lipids increased the particle size which may be due to the entrapment of air in the nanoparticles resulting in the formation of a porous structure (Figure 2-3).

Nevertheless, an even further increase in the concentration of the liquid lipid resulted in a decrease in particle size of NLCs after preparation. This might be due to a decrease in viscosity which would ultimately enhance the process of emulsification. However, it is suggested that the effect of viscosity can be neglected for glyceryl behenate, medium chain triglycerides and liquid oils as they have similar viscosities at temperatures above 70° C (Jores, Mehnert et al. 2004). Therefore, the size reduction could be due to surface modification properties of lauroglycol-90 and capryol-90 which are also considered as pharmaceutical co-surfactants and penetration enhancers (Gwak and Chun 2002; Borhade, Nair et al. 2008). The zeta potential values have decreased slightly in the presence of the liquid lipids which indicate that formulations still have good physical stability.

2.3.4. Scanning electron microscopy (SEM) of the developed SLNs and NLCs

SLNs and NLCs were morphologically examined by SEM (Figure 2-4). From the images it can be seen that both SLNs and NLCs were spherical in shape. Interestingly, they retained their shape after freeze drying and upon storage. This may potentially indicate the stability of the SLNs and their crystalline lattice upon lyophilisation. However, a more detailed stability investigation would be required to confirm this. NLCs particularly differ in morphology from SLNs as they are more porous and composed of many layers compared to smooth SLNs. This property might be due to air entrapment in the system as discussed earlier and may enhance
drug release from nano-confinements at the site of action. Also, the presence of this multi-layered structure may be a contributing factor to the high EE% of NLCs as it provides more space for the drug to be incorporated. The nanoparticles retained their shape after freeze drying but could not be dispersed uniformly after lyophilisation (Figure 2-4C).

Figure 2-4 SEM images of (A) SLNs, (B) NLCs and (C) SLNs after freeze-drying.
2.3.5. Effect of lyophilisation on the quality of SLNs and NLCs

Lyophilisation is a common method used to prepare pharmaceutical dry powders. Although the process of freeze-drying helps in establishing formulation stability, in some cases it might affect the quality of the final product. The particles size of all the formulations was significantly increased after freeze drying (P<0.05) (Figure 2-5). This might be due to aggregation of particles during the freeze drying process (Schwarz and Mehnert 1997; Mehnert and Mäder 2001). The SEM images confirmed the presence of nanoparticles after lyophilisation (Figure 2-4C). However, the particles were aggregated leading to an increased mean diameter size after freeze-drying.

The net surface charge of SLNs which was determined by measuring the zeta potential had reduced significantly in all formulation after freeze drying (P<0.05). Statistical analysis of the two populations (zeta potential before and after freeze drying) revealed that the difference was significant (P<0.05) (Figure 2-6). This also supports the formation of bigger SLNs after lyophilisation. A reduction in zeta potential values reflects a reduction in repellent forces which keep the particles apart avoiding agglomeration and aggregation.
The particle size of NLCs remained in the nanometre size range even after freeze drying, owing to their good surface properties. The zeta potential values also remained in a close range after freeze drying, with no significant difference (P>0.05) (Figure 2-5).

![Figure 2-6 Zeta potential of selected formulations before and after freeze-drying (n=3, mean ± SD).](image)

2.3.6. Differential scanning calorimetry (DSC) studies

DSC was used as a tool to investigate the melting and recrystallisation behaviour of the materials which have crystalline structures. In general, SLNs are known to be crystalline in nature with bigger particle size, whereas NLCs are expected to be amorphous in with smaller particle size (Müller, Mäder et al. 2000; Fang, Fang et al. 2008; Seyfoddin, Shaw et al. 2010).

Figure 2-7 shows the thermograms of Compritol, ACV, SLNs and NLCs with different types and concentrations of liquid lipid added. The thermal parameters of ACV and Compritol are presented in Table 2-8 and Table 2-9.

The core lipid Compritol and ACV exhibited a sharp endothermic peak representing their melting point at 72.09 °C and 250.75 °C respectively. Table 2-8 shows that the thermograms
of SLNs and NLCs had a lower enthalpy in comparison to that of the core lipid Compritol. An increase in the liquid lipid content in NLCs resulted in a decrease in melting point of the core lipid. Lower melting enthalpy values can be due to the formation of less orderly formed crystalline lattices and defects in the crystalline lattice caused by incorporation of liquid lipids (Fang, Fang et al. 2008; Shen, Sun et al. 2010). This disturbance in crystalline lattice formation can prevent drug expulsion during storage (Seyfoddin, Shaw et al. 2010). Another reason for a decrease in melting enthalpy is the small particle size of the formulations and increase in the surface area which makes the heat flow through the material slower compared to larger crystals (Fang, Fang et al. 2008). DCS thermograms of SLNs and NLCs showed slight broadening of the Compritol endothermic peak. This might be due to excipients undergoing a heating and cooling cycle, the smaller particle size resulting in higher surface area, the presence of impurities in liquid lipids and the surfactant used (Patlolla, Chougule et al. 2010).

![DSC thermograms of ACV, Compritol 888 ATO, S (3), N (1-5) formulations.](image)

The melting peak of ACV did not disappear but had reduced enthalpy values and was significantly shorter in SLNs and NLCs (P<0.05). This indicates physical homogeneity,
partial drug entrapment and a degree of interaction between ACV and the lipid matrix (Fang, Fang et al. 2008). It can be suggested that during SLNs formation, crystallisation of ACV was inhibited (Liu, Pan et al. 2005; Lv, Yu et al. 2009). A substance has higher energy in the amorphous state with better solubility, dissolution and bioavailability (Corrigan, Healy et al. 2003; Morissette, Almarsson et al. 2004; Lv, Yu et al. 2009).

Table 2-8 Thermal parameters of Compritol in different preparations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Melting point (°C)</th>
<th>Onset (°C)</th>
<th>Enthalpy (J g⁻¹)</th>
<th>Crystallinity index (CI%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compritol</td>
<td>72.09</td>
<td>68.74</td>
<td>145.00</td>
<td>100</td>
</tr>
<tr>
<td>S(3)</td>
<td>71.31</td>
<td>66.83</td>
<td>138.0</td>
<td>95.17</td>
</tr>
<tr>
<td>N(1)</td>
<td>71.35</td>
<td>67.90</td>
<td>127.0</td>
<td>87.58</td>
</tr>
<tr>
<td>N(2)</td>
<td>71.06</td>
<td>67.40</td>
<td>125.1</td>
<td>86.27</td>
</tr>
<tr>
<td>N(3)</td>
<td>70.13</td>
<td>66.22</td>
<td>120.0</td>
<td>82.75</td>
</tr>
<tr>
<td>N(4)</td>
<td>69.18</td>
<td>62.02</td>
<td>142.5</td>
<td>98.27</td>
</tr>
<tr>
<td>N(5)</td>
<td>69.13</td>
<td>63.46</td>
<td>116.3</td>
<td>80.20</td>
</tr>
</tbody>
</table>

Table 2-9 Thermal parameters of ACV in different preparations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Melting point (°C)</th>
<th>Onset (°C)</th>
<th>Enthalpy (J g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV</td>
<td>250.75</td>
<td>248.88</td>
<td>133.9</td>
</tr>
<tr>
<td>S(3)</td>
<td>251.82</td>
<td>249.89</td>
<td>4.001</td>
</tr>
<tr>
<td>N(1)</td>
<td>249.19</td>
<td>245.70</td>
<td>4.195</td>
</tr>
<tr>
<td>N(2)</td>
<td>250.27</td>
<td>247.99</td>
<td>10.10</td>
</tr>
<tr>
<td>N(3)</td>
<td>250.40</td>
<td>248.54</td>
<td>19.14</td>
</tr>
<tr>
<td>N(4)</td>
<td>252.89</td>
<td>250.77</td>
<td>5.548</td>
</tr>
<tr>
<td>N(5)</td>
<td>251.43</td>
<td>249.47</td>
<td>10.94</td>
</tr>
</tbody>
</table>

2.3.7. In vitro drug release studies

The in vitro drug release profiles of SLNs and NLCs are shown in Figure 2-8. ACV in PBS-pH 7.4 showed that the entire drug permeated into in to the receptor compartment after four hrs. A relatively fast release rate was seen in all formulations indicating that drug release is mainly a surface phenomenon. Drugs which are more lipophilic tend to have more efficient
entrapment and are embedded in the lipid core. It has been reported previously that ACV is adsorbed on to the surface of SLNs and that the release process is a result of drug nanocrystal dissolution from the nanoparticle surface (Lukowski and Werner 1998). Our findings, however, show that the release of drug from SLNs was slower than that of the control ACV suspension and confirms entrapment of a portion of the drug into the lipid core. On the other hand, the release of drug from NLCs was faster than that from SLNs. This might be due to the porous nature of NLCs. Liquid lipid incorporation into the structure of nanocarriers resulted in porous and multilayered particles which enhanced the drug release. Also, diffusion through the liquid lipid was faster than through solid lipid and both diffusion and erosion are suggested to be involved in the release mechanisms (Shen, Sun et al. 2010). Another contributing factor is the enhanced wettability of NLCs due to the presence of the liquid oils and the porosity of the nanoparticles. The SLNs surface is smooth and rigid resulting in a slower diffusion and release. The images obtained from SEM confirm these observations (Figure 2-4).

![Cumulative Drug Release](image)

**Figure 2-8 In vitro drug release of ACV from various formulations (n=3, mean ± SD).**

### 2.3.8. Drug permeation through the cornea

The permeation profile of ACV through bovine eye cornea from selected SLNs and NLCs formulations can be seen in Figure 2-9. A moderate permeation rate across the cornea was
noticed for ACV in solution. A rather long lag time was observed for all the samples which might reflect transport across a very thick bovine cornea. The central corneal thickness of a bovine eye is about 1015 ± 104 μm which is almost twice as thick as that of the human eye (Doughty, Petrou et al. 1995). The rate of the permeation of all formulations across the cornea was much faster three hrs post administration which could be due to partial loss of functionality in an experimental in vitro environment. The permeation rates of NLCs formulations (N4 and N5) were higher than free drug in solution. However, the SLNs formulation had a permeation rate of about 11-fold lower than that of the ACV suspension which is evident from the apparent permeability coefficient values (Table 2-10).

![Figure 2-9 Corneal penetration profiles of ACV through bovine cornea from various formulations (n=3, mean ± SD).](image)

**Table 2-10** Release parameters, apparent permeability coefficient (Papp) and hydration levels (HL) obtained from the ex vivo corneal permeation experiment (n=3, mean ± SD).

<table>
<thead>
<tr>
<th>Samples</th>
<th>P&lt;sub&gt;app&lt;/sub&gt;</th>
<th>HL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV</td>
<td>8.78 ± 0.01</td>
<td>80.08 ± 0.68</td>
</tr>
<tr>
<td>S(3)</td>
<td>0.77 ± 0.93</td>
<td>79.62 ± 1.19</td>
</tr>
<tr>
<td>N(4)</td>
<td>14.57 ± 1.76</td>
<td>76.95 ± 5.00</td>
</tr>
<tr>
<td>N (5)</td>
<td>13.46 ± 4.96</td>
<td>79.83 ± 2.89</td>
</tr>
</tbody>
</table>
Chapter 2 Formulation and characterisation of ACV containing SLNs and NLCs

To explain the reasons for these findings, bovine corneas were examined by SEM after conducting the permeation studies. It was observed that SLNs are capable of forming a film on the surface of the cornea from which a slow release of the drug may occur (Law, Huang et al. 2000; Shen, Sun et al. 2010). Interestingly, in the SEM image of the endothelial surface of the cornea treated with NLCs, nanoparticles that had penetrated through the corneal barrier were identifiable (Figure 2-10). The presence of liquid lipids which act as penetration enhancers may also attribute to the enhanced corneal penetration of NLCs. It is suggested that the high permeation rate observed from NLCs was the result of the cumulative amount of drug released from the NLCs into the receptor compartment after they had crossed the cornea together with the amount of drug permeated through the membrane after it released from the nanoparticles in the donor compartment. Our results showed that SLNs possessed a higher zeta potential than that of NLCs. Since cornea is a biological membrane and carries negative charges due to presence of negatively charged residues of proteins the surface charges of NLCs could be another factor that may have contributed in improving the drug’s permeability. It is evident that SLNs and NLCs are capable of enhancing drugs corneal penetration through a combination of the following mechanisms: (1) due to their lipophilicity, they increase the residence time on the surface of the cornea preventing them from being washed away by tear fluid, (2) they can form a film on the corneal epithelial surface through which the drug is released slowly and (3) due to their nano-size range, they are able to penetrate through the corneal cells and release the drug in the posterior chamber of the eye. NLCs have reportedly increased corneal residence time which will result in higher ocular bioavailability, whereas drug in solution will be washed away too quickly before reaching the $C_{\text{Max}}$ (Law, Huang et al. 2000; Shen J and W 2010). Therefore, enhanced corneal penetration and controlled release was obtained for NLCs and SLNs respectively. Enhanced corneal
penetration is required to allow sufficient drug to be delivered beyond the cornea and a sustained release profile is essential to supply the drug for a prolonged period of time.

Figure 2-10 SEM images of the cornea during and after treatment with NLCs and SLNs formulations.

These principles are similar to those of transdermal drug delivery (Fang, Fang et al. 2008) but one has to remember that drug removal by blinking and tear fluid is a crucial limiting factor in ocular drug delivery which is not accounted for ex vivo ocular drug release models (Seyfoddin, Shaw et al. 2010). An ocular drug delivery system which increases the corneal residence time may have a slower ex vivo corneal penetration profile; however, by resisting ocular removal it will ultimately have a better in vivo release profile (Law, Huang et al. 2000; Abdelkader, Ismail et al. 2011).

The percentage corneal hydration level (HL%) is a parameter which can be used to evaluate damage to the cornea. A normal cornea has a HL value of between 76-80% (Saettone, Chetoni et al. 1996; Monti, Chetoni et al. 2002; Liu, Zhang et al. 2011). Any value between
83-92% indicates epithelial or endothelial damage (Saettone, Chetoni et al. 1996; Monti, Chetoni et al. 2002; Liu, Zhang et al. 2011). As shown in Table 2-10 all the three formulations had HL values between 76-80% and did not cause damage to the cornea.

2.4. Conclusion

The hot o/w microemulsion technique is suitable to produce SLNs and ACV can be successfully incorporated into SLNs. SLNs can be modified to NLCs showing higher EE. The high entrapment efficiency (91.64 ± 5.85), nano-scale size range (319.76 ± 9.55) and good drug corneal permeation profile obtained from NLCs indicate that these carriers could be used as potential ocular drug delivery systems for ACV. Therefore, formulation N(4) was selected for further investigations in the upcoming chapters.
Chapter 3. Modulation of Acyclovir Release from Nanostructured Lipid Carriers (NLCs) through Drug-Carrier Complexation

Chapter Three

“It is a bad plan that admits of no modification.”
— Publilius Syrus
3.1. Introduction

Nanostructured Lipid Carriers (NLCs) are structural modifications of previously developed Solid Lipid Nanoparticles (SLNs) with higher drug loading, faster drug release and better stability profile (Müller, Shegokar et al. 2011; Seyfoddin and Al-Kassas 2013). In the previous chapter, it was demonstrated that acyclovir (ACV) can be successfully loaded into SLNs/NLCs (Seyfoddin and Al-Kassas 2012). However, due to the physiochemical properties of ACV, the drug release from the lipid carriers was found to be simply a surface phenomenon (Lukowski and Werner 1998; Seyfoddin and Al-Kassas 2012). Polar and non-polar drugs which are either hydrophilic or lipophilic tend to have more efficient entrapment and are embedded in the lipid core of SLNs whereas, compounds of medium polarity such as ACV are mainly adsorbed onto the surface and therefore, the release process is a result of drug nanocrystal dissolution from the nanoparticle surface (Lukowski and Werner 1998; Müller, Mäder et al. 2000; Seyfoddin and Al-Kassas 2012). In Chapter 2 the in vitro release of drug through an artificial membrane from SLNs was slower than that of ACV suspension which confirms the entrapment of a portion of the drug into the lipid core (Seyfoddin and Al-Kassas 2012). The incorporation of a liquid lipid in the process of developing NLCs resulted in a significant increase in drug loading compared to SLNs. However, the drug release rate was still fast owing to the porous nature of NLCs as well as their increased wettability due to added surfactant.

