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**NANOSUSPENSION BASED ELECTROLYTE SENSITIVE IN SITU GEL FOR  
TOPICAL OCULAR DELIVERY OF NATAMYCIN**

A Thesis

Presented for the degree of

Master of Science in Pharmaceutical Science

With emphasis in Pharmaceutics and Drug Delivery

The University of Mississippi

by

POORVA H JOSHI

May 2019

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## ABSTRACT

Natamycin (NT) is a commercially available antifungal drug used for the treatment of fungal keratitis, an infection which affects the clear corneal surface and its associated layers. Currently, NT is available commercially as a 5% w/v ophthalmic suspension, to be administered topically. The main objective of the present investigation was to develop and evaluate the natamycin loaded nanosuspension (NT-NS), as well as its corresponding *in-situ* gel (NT-NS-GG), for the treatment of fungal keratitis. NT-NS was prepared using homogenization technique and optimized based on size, PDI, zeta potential (ZP), assay and process stability. Further, optimized NT-NS was modified into an *in-situ* gel with the addition of 0.2% gellan gum as a gelling agent and evaluated for rheological properties. *In vitro* release and trans corneal permeation studies were performed. Differential scanning calorimetry studies showed no interaction between the drug and other excipients being formulated into a NS. Particle size, PDI, ZP and assay of the optimized NT-NS formulation (formulation LP1) were  $586. \pm 61.3$ ,  $0.447 \pm 0.12$ ,  $-38.97$  and  $107 \pm 0.6\%$ , respectively. LP1 formulation was stable for up to 4 weeks under refrigerated and at room temperature conditions. *In-situ* gels showed satisfactory rheological properties, prolonged drug release and permeation through rabbit corneas. The results suggest that NT loaded NS could be an alternative topical ocular dosage form.

## **DEDICATION**

I dedicate this Master of Science degree to my parents and my friends back in India for their constant support and belief in me. I am thankful to the University of Mississippi for giving me an opportunity to study at this university.

## LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
DPBS	Dulbecco's phosphate buffer saline
DSC	Differential Scanning Calorimetry
GFT	Gel formation time
GG	Gellan Gum
GRT	Gel retention time
HPLC	High Pressure Liquid Chromatography
IPBS	Isotonic phosphate buffer saline
NLC	Nano lipid carriers
NS	Nanosuspension
NT	Natamycin
NT-NS	Natamycin nanosuspension
NT-NS-GG	Natamycin Nanosuspension <i>in situ</i> gel
PDI	Polydispersity index
PVP	Polyvinyl pyrrolidone
RM $\beta$ CD	Randomly methylated $\beta$ cyclodextrin
STF	Simulated Tear Fluid
UV	Ultraviolet detection

ZP

Zeta potential

## **ACKNOWLEDGEMENT**

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## CHAPTER I

### INTRODUCTION

Eyes are one of the most important and complex sensory organs; they act as a gateway to collect external images and transmit them to the brain as signals through the optic nerve [1]. This maintains a connection between the body and the surroundings. Its visual capabilities may be affected by bacterial, viral or fungal infections of the eye as well as age related disorders. The complex anatomy, physiology and biochemistry of the eye, renders this organ highly impervious to drugs/treatment [1].

The eyes can broadly be classified into two segments: the posterior segment and the anterior segment. The anterior segment consists of the *cornea, conjunctiva, aqueous humor, iris, ciliary body* and *the lens*. The *sclera, choroid, Bruch's membrane, retinal pigmented epithelium, neural retina* and *vitreous humor* make up the posterior segment of the eye [1]. The anterior segment of the eye occupies one-third of the eye.