Another major problem with SLNs and NLCs is the unpredictability of drug expulsion upon storage (Pietkiewicz, Sznitowska et al. 2006). Lipophilic drugs have the highest tendency to escape, primarily because they are not successfully incorporated in the core of the nanoparticles but adsorbed onto the surface (Pietkiewicz, Sznitowska et al. 2006). For hydrophilic drugs, once entrapped in the lipid core, expulsion should theoretically not occur.
or at least be slower in practice. Drug expulsion can also be due to ongoing crystallisation or transformation of the solid lipid from a high energy level after emulsification to a lower energy level during storage. This phenomenon can be prevented by addition of cyclodextrins in order to produce amorphous nanoparticles (Wissing, Kayser et al. 2004).

Therefore, in order to modulate the drug release profile, the aim of this chapter was to complex ACV with several polymeric and non-polymeric carriers. Masking the physiochemical properties of active pharmaceutical ingredients (APIs) is a challenging task in the pharmaceutical industry (Masuda, Yoshihashi et al. 2012). The hypothesis is that a molecule of ACV complexed to a carrier will have different physicochemical properties (eg. increased solubility) and might behave differently in the hot microemulsion environment, leading to modification of the drug release mechanism from the developed NLCs/SLNs. Cavalli et al. (1999) reported that it is possible to modulate the drug release kinetics by incorporating a drug-carrier complex into SLNs. Two poorly soluble steroids (hydrocortisone and progesterone) were chosen as model drugs and their inclusion complexes with β-cyclodextrin (βCD) and 2-hydroxypropyl β-cyclodextrin (2HPβCD) were prepared by a co-precipitation method (Cavalli, Peira et al. 1999). The change in drug release from SLNs containing the drug-βCD complex could be due to a difference in the solubility of the complex in the lipid matrix (Cavalli, Peira et al. 1999). Cirri et al. (2012) also reported the incorporation of a polymeric cyclodextrin complex of a poorly soluble compound, ketoprofen, into NLCs. Their aim was to form a βCD inclusion complex of ketoprofen to increase its bioavailability by enhancing drug solubility and to combine this with the skin penetration potential of NLCs (Cirri, Bragagni et al. 2012).

**Use of cyclodextrins in drug delivery**

Cyclodextrins (CDs) were first discovered over 100 years ago (Villiers 1891). They are chemically α-1, 4-glucans composed of six to more than one hundred glucose units (Qi,
Mokhtar et al. 2007; Laza-Knoerr, Gref et al. 2010). Their structure resembles a cylindrical cavity with a hydrophilic exterior and a hydrophobic interior capable of accommodating poorly soluble lipophilic drugs and therefore improving their water solubility (Figure 3-1). Compounds of medium polarity such as ACV can also enter the CD cavity depending on their size (Ghera, Perret et al. 2009). In addition, the complexation provides physical and chemical protection to labile entities thus improving their stability (Ghera, Perret et al. 2009; Perret, Duffour et al. 2013). The drug-CD inclusion complex is often in the form of a stable crystalline entity. CDs are also used to facilitate the drug entrapment in different types of nanoparticles (Cavalli, Donalisio et al. 2009). Finally, they are also used to mask the unpleasant taste of a drug, reduce toxicity, avoid drug-excipient interaction and most importantly enhance the drug’s bioavailability (Laza-Knoerr, Gref et al. 2010).

Figure 3-1 Structure of β-CD (Adopted from: Qi, Mokhtar et al. 2007).

**In vivo safety of cyclodextrins**

The *in vivo* safety of CDs has been extensively reviewed (Tetsumi and Kaneto 1997; Thompson 1997; Loftsson and Järvinen 1999). There are concerns about the use of CDs for parenteral administration, however their topical administration is more feasible. The systemic and gastrointestinal absorption of CDs after topical ophthalmic administration is minimal and insufficient to cause internal toxicity. If a patient is given two drops of an isotonic solution of CDs thrice a day in the form of an eye drop which contains 20-25% w/v CDs, the total daily
dose would be about 150 mg which, under normal circumstances, would be 1/10th of its normal allowable daily usage (Loftsson and Järvinen 1999). Ocular irritation can occur if CDs penetrate through the surrounding ocular tissue (Loftsson and Järvinen 1999). However, the chances of this happening are limited due to the large molecular size of CDs which render them impermeable to such barriers. Nevertheless, some methylated and very lipophilic derivatives of CD have been found to be ocular irritants (Jansen, Xhonneux et al. 1990), most likely through the extraction of lipophilic components from the cornea or other surrounding tissues (Jansen, Xhonneux et al. 1990; Ohvo and Slotte 1996; Irie and Uekama 1997; Thompson 1997). Thus, eye lid crusting has been observed after large doses of CD eye drops have been administered (Loftsson and Järvinen 1999).

The general aim of this chapter was to identify a suitable carrier and investigate the effect of the drug-carrier complexation on aqueous solubility, dissolution and entrapment efficiency of the drug in order to control the drug release from NLCs (N(4)) developed in the Chapter 2.

3.1. Materials and methods

3.1.1. Materials

ACV powder (Jai Radhe Sales, India), Compritol® 888 ATO, stearic acid (Scharlau chemie, EU), Tween® 40, (Sigma, Germany), Capryol® 90 and Lauroglycol® 90 (Gattefosse, France) were used in the preparation of SLNs and NLCs as described in the previous chapter. β-Cyclodextrin, hydroxypropylmethylcellulose (HPMC), ethylcellulose (EC), sodium alginate and polyethyleneglycol (PEG 6000) were purchased from Sigma-Aldrich, USA. Polyvinyl pyrrolidone (PVP-K30) was kindly gifted by BASF, Germany. Phosphate buffered saline (PBS) and all other chemicals used were of analytical grade.
3.1.2. Determination of the aqueous solubility of ACV

The aqueous solubility of ACV was determined by adding an excess amount of the drug to 5 mL of water in screw-capped glass vials which were placed in a shaking water bath at 150 rpm and 37 ± 5 °C for 24 hrs (Shao, Park et al. 1994). The solution was then centrifuged, filtered through a 0.22 μm Millipore syringe filter and the concentration of ACV was determined by HPLC as reported in the previous chapter (Section 2.2). A pH-solubility curve for ACV was constructed by varying the pH from 1.15-11. For this purpose, phosphoric acid/sodium dihydrogen phosphate dehydrate buffer was used for pH 1.15-3, acetic acid/sodium acetate buffer for pH 4-5, sodium dihydrogen phosphate/disodium hydrogen phosphate buffer for pH 6-8, and PBS for pH 9-11. All samples were treated as above for 24 hrs and analysed for drug content using HPLC.

3.1.3. Preparation of drug-carrier complexes

3.1.3.1. Preparation of physical mixtures

The physical mixtures of specific molar ratios of ACV and each individual carrier were prepared by mixing the powders with a spatula and grinding the mixture in a mortar.

3.1.3.2. Melt grinding method

This method was used for polymers with low melting points (PEG 6000 and HPMC). The polymer was melted at 5 °C above its melting temperature. An appropriate amount of drug based on specific molar ratios was added to the molten carrier under magnetic stirring at 600 rpm for 20 mins or until a homogenous mixture was formed. The mixture was then solidified at room temperature, pulverised, sieved through mesh #60 to remove any coagulates and stored in a silicone desiccator overnight to remove any residual moisture. The homogeneity
of the drug-carrier molten mixture was assayed by taking a weighed quantity of samples from three different batches and quantifying the drug content.

3.1.3.3. Solvent evaporation method

The solvent evaporation method was used for PVP-K30, HPMC, EC and sodium alginate. These polymers in particular have a high melting point which made preparation by the melt grinding method difficult with laboratory grade equipment. Briefly, drug and polymer were dissolved in a minimal volume of the solvent allowing equilibration under magnetic stirring (6000 rpm) for 20 mins. The solvent was then removed by stirring the mixture at 40 °C for one hr. Solidified samples were stored overnight in a desiccator to remove extra moisture, ground and sieved through mesh #60. All samples were stored in a desiccator until further use.

Methanol was used as the solvent in the preparation of PVP and EC complexes. The mixtures were stirred for 20 mins, dried by stirring at 40 °C for one hr and treated further similar to previous samples. Sodium alginate has a high melting point (300 °C) and is insoluble in ethanol or other organic solvents. Therefore, it is impossible to prepare a drug-sodium alginate complex with either method. Complexation with sodium alginate was therefore attempted by dispersing the drug in an excess volume of ethanol. The resultant dispersion was added to sodium alginate and the mixture was triturated until dry.

3.1.3.4. Preparation of ACV-βCD inclusion complex by the lyophilisation method

ACV-βCD freeze-dried complexes were prepared by dispersing specific molar ratios of the two substances (1:1, 1:2 and 1:6) in MilliQ water followed by sonication for 15 mins in an ultrasound bath and shaking at 37 ± 5 °C for 24 hrs in a shaking water bath at 150 rpm. The
resultant mixture was freeze dried for 24 hrs until a dry powder was obtained (Rossel, Sepúlveda Carreño et al. 2000).

3.1.4. Preparation of NLCs containing ACV-βCD inclusion complexes

Nanoparticles were prepared by hot o/w microemulsion reported previously in Chapter two (Section 2.2.3). The ACV-βCD complex was first dispersed in an appropriate amount of liquid lipid and was then mixed with Compritol. The lipid mix (2:1 solid: liquid lipid) was melted at 10 °C above its melting point and the aqueous phase containing the surfactant was heated separately 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 stirring was continued for 7 mins or until the formation of nanoparticles. 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 MilliQ water and were deep-frozen overnight. Subsequently, nanoparticles were lyophilised (Labconco-7806020, USA) at -20 °C at a pressure below 0.133 mbar for 24 hrs to obtain a dry powder. Nanoparticles were characterised as previously described (sections 2.2.3 and 2.2.6 of Chapter 2).

3.1.5. Solid characterization and analysis

3.1.5.1. Hot stage microscopy (HSM)

A polarizing microscope (Leica Light transmission Microscope, Germany) equipped with a hot stage (FP82HT, Mettler Toledo FP900 processor, USA) was used to assess the melting miscibility and thermal transitions of drug-carrier samples. A weighed amount of pulverised
sample was placed on an appropriate glass container, covered with a specific glass lid and heated from 30 to 300 °C at 10 °C min⁻¹.

3.1.5.2. Scanning electron microscopy (SEM)

SEM was used to examine the morphology of the drug, βCD and their complexes. Samples were coated with gold in a (Polaron SC 7640 sputter coater (Quorum Technologies, UK) and analysed by a Philips XL30S FEG with a SiLi (Lithium drifted) Super Ultra Thin Window EDS detector.

3.1.5.3. Differential scanning calorimetry (DSC)

A DSC (Q1000 V9.9 Build 303, Universal V4.5A TA Instruments, USA) was used to evaluate the physical state of the complexes. A mass of 5 mg of samples was placed in an aluminium pan which was hermetically sealed, while an empty pan served as a reference. The thermal behaviour of samples was determined in the range of 10 to 280 °C at a heating rate of 10 °C min⁻¹.

3.1.5.4. Fourier transform infrared spectroscopy (FTIR)

FTIR (Tensor 37 FTIR, Bruker, USA) was used for the infrared analysis of samples. About 1-2 mg of the samples was weighed and examined at transmission mode over the wavenumber range of 4000 to 400 cm⁻¹. FTIR spectrum of pure ACV, βCD and inclusion complexes were obtained.

3.1.5.5. X-ray powder diffraction (XRD)

XRD studies were performed using a D2 Phaser Desktop X-ray Diffractometer, Bruker, USA. The samples were placed in the sample holder and scanned in a range of diffraction angles (2θ) between 7 to 40°. The radiation was generated by a Cu Kα filter with a
wavelength of 1.54178 Å. Diffractograms were obtained at 40 kV voltages and 40 mA current with a scanning speed of 0.02 ° min⁻¹.

3.1.6. Dissolution of ACV from the complexes

A dissolution testing system (Hanson SR8-Plus, USA) was used in order to compare drug dissolution profiles of different drug-carrier complexes. An appropriate amount of powdered samples containing 12.5 mg of ACV were placed in the flasks. The dissolution medium (PBS pH 7.4, 50 mL) was paddle stirred at 50 rpm at 37 ± 5 °C. At appropriate time intervals (10, 20, 30, 40, 50, 60, 70 mins), 1 mL samples were withdrawn, filtered and analysed by HPLC. An equal volume of dissolution medium was replaced to maintain sink conditions.

3.1.7. Drug release from the nanoparticles

Drug release from nanoparticles was determined in Franz cells using the methodology described in Section 2.2.8.

3.2. Results and discussions

3.2.1. Solubility profile of ACV

ACV is a compound of medium polarity with poor lipophilic and hydrophilic properties. The saturated aqueous solubility of ACV was found to be 1.16 ± 0.064 mg mL⁻¹ which closely resembles the data given in the literature (1.3 mg mL⁻¹) (Schaeffer, Beauchamp et al. 1978; Miranda, Krasny et al. 1982). The pH solubility curve for ACV was constructed to identify the region of higher solubility (Figure 3-2). It is apparent from Figure 3-2 that, ACV has lowest solubility in the physiological pH range (7.3-7.4). ACV is a compound of medium polarity with 2 pKa values of 2.27 and 9.2 corresponding to the hydroxyl and amine groups
Chapter 3 Modulation of ACV release from NLCs through drug-carrier complexation

(Figure 1-2) making it both a weak acid and a weak base (Laskin, De Miranda et al. 1982; Huang, Du et al. 2011).

![Figure 3-2 pH solubility curve of ACV in different buffers at 25 °C.](image)

3.2.2. Characterisation of solid dispersions and complexes

3.2.2.1. Solubility enhancement using PEG, PVP and βCD

A 1:1 molar mixture of ACV: PEG and ACV: PVP resulted in an increase in the aqueous solubility of drug from $1.16 \pm 0.064$ to $3.03 \pm 0.002$ and $3.00 \pm 0.14$ mg mL$^{-1}$ respectively. The ACV: βCD (1:1) freeze-dried complex showed a moderate increase in aqueous solubility (from $1.16 \pm 0.064$ to $2.10 \pm 0.008$ mg mL$^{-1}$) while the physical mixture only showed a slight increase (to $1.23 \pm 0.21$ mg mL$^{-1}$) indicating there might be weaker intermolecular interactions. Therefore, it is worthwhile to attempt to modify the physiochemical properties of ACV through drug-carrier complexation with βCD.
3.2.2.2. Hot stage microscopy (HSM)

HSM is a useful technique for screening polymers as candidates for producing drug-carrier complexes often known as solid dispersions/solutions. The technique allows for the observation of thermal transitions over a temperature range, detect crystalline status of the drug in dispersions and access drug-carrier miscibility in the molten state (Lloyd, Craig et al. 1997; Vitez, Newman et al. 1998; Law, Krill et al. 2001; Moneghini, Kikic et al. 2001; Martínez-Ohárriz, Rodríguez-Espinosa et al. 2002; Bikiaris, Papageorgiou et al. 2005). It often serves as the first technique in predicting the type of solid dispersion and in determining whether an amorphous state is achieved. Table 3-1 shows the thermographic events of the physical mixture and complexes of PEG 6000 and ACV. The drug appears to be in the crystalline state as the polymer starts to melt at 60 °C. Subsequently, the drug crystals are dissolved completely in the molten polymer at 70 °C. This phenomenon was not observed in the case of the physical mixture and the drug crystals only started to melt at 225 °C (near the actual melting point of ACV). The dissolution of the drug in the molten polymer does not necessarily indicate formation of an amorphous complex but indicates possible intermolecular interactions even if the drug is still in the crystalline state. Another explanation would be that ACV tends to get solubilised in the molten PEG 6000.

Table 3-1 Hot stage microscopy of PEG 6000 physical mixtures and complexes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (mg)</th>
<th>Temperature (°C)</th>
<th>HSM photograph</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical Mixture PEG 6000: ACV (1:3)</td>
<td>2.3</td>
<td>30</td>
<td></td>
<td>Polymer starts to melt</td>
</tr>
<tr>
<td>Physical Mixture PEG 6000: ACV (1:3)</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid dispersion</td>
<td>Polymer behaviour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 6000: ACV (1:1)</td>
<td>65</td>
<td>Polymer completely melted, drug crystals visible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>Drug particles melted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.55</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 6000: ACV (1:2)</td>
<td>60</td>
<td>Polymer starts to melt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Drug starts to melt in the polymer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>Drug particles completely melted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.64</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG 6000: ACV (1:3)</td>
<td>63</td>
<td>Polymer starts to melt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>Some drug particles are still visible probably due to higher amount of drug being present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>All drug particles have melted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.47</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>Description</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>PEG 6000 melting onset = 63 °C, More crystals of drug visible than previous formulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>It appears that some quantity of the drug has started to dissolve in the polymer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>170</td>
<td>Drug dissolving slowly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>215</td>
<td>At 200 °C drug started to dissolve faster. At 215 °C all drug was dissolved in the polymer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Melting point onset = 62.2 °C, All polymer melted at 65 °C, More drug crystals than previous formulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>At 90 °C drug is slowly dissolving in the melted PEG 6000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>220</td>
<td>At 207 °C some ACV is still in crystalline form. At 218 °C drug melts fast and at 220 °C drug is melted or dissolved</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3-2 shows the thermographic events of PVPK30 and βCD complexes. Similar events matching the PEG 6000 solubilising phenomena were observed for PVPK30 although in a less prominent manner. The HSM examination of βCD physical mixtures and complexes on the other hand, revealed interesting outcomes. Results in Table 3-2 suggest that ACV is in an amorphous state when complexed to βCD in 1:1 molar ratio. After βCD had melted, no drug crystals were visible. This indicates the formation of an inclusion complex. For all other polymers/carriers screened, the drug was in a crystalline state in the molten polymer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (mg)</th>
<th>Temperature (°C)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical mixture PVPK30: ACV (1:3)</td>
<td>1.81</td>
<td>160</td>
<td>Polymer melted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>255</td>
<td>Drug crystals melted</td>
</tr>
<tr>
<td>Solid dispersion PVPK30: ACV (1:1)</td>
<td>4.10</td>
<td>160</td>
<td>Polymer melted</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>Drug crystals melted</td>
</tr>
<tr>
<td>Physical mixture βCD: ACV (1:1)</td>
<td>1.2</td>
<td>63</td>
<td>βCD and drug crystals visible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>βCD melted, some drug crystals visible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>220</td>
<td>All drug melted</td>
</tr>
<tr>
<td>Solid dispersion βCD: ACV (1:1)</td>
<td>1.1</td>
<td>60</td>
<td>Mesh network appearance with no drug crystals recognisable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>155</td>
<td>βCD melted, no drug crystals visible</td>
</tr>
</tbody>
</table>

3.2.2.3. Drug dissolution from the complexes

The purpose of this experiment was to study the release behaviour of the drug from various complexes. Several dissolution apparatus have been utilised to test ocular delivery systems such as a custom made 250 mL Ehrlenmeyer flask (Qiu, Guillory et al. 1993), flow though cell method (Lee and Yalkowsky 1999; Jachowicz and Czech 2008), a controlled release analytical system (CRAS) with reservoir volume as low as 8-30 μL to mimic physiological
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conditions (Stevens, Missel et al. 1992), a USP dissolution apparatus n.1 with a 500mL vessel (Gavini, Chetoni et al. 2004) and a USP dissolution apparatus n.1 with a 50 mL vessel (Badawi, El-Laithy et al. 2008). In this study the USP apparatus n.1 was used to determine the dissolution profiles of different complex systems. It should be noted that the aim was not only to improve drug dissolution to enhance ocular bioavailability but also to improve the drug loading and release profile.

Figure 3-3 displays the drug dissolution profiles of several drug-carrier complexes. The FTIR and HSM data have shown that ACV is in the crystalline form in PEG 6000, PVPK30 and sodium alginate complexes. For all the systems, drug dissolution was slower than for ACV pure powder. An increasing trend in dissolution was observed when the drug to polymer ratio was increased. It is suspected that complex formation reduced drug wettability and therefore dissolution. Table 3-3 shows the cumulative release percentage of ACV from βCD inclusion complexes at different time points, with the enhancement of drug dissolution in the case of ACV: βCD 1:1 inclusion complex clearly visible. This could be attributed to the decreased drug crystallinity, increased wettability, reduced particle size, higher energetic amorphous state and the formation of inclusion complexes (Sachan, Pushkar et al. 2010).

The 1:2 complex had a higher dissolution rate than pure ACV; however the 1:6 complex had a slower dissolution rate. Considering the molecular weights of ACV and βCD it appears that a 1:1 molar ratio is sufficient to accommodate all the ACV molecules and any increase in the βCD ratio will result in a slower drug dissolution rate. Therefore, the 1:1 ACV: βCD inclusion complex was used for further investigation and incorporation into NLCs.
Figure 3-3 Drug dissolution profiles of selected drug-carrier complexes.
Table 3-3 Dissolution profile of ACV: βCD (1:1, 1:2 and 1:3) inclusion complexes at pH 7.4.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Cumulative drug release (%) of complexes at a ratio of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:0</td>
</tr>
<tr>
<td>10</td>
<td>48.72 ± 11.60</td>
</tr>
<tr>
<td>20</td>
<td>56.97 ± 11.83</td>
</tr>
<tr>
<td>30</td>
<td>65.90 ± 16.44</td>
</tr>
<tr>
<td>40</td>
<td>71.17 ± 11.61</td>
</tr>
<tr>
<td>50</td>
<td>75.06 ± 8.73</td>
</tr>
<tr>
<td>60</td>
<td>79.37 ± 7.34</td>
</tr>
<tr>
<td>70</td>
<td>79.55 ± 9.78</td>
</tr>
</tbody>
</table>

3.2.2.4. Scanning Electron Microscopy (SEM) of βCD inclusion complexes

SEM was used to examine the surface morphology of ACV-βCD samples. Figure 3-4 displays the SEM micrographs of ACV and ACV: βCD 1:1 inclusion complexes with ACV appearing as needle shaped crystals. The inclusion complex illustrated a mesh-like network. Interestingly, ACV crystals were not identifiable in the inclusion complex micrographs since they were molecularly dispersed in βCD and formed an inclusion complex. Also, the mesh-like structure appears porous which could potentially enhance drug dissolution.
3.2.2.5. Differential Scanning Calorimetry (DSC) of ACV: βCD inclusion complexes

DSC can be used as an analytical technique to detect complex formation and changes in the polymorphic status of crystalline substances (Law, Krill et al. 2001; Moneghini, Kikic et al. 2001; Sachan, Pushkar et al. 2010; Lembo, Swaminathan et al. 2012). Figure 3-5 displays DSC thermograms obtained for pure ACV, βCD, ACV: βCD 1:1 physical mixture and the inclusion complex. For ACV, an endothermic peak was observed at 250.7 °C. For βCD an endothermic peak was observed at 130.16 °C. The physical mixture showed both endothermic events for ACV and βCD suggesting that ACV remained in the crystalline state. However, the endothermic peak for ACV shifted and had reduced intensity in the inclusion complex suggesting that the drug penetrated into the βCD cavity by replacing water molecules and was therefore unable to crystallise (Rossel, Sepúlveda Carreño et al. 2000; Sachan, Pushkar et al. 2010; Lembo, Swaminathan et al. 2012).
3.2.2.6. Fourier transform infrared spectroscopy (FTIR) studies of ACV: βCD inclusion complexes

The FTIR spectra of powders of crude ACV, βCD and ACV: βCD complexes were obtained (Figure 3-6). Important regions of interest for ACV pure powder include: CH- stretching observed at 3514.73 cm\(^{-1}\), NH- of the primary amine at 3436.67 cm\(^{-1}\), NH-, OH- at 3176.22 cm\(^{-1}\), CH- aliphatic stretching at 1629 cm\(^{-1}\) and -C=O at 1483 cm\(^{-1}\).

For βCD the bands between 1500-400 cm\(^{-1}\) are important in discussing the complex formation of cyclodextrins with transmission metal complexes by H-bridge formation (Egyed 1990). In this region none of the modes arise from a single type of molecular vibration where in most cases C-O-H bending mode contributes to a negligible degree (Egyed 1990).
- There are three characteristic bands for the primary OH group which might be essential in distinguishing between different types of cyclodextrin OH groups (Egyed 1990).

- Bands at 1334 and 1251 cm\(^{-1}\) are identified as the complex modes of CH\(_2\)OH groups (Egyed 1990).

- An aromatic band consisting of C-1-H deformation coupled to other motions is seen at 853 cm\(^{-1}\) (Egyed 1990). It is suggested that this aromatic band can be used as the marker of CH\(_2\)OH position (Egyed 1990).

- The band at 1151 cm\(^{-1}\) is usually seen for most saccharide structures and refers to the pyranose ring vibrational mode with some C-OH bending components (Cael, Koenig et al. 1974; Egyed 1990).

- The band at 2926 cm\(^{-1}\) may present primary OH groups bonded to the ring oxygen internally or to each other inter-molecularly. The OH stretching region of βCD is very complicated because the primary and secondary OH groups maybe bonded inter- or intra-molecularly and H-bridges may also be formed between the molecules of water complexed in the cavity, or between the crystallised water molecules belonging to βCD (Egyed 1990).
ACV and βCD had distinct diffractograms, with the one of the complex differing significantly from those of the pure compounds. In addition to the ACV peaks, the complex showed appearance of new peaks which was an indication of complex formation (Rossel, Sepúlveda Carreño et al. 2000). In the finger print region of ACV: βCD (1:1) solid complex (1000-400 cm$^{-1}$) there was a new peak at 822 cm$^{-1}$ which probably indicates a new aromatic
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C-H bending (Figure 3-6f). This indicates an interaction between the aromatic ring of the drug and the hydrophobic cavity of βCD (Ahmed, Naggi et al. 1991). Moreover, the C-H stretching of ACV is missing at 3514.73 cm\(^{-1}\) which is yet another indication for drug entrapment in the hydrophobic cavity of βCD. A closer look at the FTIR spectra of samples revealed that there are numerous new peak formations which indicate interactions between the drug and βCD (Rossel, Sepúlveda Carreño et al. 2000). The changes in the 2950-3050 cm\(^{-1}\) region is suggested to be due to the alteration in valence bands of the C–H in plane vibrations of the aromatic ring (Wen, Tan et al. 2004). It has been reported that differences found in the 1480–1600 cm\(^{-1}\) regions are attributed to the skeleton vibrations of the C=C bonds in the aromatic ring (Wen, Tan et al. 2004) which is in accordance with the results obtained here. The intensity and shape of many peaks have changed indicating that the vibration of ACV was restricted possibly due to the formation of inclusion complexes (Wen, Tan et al. 2004). Comparison between the FTIR spectrum of ACV and its 1:1 physical complex with βCD revealed that the C-H aromatic stretching at 3514.73 cm\(^{-1}\) is missing in the complex. This further indicates involvement of the primary amine group of ACV with the lipophilic portion of βCD and formation of an inclusion complex.

3.2.2.7. X-ray powder diffraction of βCD inclusion complexes

The mode of interaction between ACV and βCD was also studied by XRD. A highly crystalline compound shows sharp peaks with high intensity versus half width at half-maximum ratio (1/hwhm) (Lembo, Swaminathan et al. 2012). On the other hand, higher peaks are indicative of poorly crystalline materials (Lembo, Swaminathan et al. 2012).

Figure 3-7 shows the XRD diffractograms of various samples. The one obtained for pure ACV shows the crystalline state of the drug. The diffractogram for the ACV-βCD physical mixture revealed that the drug was still in the crystalline form with the drug peaks easily
identified. Therefore, physical mixing of ACV and βCD powders did not lead to the formation of inclusion complexes.

![X-ray diffractograms of ACV, β-cyclodextrin (βCD), ACV-β-cyclodextrin inclusion complexes (ACV:βCD (1:1), ACV:βCD (1:2) and ACV:βCD (1:6)), NLCs containing ACV:βCD inclusion complex (NLCβCD).](image)

The examination of the diffractograms obtained for ACV:βCD complexes showed that the crystallinity of ACV has decreased after being confined in the cavity of the βCD. All the three different ratios of ACV:βCD lyophilised complexes resemble an amorphous powder and it appears that there is no obvious advantage of using higher ratios of βCD. All the major peaks for the crystalline ACV are absent. Therefore, the drug is either in an amorphous form or possible hydrogen binding interaction has occurred between the drug and the carrier (Lembo, Swaminathan et al. 2012). The XRD diffractogram was also obtained for NLCs containing the ACV:βCD inclusion complex. The lipid structure of NLCs has an irregular
lattice configuration due to the presence of liquid lipids in the structure. The diffractogram for NLCs shows that peaks are of much lower intensity and the particles are in an amorphous state.

3.2.3. Nanoparticle characterisation and drug release

In order to modulate the drug release from the developed NLCs, the ACV:βCD (1:1) inclusion complex was encapsulated and compared with the pure drug. NLCs containing the ACV-βCD inclusion complex had a lower entrapment efficiency of 82.66 ± 3.64% compared to that previously achieved using the free drug (91.64 ± 5.85%). The reduction in entrapment is because the molar fraction of drug within the formulation is reduced when in the inclusion complex. The mean particles size of the ACV-βCD complex containing NLCs was increased from 319.76 ± 9.55 to 364.23 ± 17.65 nm and from 412.13 ± 52.7 to 813.06 ± 57.21 nm for freeze dried samples when compared to NLCs containing the pure form of the drug. The zeta potential was reduced from -28.13 ± 0.64 to -24.2 ± 0.17 in average.

According to our previous findings (Section 2.3.7), entrapment of ACV in SLNs is primarily a surface phenomenon which results in an initial burst release of the drug when in contact with the release medium (Lukowski and Werner 1998; Seyfoddin and Al-Kassas 2013). Figure 3-8 shows that the in vitro drug release profile of NLCs containing either the ACV-βCD complex or the pure drug as well as the ACV solution and the ACV-βCD freeze dried complex in PBS (pH 7.4). In both control solutions, the drug was released very fast with about 70% of the drug released in the first 5 hrs. It was expected that the ACV: βCD inclusion complex would show a slightly faster release due to its higher aqueous solubility compared to free ACV. However, the ACV suspension in PBS had a faster release profile. This could be due to the fact that ACV is soluble in slightly basic medium. Therefore, the
effect of βCD complexation would have been prominent if the release medium was unbuffered.

On the other hand, a sustained release profile was obtained for the NLCs containing the ACV-βCD complex compared to the fast release of pure ACV from NLCs. There was no burst effect and only 13% of the drug was released during the first hr with only 35% being released after 5 hrs. This was a great improvement as in the case of pure ACV containing formulations, 20% of the drug was released in the first hr followed by 57% after 5 hr (Seyfoddin and Al-Kassas 2013). This indicates that complexation with βCD has resulted in a homogenous distribution of the drug within the nanoparticles and that the release is not just a surface phenomenon. Other release mechanisms such as erosion and bulk diffusion have thus contributed in achieving a sustained release profile. This could be due to the modified solubility of the ACV-βCD inclusion complex in the lipid matrix (Cavalli, Peira et al. 1999).

Formulation of lipid nanoparticles containing βCD inclusion complexes of other drugs like progesterone and hydrocortisone have also resulted in a slower release profile than the nanoparticles containing pure drugs (Cavalli, Peira et al. 1999). Therefore, it is evident that the release profile can be modulated by using βCD inclusion complexes. This enables us to benefit from the combined advantages of lipid nanoparticles and cyclodextrin in ocular drug delivery.
Chapter 3 Modulation of ACV release from NLCs through drug-carrier complexation

Figure 3-8 In vitro drug release profiles of ACV suspension in PBS pH 7.4 (ACV), ACV:βCD (1:1) complex in PBS pH 7.4 (ACV:βCD), NLCs encapsulated ACV:βCD (1:1) complex (NLCL90βCD) and NLCs containing the pure drug (NLCs). (n=3, mean ± SD).

3.3. Conclusion

In conclusion, several drug-carrier complexes were screened with ACV-βCD inclusion complexes showing the most promising results. The formation of ACV-βCD inclusion complexes was confirmed with HSM, SEM, DSC, FTIR and XRD studies which resulted in an increased aqueous solubility of ACV from 1.16 ± 0.064 to 2.10 ± 0.008 mg mL\(^{-1}\).

The ACV-βCD inclusion complexes were then successfully incorporated (EE%, 82.66 ± 3.64%) into NLCs with formulation code N (4) developed in Chapter 2. The resultant \textit{in vitro} drug release profile from these NLCs (NLC- βCD) was slower than that of formulations S (3) and N (4) developed in the previous chapter. The complexation of ACV with βCD also resulted in an increased \textit{ex vivo} corneal permeability of ACV.

Chapter Four

To give anything less than your best, is to sacrifice the gift.

— Steve Prefontaine
4.1. Introduction

Chapters 2 and 3 have demonstrated that Solid Lipid Nanoparticles (SLNs) and their latest structural modified derivatives, Nanostructured Lipid Carriers (NLCs), are capable of providing sustained drug release profiles (Seyfoddin, Shaw et al. 2010; Seyfoddin, Young et al. 2010; Seyfoddin and Al-Kassas 2012). The resultant particles had satisfactory pharmaceutical characteristics but, they were negatively charged (Table 2-7) and had a rather fast drug release profile (Figure 2-8). Because 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 drug corneal penetration. Also, in theory, coating of nanoparticles with a natural polymer should delay the drug release profile. Moreover, caging of the nanoparticles within a polymer improves system stability. For this purpose formulation N(4) which showed the best results in Chapter 2 and 3 was chosen to be coated with chitosan (refer to Table 2-1 for sample composition).

Chitosan has been used in drug delivery for several purposes (Figure 4-1). It was first used as an excipient in tablet manufacturing and as a vehicle in parenteral formulations but soon found applications in the design of sustained release dosage forms for oral, ophthalmic, nasal, transdermal and implantable devices (Felt, Buri et al. 1998; Ilium 1998).

![Chemical structure of chitosan](Taken from (Felt, Buri et al. 1998)).
Chitosan is a deacetylated form of chitin which is a polycationic biopolymer and is the most abundant polysaccharide in nature beside cellulose. It is a structural component of the exoskeleton of crustacea and insects and also occurs in some fungi (Roberts 1992; Felt, Buri et al. 1998; Illium 1998; Kurita 1998; Alonso and Sánchez 2003). The main pharmaceutical source of chitin is from shells of shrimp, lobster and crab which is converted to chitosan by alkaline deacetylation (Felt, Buri et al. 1998). Chitosan is an important biopolymer in the pharmaceutical field as it is nontoxic (Knapczyk, Krówczyński et al. 1984), biocompatible (Hirano, Seino et al. 1990; Struszczyk, Wawro et al. 1991), biodegradable (Struszczyk, Wawro et al. 1991), cationic and abundant (Felt, Buri et al. 1998).

The main purpose of novel ocular drug delivery system is to increase precorneal residence time and/or improve corneal penetration of drugs. Previous efforts include the development of viscosified solutions using biodegradable polymers (Felt, Buri et al. 1998; Kaur, Kanwar et al. 2002). However, several studies have shown that viscosity enhancement alone cannot enhance corneal penetration and each polymer has a characteristic effect on the cornea independent of its viscosity (Saettone, Giannaccini et al. 1984; Zaki, Fitzgerald et al. 1986; Felt, Buri et al. 1998; Kaur, Kanwar et al. 2002). Cationic polymers have superior bioadhesive properties since their electrostatic interaction leads to the development of molecular interaction forces with negative charges of the mucus and the corneal epithelium (Park and Robinson 1984; Park and Robinson 1985; Lehr, Bouwstra et al. 1992; Calvo, Remunan-Lopez et al. 1997; Calvo, Vila-Jato et al. 1997; Felt, Buri et al. 1998). Viscous ophthalmic chitosan solutions are easily manufactured and possess excellent ophthalmic tolerance when administered topically (Felt, Furrer et al. 1999). Gamma scintigraphy has shown that even small concentrations of chitosan can increase the corneal contact time significantly when compared to a simple drug solution (Felt, Furrer et al. 1999). Interestingly, the molecular weight of chitosan has not shown to be of significant importance.
Chapter 4 Chitosan coated NLCs for ocular drug delivery of ACV

in precorneal retention, enabling the use of low molecular weight chitosans (Felt, Furrer et al. 1999). Further, the results of precorneal drainage have been reported to be very similar irrespective of the chitosan concentration (0.5 to 1.5% w/v). Therefore, it has been suggested that, the enhanced corneal retention time using chitosan could be a saturable bioadhesive mechanism independent of molecular weight and concentration (Felt, Furrer et al. 1999; Alonso and Sánchez 2003).

Another benefit of using chitosan is its biodegradability as it is susceptible to enzymatic breakdown by the lysozyme secreted from the apical portion of the lachrymal acinar cells (Felt, Buri et al. 1998). Chitosan is then degraded to N-acetylg glucosamine which is a monomeric unit present in the core of certain human glycoproteins such as heparin and hyaluronic acid (Muzzarelli 1994; Felt, Buri et al. 1998; Alonso and Sánchez 2003). Ocular tolerability of chitosan has been investigated and results of different studies have shown no corneal irritation or epithelial damage (Calvo, Vila-Jato et al. 1997; Felt, Furrer et al. 1999). In addition, chitosan has proven to be a versatile biomaterial with wound healing and antimicrobial properties (Rabea, Badawy et al. 2003; Zheng and Zhu 2003; Denis, Dai et al. 2012; Li, Chen et al. 2012).

These favourable characteristics have made chitosan a popular choice of biomaterial in ophthalmic drug delivery (Alonso and Sánchez 2003; de Campos, Diebold et al. 2004; de Salamanca, Diebold et al. 2006; de la Fuente, Raviña et al. 2010). Chitosan has been used to prepare vesicular drug delivery devices, as a permeation enhancer and as a coating polymer in the preparation of surface modified ophthalmic drug delivery systems. Several ocular drug delivery systems such as microspheres, colloids and nanoparticles have been developed to deliver a wide range of drugs and bioactives (Genta, Conti et al. 1997; De Campos, Sánchez et al. 2001; Alonso and Alonso 2004; de la Fuente, Seijo et al. 2008; Motwani, Chopra et al. 2008; de la Fuente, Raviña et al. 2010). The second generation of colloidal drug carriers
constitutes of particles coated with bioadhesive polymers, which could improve the transport of drugs associated with nanoparticles (Alonso and Alonso 2004). Despite the versatility of nanoparticulate drug delivery systems coated with chitosan, only those with an optimal release are suitable for ophthalmic use since the carrier contact with the absorption site (corneal epithelium) is short and limited (Alonso and Sánchez 2003). 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 (Calvo, Vila-Jato et al. 1997).

The general aim of this chapter was to coat the negatively charged NLCs (N (4)) developed in Chapter 2 with low molecular weight chitosan (LCH) for ocular delivery of acyclovir (ACV). The main objectives of coating NLCs with chitosan were hereby to: (1) prolong the precorneal residence time, (2) increase corneal permeability, (3) reduce system leakage and (4) increase the overall formulation stability.

4.2. Materials and methods

4.2.1. Materials

ACV powder (Jai Radhe Sales, India), Lauroglycol® 90, Compritol® 888 ATO 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) purchased from Sigma-Aldrich, NZ and a fluorescence microscope (Axioplan2, Zeiss, Germany) were used in the measurement of precorneal residence time of the formulations. Phosphate buffer solution (PBS) and all other chemical used were of analytical grade.

4.2.2. HPLC method of detection

As described in Section 2.2.2.
4.2.3. Preparation of NLCs

NLCs N(4) were prepared as described in Section 2.2.3. 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 the NLCs was 0.01 mg mL⁻¹.

4.2.4. Preparation of chitosan coated NLCs

Initially, a 1% w/v stock solution of chitosan was prepared in a 0.2 M acetate buffer pH 5. The solution was stirred over night for complete dissolution and diluted to obtain the following concentrations: 0.1, 0.25, 0.5 and 1% w/v. Both freeze-dried and fresh NLCs were coated by dispersing them in different concentrations of chitosan solutions at a 1:5 (w/v) ratio and stirring at 600 rpm for 20 mins at ambient temperature.

4.2.5. 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). Entrapment efficiency (EE%) was determined by measuring the amount of drug entrapped in nanoparticles by HPLC as described in Section 2.2.7.

4.2.6. Ex vivo model for measurement of precorneal residence time of formulations

A system based on the ex vivo method for evaluation of precorneal residence of topical ophthalmic formulations was used with some modifications (Liu and Wang 2009). It consisted of a perfusion chamber, two precision pumps to pump in/out the perfusion solution at 4 µl mL⁻¹ and a fluorescence microscope (Figure 4-2). 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
Chapter 4 Chitosan coated NLCs for ocular drug delivery of ACV

The surface of the cornea followed by 5 µL of fluorescein containing NLCs. Subsequently, a 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 the rate of 4 µl mL⁻¹. Fluorescent images of the cornea were taken at 30 sec intervals for 60 mins and the fluorescein intensity was measured with ImageJ software. Measurements were obtained in triplicate.

4.2.7. *In vitro* drug release study

The *in vitro* drug release studies were carried as described in Section 2.2.8.
4.2.8. *Ex vivo* corneal permeation

The methodology reported in Section 2.2.9 was followed to measure corneal permeation of chitosan coated NLCs.

4.2.9. Stability studies of NLCs

The stability of ACV loaded chitosan coated and uncoated NLCs was assessed at three different environmental conditions: (1) 4 ± 5 °C, (2) 25 ± 5 °C, 60% RH and (3) 40 ± 5 °C, 75% RH. A volume equal to 5 mL of both freshly prepared and lyophilised NLCs was used. At different time intervals the samples were collected and analysed for particle size and zeta potential after appropriate dilution using the method described earlier. A differential scanning calorimeter (DSC Q1000 V9.9 Build 303, Universal V4.5A TA Instruments, USA) was used to evaluate the physical state of particles at the end of the stability study period. A mass of 5 mg of the lyophilised sample was placed in an aluminium pan which was hermetically sealed while an empty pan was used as the reference. The thermal behaviour of the sample was determined in the range of 10-280 °C at a heating rate of 10 °C min⁻¹. The crystallinity index of each sample was calculated using the below formula by assuming the melting enthalpy of Compritol 888 ATO as 100% (Shen J and W 2010):

\[
Crystallinity\ Index\ (CI\%) = \left( \frac{\text{Enthalpy}_{\text{NLCs}}}{\text{Enthalpy}_{\text{Bulk lipids}} + \text{Concentration}_{\text{Lipid phase}}} \right) \times 100
\]

The percentage of ACV remaining in the formulations at 4°C, 25°C, and 40°C over 90 days was determined by analysing the quantity of the drug in the supernatant by HPLC after centrifuging the sample vials at 25,000 rpm for 20 mins followed by filtering through a 0.22 μm Millipore filter unit. The organoleptic properties of the formulation such as physical appearance and odour were noted alongside suspension characteristics including sedimentation and redispersability.
4.3. Results and discussion

4.3.1. Effects of chitosan coating on NLCs

Chitosan is expected to cover the surface of the NLCs in a saturable process with the negatively charged lipid Compritol providing a 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 for a longer shelf life (Freitas and Müller 1998; Freitas and Müller 1999).

The separation and purification of coated NLCs from the chitosan solution is not required for ophthalmic use (Luo, Zhao et al. 2011). The mucoadhesive natural polymer, chitosan, by itself is used in ophthalmic solutions with high viscosity to enhance corneal residence time (Felt, Buri et al. 1998; Ilium 1998; Felt, Furrer et al. 1999; Alonso and Sánchez 2003). Therefore, retaining the coated particles in the chitosan solution would eventually be of benefit to the delivery system by utilising the potential of the viscosified solution and the bioadhesive coated particles simultaneously.

Table 4-1 shows the effect of increasing chitosan concentration on the particle size and zeta potential of freeze dried NLCs. Coating of the freeze dried NLCs with chitosan resulted in a significant increase in the particle size. 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 value (3.86 ± 0.73) leading to particle-particle attractions and aggregation. Generally, a value close to ±30 mV is considered as a suitable zeta potential threshold value for optimum stability of a nanosuspension where particle-particle repulsion forces inhibit aggregation of the nanoparticles (Freitas and Müller 1998). Increasing the concentration of chitosan resulted in an increase in the zeta potential but a decrease in particle size, although the NLCs coated with 0.25 and 0.5% w/v chitosan
solutions remained in the micro size range. Coating of freeze-dried NLCs resulted in the formation of microparticles similar to what was reported by Dharmala, Yoo et al. (2008). Therefore, to obtain nanoparticles coated with chitosan freshly prepared nano-suspensions were used.

Table 4-1 Physiochemical properties of freeze-dried NLCs coated with different LCH concentrations (n =3, mean ± SD).

<table>
<thead>
<tr>
<th>Concentration of chitosan (w/v)</th>
<th>Zeta potential (mV)</th>
<th>Particle size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-32.16 ± 3.39</td>
<td>801.33 ± 84.05</td>
</tr>
<tr>
<td>0.1</td>
<td>3.86 ± 0.73</td>
<td>4690.00 ± 86.86</td>
</tr>
<tr>
<td>0.25</td>
<td>30.26 ± 1.40</td>
<td>4439.00 ± 252.93</td>
</tr>
<tr>
<td>0.5</td>
<td>45.96 ± 1.70</td>
<td>3084.00 ± 725.53</td>
</tr>
</tbody>
</table>

Table 4-2 shows the physiochemical properties of freshly prepared coated and uncoated NLCs. The original NLCs were 323.33 ± 14.6 nm in diameter. The diameter of NLCs increased as a result of surface coating with chitosan. At a relatively low concentration of chitosan (0.1% w/v), the particle size increased significantly (P<0.05) because, the induced positive charges were not sufficient to stabilize the NLCs suspension (Li, Zhuang et al. 2009). In other words, due to the low surface charge, the particle-particle attraction forces were strong and this resulted in aggregation of the nanoparticles.
Table 4-2 Physicochemical properties of fresh NLCs coated with different chitosan concentrations (n =3, mean ± SD).

<table>
<thead>
<tr>
<th>Concentration of chitosan (% w/v)</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>323.33 ± 14.60</td>
<td>-25.50 ± 1.65</td>
<td>90.54 ± 6.74</td>
</tr>
<tr>
<td>0.1</td>
<td>529.90 ± 86.86</td>
<td>05.03 ± 1.01</td>
<td>85.63 ± 5.14</td>
</tr>
<tr>
<td>0.25</td>
<td>444.73 ± 21.16</td>
<td>17.56 ± 0.85</td>
<td>86.32 ± 4.51</td>
</tr>
<tr>
<td>0.5</td>
<td>457.30 ± 44.38</td>
<td>28.10 ± 0.72</td>
<td>84.76 ± 1.23</td>
</tr>
<tr>
<td>1</td>
<td>467.46 ± 56.25</td>
<td>28.20 ± 0.57</td>
<td>87.94 ± 1.93</td>
</tr>
</tbody>
</table>

However, as the concentration of chitosan increased, the positive surface charges also increased resulting in particle-particle repulsion forces. 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. Statistical analysis showed that the change in particle size was significant for all samples (P<0.05).

Table 4-2 also shows the ACV entrapment efficiency of the coated formulation. When compared to the uncoated particles, the decrease in the EE% was insignificant after coating. 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% (Li, Zhuang et al. 2009).

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

To date, most experimental designs for the measurement of corneal residence time are based on in vivo animal studies (Cavalli, Gasco et al. 2002; Li, Nie et al. 2008). However, Liu and
Wang (2009) developed an *ex vivo* corneal perfusion method to evaluate the corneal residence time of polymeric formulations. Freshly excised rat corneas were used as the biomembrane in this study. The perfusion medium was set to flow over the cornea which resembles the physiological tear turnover. Mucins such as MUC1 and MUC4 play an important role in the formation of a gelled mucous layer of the tear film and are responsible for maintaining the surface tension of the tear film (Liu and Wang 2009). In this study, mucin substrate was added to the surface of the rat corneas, just before the application of the nanoparticle suspensions, to resemble the ocular physiological environment. As human mucin is not commercially available, porcine gastric mucin was used as a substrate which has been shown to be a good substitute for human mucin (Nagyova and Tiffany 1999; Hägerström, Paulsson et al. 2000; Argüeso and Gipson 2001; Liu and Wang 2009).

Figure 4-4 shows the fluorescent images obtained at different time points when various nanoparticles loaded with fluorescein were applied onto the surface of the rat corneas illustrating a typical fluorescein intensity decay profile for each formulation. Images were taken from the centre of the rat excised cornea placed in the perfusion chamber. The change in fluorescent intensity over time indicates the precorneal residence of the formulations. It is expected that the fluorescent intensity will decrease over time (Liu and Wang 2009).

A 0.01 mg mL\(^{-1}\) solution of fluorescein sodium which was used as the control was completely flushed away from the cornea after only 15 mins of application with the image being completely dark at 30 min. The NLCs, however, retained the dye on the surface of the cornea for an extended period of time. As the concentration of chitosan was increased from 0.1 to 0.5% w/v the corneal residence time also increased significantly (P<0.05). The NLCs formulation coated with 0.5% w/v chitosan solution retained the fluorescent dye even after 45 mins of exposure as shown in Figure 4-4.
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<table>
<thead>
<tr>
<th>Sample</th>
<th>0 min</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>NLCs (0% w/v chitosan)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>NLCs (0.1% w/v chitosan)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>NLCs (0.25% w/v chitosan)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>NLCs (0.5% w/v chitosan)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 4-3 Precorneal resident time of the fluorescein loaded formulations over time.

Figure 4-5 shows a numerical graph of fluorescein decay as a function of time. To quantitatively analyse the data obtained from the corneal perfusion study, the fluorescent 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 have hereby contributed to the increased surface adhesion of the nanoparticles.
4.3.3. In vitro drug release studies

The in vitro drug release profiles of different formulations are shown in Figure 4-5. ACV suspension in PBS pH 7.4 passed through the artificial membrane, showing that drug perfusion through the artificial barrier is not the limiting factor. The freeze-dried NLCs had the fastest drug release profile amongst all other formulations. This could be due to the dehydration process where the nanoparticle shrinkage could have expelled the encapsulated drug molecules towards the surface resulting in the faster drug release rate (Mehnert and Mäder 2001). 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 (Alonso and Sánchez 2003). Although water
insoluble, the chitosan coating layer absorbs water and swells which provides hydrated channels for the release of the drug (Badawi, El-Laithy et al. 2008). Figure 4-5 shows that, increasing the concentration of chitosan did not influence the release parameters significantly (P<0.05).

![Figure 4-5 In vitro release profiles of ACV suspension, freshly prepared NLCs, lyophilised NLCs and NLCs coated with 0.1, 0.25 and 0.5 % w/v low molecular weight chitosan (LCH) (n=3, mean ± SD).]

**4.3.4. Ex vivo corneal penetration studies**

Figure 4-7 displays the *ex vivo* ACV corneal penetration profiles and Table 4-3 lists the corresponding permeability coefficients (P_{app}) of the different formulations. The ACV control suspension had the slowest penetration rate but inclusion of ACV into NLCs improved its corneal penetration (Seyfoddin and Al-Kassas 2012). There was a slight improvement in P_{app} of ACV from 8.78 ± 0.01 to 9.12 ± 1.16 which was not significant (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 cornea (P>0.05). However, NLCs coated with 0.5% w/v chitosan had the maximum penetration through the cornea ($P_{app}=14.74 \pm 5.029$) 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).

![Figure 4-6 Drug corneal penetration profile of ACV suspension, uncoated NLCs, and NLCs coated with 0.1, 0.25 and 0.5% w/v LCH (n=3, mean ± SD).](image)

Increase in corneal permeability of the chitosan coated NLCs could be due to the sustained release profiles reported previously (Figure 4-5). In addition to its bioadhesive nature, chitosan is also a cell membrane penetration enhancer (Majumdar, Hippalgaonkar et al. 2008), which has been attributed to the modulation of the tight junction barriers between corneal epithelial cells (Artursson, Lindmark et al. 1994) and also intracellular routes (Dodane, Amin Khan et al. 1999; Alonso and Sánchez 2003). There are 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
in intracellular pathways of epithelial cells, in a reversible manner without causing cellular toxicity (Dodane, Amin Khan et al. 1999).

Table 4-3 Apparent permeability coefficients determined from the ex vivo drug release profiles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>ACV suspension</th>
<th>Uncoated NLCs</th>
<th>NLCs coated with 0.1% w/v chitosan</th>
<th>NLCs coated with 0.25% w/v chitosan</th>
<th>NLCs coated with 0.5% w/v chitosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent permeability coefficient (P_{app})</td>
<td>8.78 ± 0.01</td>
<td>9.12 ± 1.16</td>
<td>12.22 ± 1.45</td>
<td>12.64 ± 0.69</td>
<td>14.74 ± 5.02</td>
</tr>
</tbody>
</table>

4.3.5. Stability studies of NLCs

4.3.5.1. Effect of temperature on particle size and charge of the formulations

Figure 4-8 and Figure 4-9 show the effect of storage conditions on particle size and surface charge of the formulations investigated, respectively. The average particle size of both chitosan coated (NLCs-LCH 0.5% w/v) and uncoated (NLCs) systems increased significantly during the storage period, P<0.05 and P<0.001, respectively). This coincided with a significant loss of surface charge. The particle growth and loss of surface charge was much slower at 4 °C but storage of NLCs at higher temperatures (25 and 40 °C) increased the kinetic energy of the system which led to accelerated collision of the nanoparticles, faster loss of the surface charge and subsequently increased nanoparticle aggregation (Freitas and Müller 1998; Hu, Jiang et al. 2006; Li, Nie et al. 2008). Both coated and uncoated NLCs were relatively stable after 30 days of storage at 4 °C and their particle size increased by only 6.36 and 9.34% respectively. After 60 days of storage, the particle size of the chitosan coated NLCs only increased by 6.40% whereas that of the uncoated NLCs increased by 24.64%. However, at the end of the 90 day storage period, the particle size of coated and uncoated particles had increased by 59.36 and 49.16% respectively which demonstrates formulation
instability although the particle size still falls within the accepted nano range. The particle size of the lyophilised uncoated NLCs formulation increased rapidly. The effect of temperature was significant (P<0.05) and higher temperatures resulted in more intense particle aggregation. The change in the zeta potential of the formulation at 4 °C was insignificant (P>0.05), however, this did not prevent the growth in the particle size when compared to the formulation stored at higher temperatures. All freeze dried formulations were in the micro size range after 90 days with a 319.62% increase in size at 40 °C.

The physical stability of lipid nanoparticles has been investigated extensively by measuring the particle size (photon correlation spectroscopy, PCS; laser diffraction, LD), zeta potential of the particles (ZP) and by conducting thermal analysis (differential scanning calorimetry, DSC) (Wissing, Kayser et al. 2004). It has been shown that SLNs can retain their physical stability in an aqueous dispersion for up to one year (Freitas and Müller 1998; Freitas and Müller 1999). However, accelerating factors such as light and temperature can result in increased kinetic energy of the system which could increase the chances of particle-particle collisions, coagulations and therefore result in an increase in the particle size (Freitas and Müller 1998; Wissing, Kayser et al. 2004). The major storage stability problems associated with NLCs are an increase in the particle size, gelation and drug expulsion from the lipid matrix (Mehnert and Mäder 2001).

One of the most important factors to study while evaluating the physical stability of NLCs is the tendency to aggregate upon storage (Müller, Mäder et al. 2000; Mehnert and Mäder 2001; Müller, Radtke et al. 2002; Müller, Petersen et al. 2007; Agrawal, Petkar et al. 2010). Generally, a zeta potential close to ±30 mV is considered as the threshold value for optimum stability of a nanosuspension where particle-particle repulsion forces inhibit the aggregation of the nanoparticles (Freitas and Müller 1998). In theory, the loss of surface charge results in an increase in nanoparticle diameter and physical instability (Freitas and Müller 1998). The
threshold of agglomeration in a nanoparticle dispersion occurs at a zeta potential range of $-20$ mV to $-11$ mV (Riddick 1968; Freitas and Müller 1998). According to the Derjaguin and Landau, Verwey and Overbeek (DLVO) theory, a system is regarded as being stable if the electrostatic repulsion is stronger than the attractive Van der Waals forces with the particles having to overcome an electrostatic repulsion barrier to be able to attract each other and form agglomerates (Freitas and Müller 1998). If the kinetic energy of the system during storage is high, the particles can collide with each other (Freitas and Müller 1998). This kinetic energy of a nanoparticle dispersion is affected by the storage temperature and/or exposure to light which can lead to nanoparticle aggregation and in some cases gelation (Freitas and Müller 1998).
Figure 4-7 Particle size profile of A) chitosan coated NLCs (NLC-LCH), B) NLCs and C) lyophilised NLCs (NLCs-FD) over 90 days at 4, 25 and 40 °C.
Figure 4-8 The surface charge profile of A) chitosan coated NLCs (NLC-LCH), B) NLCs and C) lyophilised NLCs (NLCs-FD) over 90 days at 4, 25 and 40 °C.
4.3.5.2. Effect of temperature on drug leakage

Another important physical stability parameter for nanoparticles is the drug leakage. The percentage of ACV remaining in the different formulations at 4, 25 and 40 °C over three months was determined by analysing the quantity of the drug in the supernatant after centrifuging the sample vials at 25,000 rpm for 20 mins. The supernatant was also filtered through a 0.22 µm Millipore filter unit. Figure 4-10 shows the drug leakage from the different formulations. The percentage of drug loss was greater when both formulations were stored at higher temperatures over time. At 4 °C the percentage of drug loss from chitosan coated NLCs was insignificant (P>0.05), but was significant from the uncoated NLCs. Table 4-4 shows the percentage of ACV remaining in the nanoparticles after 90 days of storage. The amount of drug remaining in the chitosan coated NLCs was much higher than in the uncoated particles. This is because the chitosan formed a polymeric coat on the surface of NLCs which prevented the drug from escaping and in theory retarded and controlled the release of the drug. Therefore, it seems that chitosan coating can increase the physical stability and storage shelf life of the formulation (De Campos, Diebold et al. 2004).

Table 4-4 Drug leakage of chitosan coated NLCs, NLCs and lyophilised NLCs (NLCs-FD) over the stability period.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Temperature (°C)</th>
<th>% of ACV remained after 90 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLCs-LCH</td>
<td>4</td>
<td>97.21 ± 7.23</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>91.64 ± 5.16</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>75.66 ± 7.61</td>
</tr>
<tr>
<td>NLCs</td>
<td>4</td>
<td>85.12 ± 7.56</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>75.59 ± 7.96</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>65.78 ± 9.35</td>
</tr>
<tr>
<td>NLCs-FD</td>
<td>4</td>
<td>69.45 ± 6.41</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>61.45 ± 6.32</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>51.98 ± 9.16</td>
</tr>
</tbody>
</table>
Figure 4-9 Drug leakage from chitosan coated NLCs, NLCs and lyophilised NLCs over 90 days at A) 4°C, B) 25°C and C) 40°C.
4.3.5.3. Effect of temperature on organoleptic properties of the formulations and the stability of the suspensions

Table 4-5 shows the suspension characteristics and organoleptic changes of the formulations under investigation. The chitosan coated NLCs formed a flocculated suspension which was easily redispersible upon shaking. The uncoated particles formed a deflocculated system that did not crack or cake. Therefore, in terms of suspension characteristics, both systems were stable. In comparison to the uncoated particles, the chitosan coated NLCs turned slightly yellow after 90 days of storage at 40 °C which could be due to chemical degradation of chitosan.

Table 4-5 Organoleptic changes of chitosan coated NLCs (NLC-LCH), NLCs and lyophilised NLCs (NLC-FD) over the stability period at 4, 25 and 40 °C.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Temperature (°C)</th>
<th>Suspension/Powder characteristics after 90 days of storage</th>
<th>Organoleptic properties after 90 days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLCs-LCH</td>
<td>4</td>
<td>Suspension separated, flocculated clusters, easily redispersible</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>Suspension separated, flocculated clusters, easily redispersible</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Suspension separated, flocculated clusters, easily redispersible</td>
<td>Colour turned yellow, no odour</td>
</tr>
<tr>
<td>NLCs</td>
<td>4</td>
<td>Stable, no phase separation</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>Stable, no phase separation</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Stable, no phase separation</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td>NLCs-FD</td>
<td>4</td>
<td>Free flowing</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>Free flowing</td>
<td>No colour change, no odour</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>Sticky with large agglomerates</td>
<td>No colour change, no odour</td>
</tr>
</tbody>
</table>
The lyophilised NLCs formulation was investigated in terms of its powder characteristics. This formulation retained its physical appearance even after 90 days of storage at 4 and 25 °C. However, when the formulation was stored at 40 °C, it formed large agglomerates which were not easily redispersible.

**4.3.5.4. Effect of temperature on the lipid stability**

Figure 4-11 shows the DSC thermogram of the lyophilised samples of freshly prepared NLCs along with the thermograms obtained after storing the NLCs at 4, 25 and 40 °C for three months. It is evident that there is no significant change in the melting peaks of Compritol at 70 °C and ACV at 252 °C. Table 4-6 shows the thermographic events of the pure Compritol along with freshly prepared NLCs and NLCs stored at 4, 25 and 40 °C for 3 months. The melting point and recrystallization index of Compritol as the core lipid in NLCs remained more or less unchanged throughout the study at different climatic conditions. Therefore, both the lipid and drug seem to be chemically stable during the course of this study.
Chapter 4 Chitosan coated NLCs for ocular drug delivery of ACV

Figure 4-10 DSC thermograms of the lyophilised NLCs at the beginning and end of the storage period at 4, 25 and 40 °C.

Table 4-6 The thermal parameters of the core lipid Compritol, fresh NLCs and NLCs stored for 90 days at 4, 25 and 40 °C.

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>Melting point (°C)</th>
<th>Melting onset (°C)</th>
<th>Enthalpy (J g⁻¹)</th>
<th>Recrystallization index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compritol</td>
<td>72.09</td>
<td>68.74</td>
<td>145.00</td>
<td>100</td>
</tr>
<tr>
<td>Fresh NLCs</td>
<td>69.18</td>
<td>62.22</td>
<td>125.5</td>
<td>36.37</td>
</tr>
<tr>
<td>4 °C</td>
<td>66.96</td>
<td>56.52</td>
<td>141.5</td>
<td>41.01</td>
</tr>
<tr>
<td>25 °C</td>
<td>66.47</td>
<td>56.77</td>
<td>147.4</td>
<td>42.72</td>
</tr>
<tr>
<td>40 °C</td>
<td>69.49</td>
<td>63.02</td>
<td>135.8</td>
<td>39.36</td>
</tr>
</tbody>
</table>
4.4. Conclusion

Negatively charged NLCs were successfully coated with chitosan. The chitosan coating resulted in an inversion of particle charge, a slight increase in particle size, increased precorneal residence time, enhanced *ex vivo* corneal permeability, reduced system leakage and increased formulation stability. Therefore, this formulation was chosen for further biological assessments to (Chapter 5).
Chapter 5. *In Vivo* and *Ex Vivo* Efficiency of Lipid Nanoparticles

Chapter Five

Science does not know its debt to imagination.

— Ralph Waldo Emerson
5.1. Introduction

This chapter evaluates the safety and efficacy of the developed formulations. For an ocular formulation to be marketed, it should not induce any irritation in the eye. In general, the physiological response to a chemical stimulus is irritation which is associated with objective changes (such as local redness and oedema) and subjective sensations (such as pruritus and pain) (Vinardell and Mitjans 2008). To date, most of the irritation studies for ocular formulations are based on in vivo methods and the use of laboratory animals. However, concerns about ethical issues associated with the use of animals for research and the search for more appropriate, accurate and efficient protocols have resulted in the development of several alternatives. Groups such as the Cosmetics, Toiletries and Fragrances Association, the German Federal Health Office (BGA), as well as individual industrial laboratories and academics have attempted to evaluate alternative in vitro tests for practicality and acceptable in vivo correlation (Bruner, Kain et al. 1991; Sina, Galer et al. 1995). These tests have one thing in common: the end points are subjective and validation of one assay in a laboratory is not applicable in another laboratory or testing situation (Sina and Gautheron 1994; Sina, Galer et al. 1995).

In vivo studies are necessary to confirm the in vitro findings and to evaluate the efficacy and safety of the formulation. Animal experiments play an essential role in the development of ocular delivery systems, as human studies are limited to non-invasive procedures. Parameters such as blinking rate and naso-lachrymal drainage are important factors in ocular drug delivery and cannot be simulated in an in vitro set up. Pharmacokinetic and pharmacodynamic studies are a prerequisite of the drug approval process (Worakul and Robinson 1997; Derendorf and Meibohm 1999). Therefore, ocular pharmacokinetic assessments of new drug delivery systems are essential to define the influence of precorneal
factors such as drainage, lachrimation, conjunctival absorption and protein binding as well as to determine the intraocular drug distribution (Lee and Robinson 1986). A large number of animals are required to obtain statistically valid profiles therefore, “it is not unusual that about 120-150 animals are required to complete a single dose study to define the pharmacokinetics of a drug under typical physiological conditions” (Lee and Robinson 1986). In some studies a minimum of three rabbits were used per time point (Law, Huang et al. 2000), although most researchers consider six animals per time point as a minimum requirement for obtaining statistically reliable data (Genta, Conti et al. 1997; Vega, Gamisans et al. 2008). To reduce the number of required animals and strengthen the statistical viability of the data serial sampling of the aqueous humour could also be performed (Macha and Mitra 2001), but one has to consider the traumatisation of the cornea and breach of the blood-aqueous barrier which could seriously alter drug permeation through the cornea (Lee and Robinson 1986). There is also a debate on whether or not to use both eyes of an experimental animal in pharmacokinetic studies. This depends on the extent of systemic absorption of the drug but, as a general rule, providing that at the latest anticipated time point the concentration of drug in the untreated eye is less than 10%, both eyes could be used (Lee and Robinson 1986).

There is a substantial quantity of ocular work published using rabbits as model and extensive information is available on the rabbit ocular biochemistry and physiology (Lee and Robinson 1986; Urtti, Pipkin et al. 1990; Hitoshi, Choyu et al. 1993; Järvinen, Järvinen et al. 1995; Järvinen and Järvinen 1996; Jiao 2008; Civiale, Licciardi et al. 2009; Li, Zhuang et al. 2009; Luo, Zhao et al. 2011). One important consideration is that the choice of an animal model should represent the function and performance of the delivery system in human subjects (Lee and Robinson 1986). Rabbits have 50% lower tear turnover rate than humans which makes precorneal residence time of topically applied medications somewhat longer (Lee and
In Vivo and ex Vivo efficiency of lipid nanoparticles

Robinson 1986). However, the other precorneal parameters such as tear volume and tear protein concentration are very similar to humans. In addition, the aqueous humour properties are sufficiently close and drug disposition in this physiological environment is probably similar to humans (Lee and Robinson 1986). Another contributing difference in ocular physiology of rabbits is the lower blinking rate which might interfere with the disposition of the delivery systems as the lower rate of blinking may alter the function of those systems which rely on the shear force provided by blinking (eg. in situ gel systems) (Lee and Robinson 1986).

The general aim of this chapter was to determine the safety and efficacy of the developed drug delivery system, the specific objectives were to:

1. determine the suitability and safety of the developed formulations by carrying out in vitro toxicity assays,
2. assess the antiviral properties of the delivery system using a plaque reduction assay,
3. assess the cell uptake of the developed delivery systems by fluorescent microscopy and
4. establish the in vivo corneal permeation profile of the delivery system in comparison to the available commercial ophthalmic ointment.

5.2. Materials and methods

5.2.1. Materials

Materials mentioned in Section 4.2.1 were used to prepare the formulations. Zirovax ophthalmic antiviral ointment (GlaxoSmithKline, New Zealand) was used as a control in the in vivo release study. Petroleum jelly (Multichem, NZ) was used to dilute Zovirax ointment. 26G ½ (0.45 x 13mm) and 21G 1 ½ TW (0.8mm x 38mm) needles (BD PrecisionGlide™
Needles, Singapore) were used to collect the ocular fluid samples. Guanosine (Sigma, Germany) was used as HPLC internal standard. Phosphate buffer solution (PBS) and all other chemicals used were of analytical grade.

5.2.2. Selected formulations

In this chapter, SLNs S (3) and NLCs N (4) developed in Chapter 2, NLCs containing ACV-β-cyclodextrin inclusion complexes developed in Chapter 3 and chitosan coated NLCs (NLCs-LCH 0.5% w/v) developed in Chapter 4 were accessed for toxicity. The cell uptake compared both chitosan coated NLCs (NLC-LCH 0.5% w/v) and NLCs N (4). However, only NLC-LCH 0.5% w/v was subjected to the antiviral assay and in vivo corneal permeation studies.

5.2.3. Toxicity studies

5.2.3.1. Bovine corneal opacity-permeability (BCOP) assay

Bovine eyes were collected from Auckland Meat Processors within 10 mins of slaughter time. Eyes were placed in PBS pH 7.4 at 10 °C, transferred to the laboratory and were generally used within 30 mins. The eyes were placed in rectangular plastic containers containing 10 mL of PBS and were incubated for 10 mins at (37 °C ± 5) (Figure 5-1).
Subsequently, a silicone o-ring (13 mm diam.) was placed on the centre of the cornea, 100 μL of PBS was added inside the ring and incubated for 5 mins. A volume of 100 μL of the test substance was then applied to the corneal surface inside the ring of three eyes. Four different controls were used to assess the severity of ocular irritation. A 0.5 M solution of NaOH was used as strong irritant, acetone as mild irritant, propylene glycol as slight irritant and PBS as negative control. After one and a half min of exposure, the silicone ring was removed and eyes were rinsed with approximately 10 mL of warm PBS. Eyes were incubated for a further 10 mins after which they were examined for ocular opacity, epithelial integrity and epithelial detachment. The ocular irritancy scores were obtained according to the scale shown in Table 5-1 (Bruner, Kain et al. 1991) with the final score determined by adding up the individual scores for corneal opacity, integrity and detachment. Table 5-2 shows the interpretation of the scores obtained.
Chapter 5 *In Vivo* and *ex Vivo* efficiency of lipid nanoparticles

**Table 5-1 Score table for the BCOP assay (adopted from [Bruner, Kain et al. 1991]).**

<table>
<thead>
<tr>
<th>Opacity</th>
<th>Score</th>
<th>Epithelial integrity (degree of staining)</th>
<th>Score</th>
<th>Epithelial detachment</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>None</td>
<td>0</td>
<td>No gross changes</td>
<td>0</td>
</tr>
<tr>
<td>Slight</td>
<td>1</td>
<td>Diffuse and weak</td>
<td>0.5</td>
<td>Wrinkling of surface</td>
<td>2</td>
</tr>
<tr>
<td>Marked</td>
<td>2</td>
<td>Confluent and weak</td>
<td>1</td>
<td>Loosened epithelium</td>
<td>3</td>
</tr>
<tr>
<td>Severe</td>
<td>4</td>
<td>Confluent and intense</td>
<td>1.5</td>
<td>Epithelium absent</td>
<td>4</td>
</tr>
<tr>
<td>Complete</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 5-2 Interpretation of the BCOP assay scores (adapted from [Bruner, Kain et al. 1991]).**

<table>
<thead>
<tr>
<th>BCOP assay mean score</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.3</td>
<td>Non irritant</td>
</tr>
<tr>
<td>0.3-1.9</td>
<td>Slight</td>
</tr>
<tr>
<td>2.0-3.5</td>
<td>Moderate</td>
</tr>
<tr>
<td>&gt;3.5</td>
<td>Severe</td>
</tr>
</tbody>
</table>

**5.2.3.2. Histological evaluation of the treated corneas**

The treated corneas from the BCOP test along with corneas treated similarly for a longer period of time (1, 3 and 8 hrs) were histologically examined. Corneas were excised leaving behind a thin film of sclera, placed into labelled cassettes and fixed with 10% w/v neutral buffered formalin solution for 24 hrs. Samples were stored in 70% v/v ethanol for a further 24 hrs before being processed. Corneal samples were paraffin embedded, bisected into two halves and a section of each half was placed on a single microscope slide. Samples were then stained with haematoxylin and eosin and corneal sections were examined for lesions in the epithelium, stroma (swelling and keratocyte damage) and endothelium ([Harbell 2006](#)).
5.2.3.3. MTT cell proliferation assay

A MTT stock solution (5mg mL⁻¹) was made in RPMI-1640 without phenol red, filtered through a 0.2 µm filter and stored at 2-8 °C. MTT working solution was prepared by a 1:10 dilution of the 5mg mL⁻¹ stock following the protocol below:

1. Cultured Vero cells were washed with warm RPMI-1640 without phenol red.
2. Cells were incubated with MTT working solution at 37 °C for 2 hrs.
3. After 2 hrs, the medium was discarded.
4. The converted dye was then solubilized with 1 mL of acidic isopropanol (0.04 M HCl in absolute isopropanol).
5. The dye solution with the cells was then transferred into a 1.5 mL eppendorf tube and centrifuged at 13,000 rpm for 2 min.
6. 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.

5.2.4. Determination of the antiviral efficacy of ACV loaded NLCs

Human embryonic fibroblasts and monkey kidney cells (CV-1) were used in the assay for antiviral efficiency. Cell cultures were grown and maintained in DMEM supplemented with 10% v/v FBS and 1% w/v penicillin-streptomycin. Herpes simplex virus type-1 (HSV-1) was a hospital isolate amplified in human fetal fibroblasts that stained positive for HSV-1 specific antibody. The plaque purification protocol below was used to purify the viral stock:

1. The agarose-overlay procedure was followed to obtain a 6 well plate with well-isolated plaques. Agarose was removed by gentle tapping and plaques were stained with neutral red (0.1% w/v).
2. Using a yellow pipette tip, up to 23 plaques were selected depending on how well separated they appeared and were transferred to a 24-well plate seeded with Vero
cells on the previous day (one plaque per well). One well was left uninfected as a control.

3. The plate was incubated at 37 °C in a humidified atmosphere at 5% CO₂ until the cytopathic effect was complete (approximately 5 to 10 days).

4. Cell lysis was promoted with three consecutive freeze-thaw cycles to release the virus.

5. Media from two wells were randomly transferred to a sterile 1.5 mL microcentrifuge tube.

6. The virus dispersions were centrifuged briefly (1,000 rpm for 5 min) to remove cell debris and were stored at –80 °C.

7. A viral titre assay was performed to determine the viral titre.

8. Steps 1-7 were repeated twice to collect a purified viral stock.

The virus titer was then determined by a plaque reduction assay expressed as plaque forming units pfu mL⁻¹ which is defined as the lowest concentration of virus that can infect cells.

A known titer of the virus stock was then added to infect the cells. The cells were infected by allowing them to absorb the virus for 2 hrs at 37 °C. The non-absorbed virus in solution was then removed and cells were washed with the 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 (Figure 5-2 and Figure 5-3).
CV-1 cells were seeded into 6-well plates and grown in DMEM with 10% v/v FBS overnight until the cells were about 80 to 90% confluent. CV-1 cells were washed twice with DMEM (no serum) before removing the final wash and 10-fold serial dilutions of the virus stock in DMEM were prepared (each dilution was added to duplicate wells with 1 mL per well). The virus sample was centrifuged for 2 mins at 2,000 rpm in a microfuge. A volume of 100 μL of the viral sample was carefully transferred to an eppendorf tube containing 900 μL of DMEM (1:10 dilution). Using a transfer volume of 0.3 mL, a series of further dilutions from $10^2$ to $10^7$ was generated (final volume of 3 mL in small Falcon tubes). DMEM was removed from the cells, duplicate wells were overlaid with 1 mL of each virus dilution and plates were rocked to achieve good coverage of the cells. Plates were incubated at 37 °C for 2 hrs with occasional rocking. The virus inoculum was aspirated from each well and overlaid with 3 mL of DMEM containing 5% v/v FBS and 0.5% w/v Seaplaque agar. Plates were further incubated at 37 °C for 4 to 5 days.
5.2.5. Cell uptake of fluorescein-loaded NLCs

Based on the methodology reported by Shen et al. (2010) a primary human corneal epithelial cell line (HCEC) was used in this study. Cells were plated in a 24-well plate as a monolayer on round coverslips at a density of 4×10⁴ cells/well at 37 °C in a 5% CO₂ 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 were used as the culture medium. When the cells were at least 90% confluent, they were treated with fluorescein-loaded lipid nanoparticle dispersion in a supplement free culture medium. The concentration of lipid and fluorescein in the samples was maintained at 4 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) the 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 were photographed for each slide using a confocal laser scanning microscope (FLUOVIEW FV1000, Olympus, Germany).
5.2.6. Cell uptake of ACV-loaded NLCs

HCEC cells were grown as described above with 1 mL DMEM in each well before inoculation. Each well was then inoculated with 10 µL of the test solutions. The test solutions included: Chitosan coated NLCs, uncoated NLCs and ACV solution all containing 0.3% w/v ACV. At specific time points (0.5, 1, 2, 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 12000 rpm for 10 mins. The sedimented cells were re-suspended in 150 µl PBS and freeze-thawed thrice to distort the cell membranes. A volume of 100 µl of the cell suspension was diluted with 100 µl of PBS, vortexed for 5 mins and centrifuged at 12,000 rpm for 10 mins. 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:

\[
\text{Drug uptake (µg mg}^{-1}\text{protein)} = C/M
\]

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.

5.2.6.1. Virus titre and cell infection

The virus was prepared in aliquots, stored at -80 °C and thawed at room temperature just before use. A monolayer of monkey kidney cells (CV-1) was used to determine the infectious virus titre. A plaque reduction assay was performed and the virus titre was presented as plaque forming units per mL (pfu mL⁻¹).
5.2.6.2. Plaque reduction assay in Vero cells

The efficacy of nanoparticles containing ACV was measured by a plaque reduction assay of HSV-1 replication in Vero cells. Concentrations of each formulation of drug necessary to reduce plaque formulation by 50% (EC50) was measured and compared to the 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 formulation at a range of concentrations. When plaques were formed, the monolayers were stained with 1% w/v crystal violet in 20% v/v methanol and plaques were counted using a stereomicroscope. The concentration of drug that reduced plaque formation by 50% (EC50) was calculated using Prism software by comparing drug-treated with untreated cultures.

5.2.7. In vivo evaluation of ACV loaded NLCs

This study was conducted at the Vernon Jenson Unit (VJU) of the Faculty of Medical and Health Sciences in accordance with the University of Auckland guidelines for the use of laboratory animals in research under permit number AEC R865. New Zealand albino rabbits (3-4 kg) were used and were checked for any signs of ocular damage. Rabbits were confined in standard rabbit housings 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 ACV loaded NLCs in the form of an ocular suspension and the fourth group received a 3% w/v and the fifth group a 0.3% w/v ACV suspension in buffer as a control. 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 pentobarbitone (300 mg mL$^{-1}$) into the marginal ear vein.

The formulation was instilled in the cul-de-sac of the eye by pulling the lower lid forward (Figure 5-4). After instillation, the lower lid was lifted back and forth to allow mixing of the NLCs dispersion with the lachrymal fluid (Law, Huang et al. 2000). At specific time intervals (0.5, 1, 2, 4, 6 and 24 hrs) six animals were euthanized and samples including the cornea, aqueous humour and vitreous were obtained. Samples of aqueous and vitreous humour were withdrawn by a syringe using a 26G and 21G needle respectively. For collecting aqueous humour, the needle was inserted parallel to the iris and for collection of vitreous humour, the needle was inserted at a 45° angle through the stroma. Dissected samples of cornea were rinsed with 5 mL of PBS and collected in pre-weighed vials. All samples were stored at -20° C until analysed by HPLC.

Corneal samples were collected in pre-weighed vials and the weight of the cornea was determined. An appropriate amount of the internal standard, guanosine, was added to each vial. The corneal tissue was then digested with 0.5 mL of 0.5 N sodium hydroxide solution in
a shaking water bath at 60 °C for 1 hr. The digested samples were then immediately neutralised by adding 0.54 mL of 12% v/v perchloric acid solution and centrifuged at 5000 rpm for 20 mins to obtain a clear supernatant. Accurately measured 50 µL of a 44% w/v sodium acetate solution were added to each vial and samples were filtered through a 0.22 µm filter before injecting 50 µL into the HPLC column (Law, Huang et al. 2000).

The aqueous and vitreous humour samples were prepared by following the protocol reported in the literature (Genta, Conti et al. 1997; Fresta, Fontana et al. 2001; Giannavola, Bucolo et al. 2003). An appropriate amount of the internal standard, guanosine (Sigma, Germany), 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 vortex mixed for 1 min and centrifuged at 6,000 rpm for 20 mins. The clear supernatant was filtered through a 0.22 µm filter and 50 µL were injected into the HPLC for analysis where guanosine was used as HPLC internal standard.

5.3. Results and discussion

5.3.1. Toxicity and irritation potential of NLCs

5.3.1.1. Bovine corneal opacity-permeability (BCOP) assay

Figure 5-5 shows the treated corneas with four different control materials. A 0.5 M solution of NaOH was used as a strong irritant and severe corneal opacity was observed after only a brief treatment (30 sec) with the corneal epithelium found detached and loose. Intense fluorescein staining indicated sever epithelial damage. Treatment with acetone as a mild or moderate irritant resulted in marked corneal opacity, weak and confluent fluorescein staining and a loose epithelium. Consequently, treatment with propylene glycol as a slight irritant resulted in very slight corneal opacity along with confluent and weak fluorescein staining
with the epithelium remaining intact. With the negative control, the cornea remained transparent, intact and no fluorescein staining was evident. The responses recorded for the four control materials were used as endpoints in assessing the severity of corneal irritation associated with the developed ocular formulations.

Figure 5-6 shows the bovine corneal events of the four test solutions (SLNs, NLCs, NLCs coated with 0.5% w/v LCH and βCD-ACV complex-loaded NLCs). Corneas retained their transparency and integrity in all samples and were completely impermeable to fluorescein. The test scores were calculated based on Table 5-1 and Table 5-2 with all test substances having a score below 0.5 which indicated that they do not induce corneal irritation and are well tolerated. Figure 5-7 shows a graphic representation of the cumulative BCOP scores of the control and test substances.
Chapter 5 In Vivo and ex Vivo efficiency of lipid nanoparticles

0.5M NaOH
(Strong irritant)
Severe corneal opacity
Intense fluorescein staining

Acetone
(Moderate irritant)
Marked corneal opacity
Confluent and weak staining

Propylene glycol
(Slight irritant)
Slight corneal opacity
Confluent and weak staining

PBS pH 7.4
(Negative control)
Transparent cornea
No staining of the cornea

Figure 5-5 The bovine corneal events after application of control solutions.
Chapter 5 *In Vivo* and *ex Vivo* efficiency of lipid nanoparticles

**SLNs**
- Cornea retained transparency
- No staining of the cornea

**NLCs**
- Cornea retained transparency
- No staining of the cornea

**NLC-βCD**
- Cornea retained transparency
- No staining of the cornea

**NLC-LCH**
- Cornea retained transparency
- No staining of the cornea

*Figure 5-6* The bovine corneal events after application of the test solutions.
Chapter 5 In Vivo and ex Vivo efficiency of lipid nanoparticles

Figure 5-7 Cumulative BCOP scores for the four control solutions: 0.5 M NaOH, acetone, propylene glycol and PBS (negative control) and the four formulations: SLNs, NLCs, NLCs containing ACV-βCD complex (NLC-βCD) and NLCs coated with 0.5% w/v LCH (NLC-LCH), (n=3, mean ± SD).
5.3.1.2. Histological evaluation of bovine corneas

Figure 5-9 shows the histopathological evaluation of the same control substances used in BCOP assay. The results obtained in this section strongly correlate with those obtained for the BCOP assay. The photomicrographs demonstrate the epithelial detachment phenomenon (Figure 5-9 A+B). In addition, nuclei and cytoplasm vacuolisation can also be observed under higher magnification (Figure 5-9 B). Epithelial coagulation and chromatin condensation is identifiable for acetone as a mild irritant (Figure 5-9 C+D). There was some corneal surface damage but acetone did not result in epithelial detachment. Mild epithelial coagulation and chromatin condensation was also evident for propylene glycol as a slight irritant (Figure 5-9 E+F). However, corneal surface damage could not be identified and the epithelium looked normal (Figure 5-8). The cornea treated with PBS looked normal and was used for setting a baseline for comparison of the test formulations, while the positive controls were used to assess the severity of the corneal toxicity.

Figure 5-10 to Figure 5-13 show the histopathological evaluation of the corneas treated with the four different formulations for 30 sec, 1, 3 and 8 hrs. There was no gross toxicity associated with any of the formulations tested even after extended periods of exposure. This is in close agreement with the results obtained with the BCOP assay. There was no epithelial detachment identified and the epithelial nuclei and cytoplasm were not vacuolated. Keratocytes and stroma also retained their integrity. According to these results, the developed formulations are non-toxic and are well tolerated by the cornea.
Figure 5-8 A) Full thickness cornea, B) Epithelium and upper stroma.
Figure 5-9 Histological evaluation of bovine corneas incubated with NaOH, acetone, propylene glycol and PBS.
Figure 5-10 Histological photomicrographs of bovine corneas treated with SLNs at various time points. No gross corneal damage could be detected.
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Figure 5-11 Histological photomicrographs of bovine corneas treated with NLCs at various time. No gross corneal damage could be detected.
Chapter 5 In Vivo and ex Vivo efficiency of lipid nanoparticles

Figure 5-12 Histological photomicrographs of bovine corneas treated with βCD-ACV loaded NLCs at various time points. No gross corneal damage could be detected.
Figure 5.13 Histological photomicrographs of bovine corneas treated with NLCs coated with 0.5% w/v LCH at various time points. No gross corneal damage could be detected.
5.3.1.3. MTT cell proliferation assay

The MTT assay is a convenient colorimetric assay which can be used to determine the cellular toxicity of the developed nanoparticles by estimating the number of viable cells in the microtitre tray wells (Denizot and Lang 1986; Gerlier and Thomasset 1986; Xu, Chen et al. 2009). The assay principle is that living cells are capable of reducing the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan dye which can be quantified by spectrophotometry (Denizot and Lang 1986). The MTT cell proliferation assay in this study was performed on Vero cells. For this purpose, cells were treated with increasing concentrations of the nanoparticles. Both empty and ACV loaded NLCs were examined in order to eliminate the effect of unprecedented drug toxicity.

![MTT calorimetric measurement](image)

Figure 5-14 MTT calorimetric measurement of the proliferation of cells treated with different concentrations of ACV containing NLCs, chitosan coated NLCs (NLCs-LCH) and empty NLCs.

Figure 5-14 shows the MTT calorimetric measurement of the proliferation of cells treated with different concentrations of ACV containing NLCs, chitosan coated NLCs (NLCs-LCH) and empty NLCs. The cell toxicity increased with an increase in the concentration of the NLCs. The LD$_{50}$ of ACV loaded NLCs was 1086 µg mL$^{-1}$ which closely resembles values reported for other lipid based nanocarriers (Nassimi, Schleh et al. 2009). The LD$_{50}$ of the empty NLCs was 1015 µg mL$^{-1}$ which was not significantly different to that of drug loaded
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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).

### 5.3.2. 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. Vero cells are considered to be a suitable host for the virus (Walro and Rosenthal 1997; Pope, Marcelletti et al. 1998; de Jalón, Blanco-Príeto et al. 2003). Figure 5-15 shows the morpho-pathological changes of Vero cells after being infected by HSV-1.

![Figure 5-15](image)

*Figure 5-15 (A) Monolayer of Vero cells before infection and (B) Vero cells 24 hrs after being infected by a purified colony of HSV-1.*

The ACV containing NLCs exhibited a concentration dependent plaque reduction profile. Figure 5-16 shows the dose response curve of the ACV solution 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\textsubscript{50} of ACV in solution versus ACV in NLCs was 0.018 ± 0.01 versus 0.063 ± 0.03 after 24 hrs and 0.017 ±0.02 versus 0.061 ± 0.01 after 48 hrs of treatment. Based on these results, the antiviral efficacy of ACV was increased when formulated as NLCs.

Figure 5-16 Antiviral efficacy of ACV in solution (ACV) and ACV encapsulated NLCs (NP) after (A) 24 hrs and (B) 48 hrs treatment.
NLCs are thought to enhance the antiviral efficiency of ACV by enhancing drug penetration through the 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 (Ropert and Mishal 1996; de Jalón, Blanco-Príeto et al. 2003). The enhanced antiviral property of ACV in NLCs could be due to two different mechanisms. The sustained drug release property of NLCs would have increased the exposure time of the infected cells to the drug resulting in a more efficient treatment. Also, the cell internalising property of NLCs could be another reason for enhanced antiviral efficacy of ACV since the drug in solution would not be taken up by cells in sufficient amounts to eradicate the virus within the cells.

5.3.3. Cell uptake of NLCs

Cell uptake studies are important in order to understand the mechanism by which the antiviral efficacy of ACV could be enhanced using lipid nanoparticles. Cell internalisation is the hypothesis behind enhancement of therapeutic properties of the drug. Therefore, because ACV could not be fluorescein-tagged, a fluorescein cell uptake assay was set up to obtain a qualitative set of data showing the cell internalisation ability of the nanoparticles. The ACV loaded nanoparticles were also investigated by measuring the drug concentration internalised within a primary culture of corneal epithelial cells.

Figure 5-17 and Figure 5-18 show the fluorescence uptake of the corneal epithelial cells after being treated with the developed NLCs, chitosan coated NLCs (NLCs-LCH) and the NLCs containing the ACV-βCD complex (NLC-βCD). Figure 5-17 shows the fluorescent images after incubating the cells with fluorescein solution. It is evident that the cells were confluent and they remained impermeable to fluorescein even after 12 hrs of incubation. Cells were
also treated with NLCs which apparently coated the exterior surface of the cells (Figure 5-17B). The fluorescein intensity on the cells improved as the incubation time increased because 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 5-18 reveals that they differ considerably from the images obtained after treating the cells with the control and NLCs. Decisively, addition or inclusion of chitosan or βCD to the formulations respectively allowed the nanoparticles to be internalised with fluorescein present in the cytoplasm of the epithelial cells which seems to intensify over time. This was expected as chitosans and cyclodextrins are known for their corneal penetration enhancing properties (Måsson, Loftsson et al. 1999; Kaur and Smitha 2002; Aktas, Ünlü et al. 2003; Majumdar, Hippalgaonkar et al. 2008).
Figure 5-17 Fluorescein uptake of (A) control fluorescein solution and (B) NLCs after 2, 4 and 12 hrs.
A) NLC-LCH

B) NLC-βCD

Figure 5-18 Fluorescein uptake of NLCs-LCH and NLCs-βCD after 2, 4 and 12 hrs.
Figure 5-19 shows the cellular uptake of the ACV solution and ACV loaded NLCs (coated and uncoated). The cellular uptake of chitosan coated NLCs is higher than that of uncoated NLCs or the ACV solutions. ACV in both coated and uncoated NLCs has much higher cellular uptake than free ACV in solution, therefore both systems are capable of internalising the drug and increasing its corneal penetration. The chitosan coated system had the highest drug cellular uptake because of chitosan’s cell penetration enhancing properties. The results obtained here are only a relative measure of ACV cell uptake since the Coomassie Brilliant Blue test used to quantify the amount of proteins is only semi-quantitative.

![Graph of ACV uptake concentration profile](image)

**Figure 5-19** Cell uptake concentration profile of ACV in solution (ACV), uncoated NLCs (NLCs) and chitosan coated NLCs (NLCs-LCH).

### 5.3.4. In vivo corneal permeation

The only marked 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. Novel drug delivery systems promise to increase the ocular bioavailability of drugs and by doing so would decrease the required loading dose (Ghate and Edelhauser 2006; Gaudana, Ananthula et al. 2010). The chitosan coated NLCs system under investigation here, contained 0.3% w/v of ACV which is
ten times lower than the marketed ophthalmic ointment. 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 5-20 shows the ACV concentration in the aqueous humour with the corresponding pharmacokinetic data reported in Table 5-3.

![Figure 5-20](image)

**Figure 5-20** Drug concentration profile of chitosan coated NLCs (NLCs-LCH), a 0.3% w/v ACV eye drop and a 0.3% w/v ophthalmic ointment in the aqueous humour.

**Table 5-3** Aqueous humour pharmacokinetic parameters of the formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (min)</th>
<th>AUC (min µg mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Relative AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLC-LCH</td>
<td>1.56 ± 0.43</td>
<td>60</td>
<td>275.9 ± 70.49</td>
<td>4.17</td>
</tr>
<tr>
<td>3% w/v Ointment</td>
<td>1.80 ± 0.96</td>
<td>60</td>
<td>252.7 ± 84.42</td>
<td>3.82</td>
</tr>
<tr>
<td>0.3% w/v Ointment</td>
<td>0.52 ± 0.10</td>
<td>60</td>
<td>66.07 ± 66.06</td>
<td>1.00</td>
</tr>
<tr>
<td>3% w/v ACV suspension</td>
<td>0.42 ± 0.23</td>
<td>30</td>
<td>44.46 ± 32.10</td>
<td>0.67</td>
</tr>
<tr>
<td>0.3% w/v Eye drop</td>
<td>0.34 ± 0.11</td>
<td>30</td>
<td>39.78 ± 29.62</td>
<td>0.60</td>
</tr>
</tbody>
</table>
The relative AUC for ACV in NLCs formulation increased 4.17 times when compared to the ointment and 6.93 times when compared to the eye drop. The $C_{\text{max}}$ 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 $C_{\text{max}}$ 1 hr after topical administration ($T_{\text{max}}=60 \text{ min}$); however, the eye drop control reached $C_{\text{max}}$, 30 mins post administration. This is due to the fast availability of the drug for absorption and penetration across the cornea (Järvinen, Järvinen et al. 1995). Figure 5-21 shows the aqueous humour ACV profiles for the chitosan coated NLCs when compared to a 3% w/v ophthalmic ointment and a 3% w/v ACV suspension. Although the commercial ointment had 10 times more ACV loaded, the AUC for both NLCs and ointment remained similar and the difference was insignificant ($P>0.05$) (275.9 ± 70.49 and 252.7 ± 84.42 respectively). This illustrates how inefficient the ophthalmic ointment is for this particular treatment (Chetoni, Rossi et al. 2004). 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 ($P>0.05$). The time to reach the maximum concentration ($T_{\text{max}}$) 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 $T_{\text{max}}$ for the NLCs was very similar to what has been reported for ACV loaded liposomes (Table 5-3) (Law, Huang et al. 2000). This shows that the positively charged chitosan coated NLCs had a controlled and slow penetration through the cornea (Law, Huang et al. 2000). Also, the 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 5-21 Drug concentration profile of chitosan coated NLCs (NLCs-LCH), 3% w/v ACV aqueous suspension and 3% w/v ophthalmic ointment in aqueous humour.

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 (Law, Huang et al. 2000; Fresta, Fontana et al. 2001; Giannavola, Bucolo et al. 2003; Chetoni, Rossi et al. 2004). Only one study included the commercial ophthalmic ointment as a control (Chetoni, Rossi et al. 2004) and none of the studies analysed the concentration of penetrated ACV in the posterior chamber. 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 (Tullo, Easty et al. 1982). Therefore, in this study, the concentration of ACV in the vitreous humour was also determined to establish the drug concentration profile of the formulation in the posterior section of the eye.
Figure 5-22 shows the concentration of ACV in the vitreous humour of rabbits at several time points after the administration of ACV containing chitosan coated NLCs, the ophthalmic ointment and the eye drop containing 0.3% w/v of ACV. Amongst all, the developed NLCs formulation had the highest drug concentration in the vitreous. The AUC for NLCs was 48.7 times larger than the ophthalmic ointment of the matching concentration (Table 5-4). The developed NLCs, unlike the control ointment and the eye drop, exhibited a sustained drug release profile in the vitreous humour.

Table 5-4 Vitreous humour pharmacokinetic parameters of the formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>AUC (min µg mL⁻¹)</th>
<th>Relative AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLC-LCH</td>
<td>0.5616 ± 0.23</td>
<td>48.70</td>
</tr>
<tr>
<td>3% w/v Ointment</td>
<td>2.821 ± 0.98</td>
<td>244.66</td>
</tr>
<tr>
<td>0.3% w/v Ointment</td>
<td>0.01153 ± 0.01</td>
<td>1</td>
</tr>
<tr>
<td>3% w/v ACV suspension</td>
<td>0.1127 ± 0.02</td>
<td>9.77</td>
</tr>
<tr>
<td>0.3% w/v Eye drop</td>
<td>0.0180 ± 0.013</td>
<td>1.5611</td>
</tr>
</tbody>
</table>

Figure 5-22 Drug concentration profile of chitosan coated NLCs (NLCs-LCH), a 0.3% w/v ACV eye drop and a 0.3% w/v ophthalmic ointment in vitreous humour.
When the ophthalmic ointment and aqueous suspension containing 3% w/v ACV were used, the ointment had the largest AUC (Table 5-4). When the amount of drug was increased ten times, the AUC of the eye drop was enhanced proportionally. However, it stayed much lower than that of the NLCs and the ointment. The reason for the higher AUC of the ointment in comparison to the eye drop is that it stays longer on the ocular surface due to its high viscosity and sticky nature, whereas the eye drop is quickly removed by the tear flow after application. The ointment had an AUC five times higher than NLCs but it contained ten times more drug.

![Graph](image)

**Figure 5-23** Drug concentration profile of chitosan coated NLCs (NLCs-LCH), a 3% w/v ACV eye drop and a 3% w/v ophthalmic ointment in vitreous humour.

Figure 5-24 and Figure 5-25 show the time dependent ACV concentration profile of NLCs and the control formulations in rabbit corneas excised after the withdrawal of aqueous and vitreous humour samples. The study of the drug concentration remaining on, or within, the cornea helps to examine the corneal residence time of various formulations *in vivo* (Law,
Huang et al. 2000). Figure 5-24 represents the corneal drug concentrations of the NLCs and the controls of matching concentrations (0.3% w/v).

![ACV Concentration in cornea (µg/mg) vs Time (hrs)](image)

**Figure 5-24** Drug concentration profile of chitosan coated NLCs (NLCs-LCH), a 0.3% w/v ACV eye drop and a 0.3% w/v ophthalmic ointment in the cornea.

In the case of the ophthalmic ointment and the eye drop, the corneal drug concentration decimated quickly to negligible amounts. Although the ointment seemed to retain slightly higher concentrations of the drug on or within the cornea, both failed to attain longer corneal residence times to promote higher drug permeation. The NLCs formulation on the other hand, retained a higher concentration of the drug on the corneal surface for a longer time with a considerable amount of drug still in the cornea between 2 to 6 hrs post administration. These data support our previous hypothesis that the NLCs are capable of forming an adhesive film on the surface of the cornea from which the drug is released slowly.
5.4. Conclusion

From the data shown in this chapter it can be concluded that NLCs are well tolerated and are nontoxic to the eye. They can enhance the antiviral efficiency of ACV by means of cell internalisation. The in vivo animal study showed that the chitosan coated nanoparticles are capable of enhancing the ocular bioavailability of the drug and reducing the required administered dose. The chitosan coated NLCs were also capable of delivering the drug to the back of the eye in addition to maintaining a therapeutic concentration of the drug on the corneal surface, over an extended period of time. The aqueous humour AUC of the NLCs was 4.5 times higher than that obtained from a commercial ophthalmic ointment of matching concentration which indicates the potential of NLCs to optimise topical ocular drug delivery of ACV.
Chapter 6. General Discussion, Limitations and Future Directions

Chapter Six

Each problem that I solved became a rule, which served afterwards to solve other problems.

— Rene Descartes
6.1. General discussion

Ocular drug delivery is challenged and limited not only by the complex physiology of the human eye but also by problematic pharmaceutical properties of drugs and new therapeutic moieties. Topical ocular drug delivery has been confined to the use of classical ointments and eye drops which, in best case scenarios, would only provide a drug dispersion medium with no added benefits. The ocular drug bioavailability of eye drops and ointments remain below 15% in most cases. To compensate for the low bioavailability the physician is obliged to prescribe high and frequent doses in order to achieve an optimal therapeutic response. As a result, the patient is unnecessarily exposed to toxic concentrations systemically leading to side effects, an issue which has not been addressed adequately despite the progress of science and technology. There is a growing need for the development of advanced and more efficient ocular therapeutic systems. The pharmaceutical industry is especially interested in marketing controlled released novel drug delivery systems which could be patented to enhance the bioavailability of existing or problematic drugs. Moreover, most of the recently discovered therapeutic molecules and moieties are water insoluble candidates requiring a nanoparticulate system to transport them across the cornea to the primary site of action.

Topical ocular drug delivery has been commercially limited to the formulation of aqueous eye drops for hydrophilic drug candidates and ocular ointments for hydrophobic drugs. Even with the most pharmaceutically compatible drug candidates, none of the aforementioned dosage form designs offer any particular advantage except for providing a simplistic vehicle to administer the required drugs. The lack of a suitable topical ocular drug delivery system has forced the formulation scientists to increase the loading dose of the medicament (eg. 3% w/v ACV ophthalmic ointment) in order to achieve the minimum ocular therapeutic concentration. Novel ocular drug delivery systems have the potential to optimise
conventional dosage forms (eye drops and ointments) offering advantages such as site specific treatment, decreased systemic toxicity, controlled release, improved permeability and extended corneal residence time. Many ocular drug delivery systems have been extensively examined over the past two decades amongst which solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), liposomes, niosomes and other polymeric nanoparticles have been widely reviewed due to the added advantages that they present over classical ocular dosage forms such as increased corneal permeation and ocular bioavailability.

The aim of this thesis was to design and develop a lipid based nanoparticulate ocular drug delivery system to encapsulate and deliver ACV which is a compound of medium polarity with poor hydrophilic or lipophilic properties.

In Chapter 1, the literature was reviewed to assess the suitability of SLNs for ocular drug delivery. The barriers to topical drug delivery were identified and limitations of the current therapeutic regimens were highlighted. Methods of fabrication and characterisation of SLNs were discussed in detail. Structural modifications of SLNs containing liquid lipids (NLCs) were then discussed in detail highlighting the benefits provided. The chemicals used in the fabrication of SLNs, such as physiological lipids, are biodegradable and nontoxic. The structure and properties of these carriers can be tailored in order to suit the needs of patients, clinicians and pharmacists. The concept of personalized medicines is truly applicable here where, instead of having a common and unspecific single drug dosage form for the masses, the site specific nanoparticles could be tailored to suit the individual needs of the patient. Recently, the FDA and EMEA have taken promising steps to lead academic research into more industrial and commercial aspects (Boisseau, Kiparissides et al. 2005). To commercialise novel drug delivery systems, more contribution from scientists and the pharmaceutical industry is needed to refine the academic findings into commercially successful products.
Chapter 2 of this thesis was concerned with the fabrication and characterisation of SLNs and NLCs. Several fabrication techniques were evaluated before a hot oil-in-water microemulsion met the technical expectations. One of the advantages of SLNs over other delivery systems is the simplicity of the formulation techniques and the feasibility of industrial scale up. The effects of several formulation factors such as type, ratio and combination of lipids and surfactants on the quality of the final product were studied. The SEM images obtained showed that SLNs were spherical and within the optimal ocular drug delivery size range (465.86 ± 7.15 nm). However, the drug entrapment efficiency was limited (11.14 ± 0.67%) and the drug release from SLNs was a pure surface phenomenon associated with a high burst release. To increase the ACV entrapment efficiency SLNs were structurally modified to NLCs by adding liquid lipids (lauroglycol 90). The liquid oil droplets in the structure of NLCs create a porous structure with the capacity to accommodate more drug. The entrapment efficiency was improved to 91.64 ± 5.85. However, the in vitro drug release rate was still faster than desired. Therefore, the complexation approach (Chapter 3) and the coating approach (Chapter 4) were followed to retard the drug release rate from NLCs.

In Chapter 3 the physicochemical properties of the drug were masked through complexation with βCD to modulate and control the drug release from NLCs. Several carriers were investigated in terms of their chemical interaction with ACV and their effect on the solubility of the drug was studied through dissolution experiments. The ACV-βCD inclusion complex increased the aqueous solubility of the drug and was ultimately incorporated into NLCs. This resulted in a slower release profile. However, due to possible βCD local toxicity through extraction of cellular lipophilic components such as cholesterol, a coating approach to retard the drug release was also followed in Chapter 4. The NLCs developed in Chapter 3 had good pharmaceutical characteristics but were negatively charged which would limit their precorneal resident time. As corneal epithelial cells bear negative charges, by converting the
surface charge of NLCs, one can significantly increase the precorneal residence time of the formulation to enhance drug corneal permeation.

NLCs were therefore coated with the cationic mucoadhesive polymer chitosan. The effect of different concentrations of chitosan on the NLCs such as particle size, zeta potential and entrapment efficiency were investigated. An *ex vivo* set up which mimicked the corneal tear removal was used to measure the corneal residence time of the formulations on the cornea. The precorneal retention time of NLCs was significantly increased when coated with an increasing concentration of chitosan. Chitosan coating was a saturable process and concentrations more than 0.5% w/v did not increase the cationic charge of NLCs any further. The *in vitro* drug release showed a significantly slower profile with no burst effect. Furthermore, an *ex vivo* study revealed improved corneal permeation.

From the results obtained, the chitosan coated NLCs formulation (0.5% w/v) was chosen for the biological investigations in Chapter 5. It was necessary to assess the antiviral efficacy and ocular tolerability of the formulation before an *in vivo* animal pharmacokinetic study could be undertaken. It was found that the encapsulation of ACV in NLCs resulted in 3.5 fold reduction in the required dose to achieve 50% reduction in viral plaques. NLCs also improved the cellular uptake of the drug. *In vitro* corneal toxicity assays were used in this study to minimise the need for laboratory animals. Based on the results obtained from the MTT proliferation assay, the BCOP assay and histopathological examination of the treated corneas, none of the developed formulations showed any corneal toxicity. Finally, an *in vivo* animal experiment was conducted to assess the efficacy of the chitosan coated NLCs in comparison to the commercially available ACV ophthalmic ointment. The encapsulation of ACV in chitosan coated NLCs resulted in a 4.5 fold increase in ocular bioavailability of the drug.
6.2. Limitations and future direction

The research question in this thesis was whether or not a lipid based drug delivery system could enhance the ocular bioavailability of ACV as a poorly soluble drug candidate. Ideas were explored and an effort was made to adequately answer the research question in a timely manner. Time was the main limiting factor in this work and future research could explore the following directions:

1. Other methods of nanoparticle preparation such as high pressure homogenisation, spray drying and solvent evaporation methods could be investigated to produce smaller particles.

2. More investigation is needed in order to optimise the lyophilised nanoparticles to increase the shelf life.

3. An *in vivo* model of ocular *herpes keratitis* infection on New Zealand white rabbits could be developed to assess the therapeutic efficacy of the new drug delivery system.

4. The effect of sterilization techniques such as heat and gamma radiation on the physical stability of the nanoparticles needs to be investigated.

6.3. Conclusion

In conclusion, a novel lipid based nanoparticulate drug delivery system 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:

- 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,
- enhance the antiviral efficacy of the drug,
- illustrate no toxicity and suitability for ocular drug delivery
- demonstrate good physical stability and shelf life,
- show increased drug cellular uptake 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 optimise topical ocular drug delivery. The commercial potential and ease of scale up manufacturing process support the recognized pharmaceutical advantage of lipid nanoparticles over conventional ophthalmic dosage forms. The nanoparticulate formulation designed, developed and optimised in this thesis 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.
References


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