#### **Anatomy and Physiology of the eye.**

##### **Anterior segment**

The cornea is known to be the largest penetration barrier, inhibiting the passage of drug into the eye, avascular in nature and comprising of 5 layers. The primary diffusional barrier is the corneal epithelium, which controls the entry of drug into the eye [2]. The external surface of the cornea is exposed to the environment, and is covered by a tear film; its inner surface is in contact with the aqueous humor [1]. The efficiency of drug penetration across the cornea is governed by

various factors, such as the integrity of the cornea, the properties of the drug and the type of formulation the drug is administered in [2].

The conjunctiva is a thin, semitransparent, elastic, mucous secreting tissue forming the lining of the upper and lower eyelids [1]. A thin vascular layer known as the ciliary body contains the ciliary muscle responsible for adjusting the shape of the lens to focus on an object [3]. The epithelial cells of the ciliary body continuously produce an alkaline ocular fluid, at a rate of 2.5 $\mu$ L/min, known as the aqueous humor which supplies nutrients to the cornea and lens

Located at the posterior region of the cornea, the iris consists of 3 layers. the endothelium, stroma and the epithelium [1]. The central aperture is a pupil which is controlled by the iris, regulating the amount of light entering the eye [4].

The ciliary body is located anterior to the iris and has three major functions: secreting aqueous humor, adjusting focus and draining aqueous humor [1].

Located behind the iris and the pupil, the lens is transparent and biconcave in nature. The anterior part is in contact with the aqueous humor while the posterior part is in contact with the vitreous humor [1].

### **Posterior segment of the eye**

The sclera also known as the ‘white of the eye’ is an elastic tissue present below the conjunctiva. It gives the eyeball its shape and protects the internal organs of the eye from damage [5]. The sclera is made up of a network of collagen fibers responsible for the scattering of the visible light.

Present between the peripheral sclera and the inner retinal membrane, the choroid is made of the outer suprachoroid, which is 6-10 layers thick, the vascular layer below the suprachoroid and the Bruch’s membrane which is the innermost layer consisting of

photoreceptor cells [1]. Each eye of an individual contains approximately 3.5 million non-dividing retinal pigmented epithelium cells (RPE). It plays a vital role in the functioning of photoreceptors and is essential for maintaining visual function [1].

Light sensitive neural cells line the inner lining of the eyeball which makes up the neural retina. The neural retina interacts with the external environment by transmitting sensory information to the brain. Rods and cones are collectively known as the photoreceptor cells. These cells capture and convert the collected photons into neural signals. The cone cells help in distinguishing between black and white colors in dim light while the rod cells differentiate colors in bright light [1].

A gel like fluid is present between the lens and the retina known as the vitreous humor. The vitreous humor is 99.9% water, 0.01% of collagen fibrils, hyaluronic acid and ions, and aids in maintaining the structure of the eye.

### **Topical dosage forms**

Topical administration is preferred for treating disorders of the anterior segment of the eye as it offers a few major advantages [6]. It helps in localizing the drug effects by minimizing systemic exposure. It's ease of administration makes it convenient for self-administration by patients and is a painless method which in turn leads to increased patient compliance [6]. Due to these reasons ophthalmic drugs are primarily administered topically, in the form of solutions, suspensions, and ointments. However, ocular tissues are biologically protected from external toxic elements by a variety of mechanisms [6]. The most prominent mechanism is tear secretion, which continuously flushes the surface of the eye. There is also the surface epithelium which is almost impermeable, and possesses an active efflux-transport system [6]. The major goal in ocular therapeutics is to circumvent these structural obstacles and protective mechanisms in

order to elicit the desired pharmacological responses [7]. Physiological barriers to topically administered drugs are present mainly in the precorneal and corneal regions. Precorneal factors such as lacrimation, dilution, and conjunctival absorption cause loss of more than 90% of the topically administered formulation [1]. Due to these formidable physiological barriers, frequent dosing is often necessitated to achieve satisfactory therapeutic results. This results in a pulsating dosing pattern with extreme fluctuations of drug concentration in the ocular region. These extreme fluctuations may cause local or systemic adverse effects. For maximum bioavailability of the topically administered drug, it is thus important that duration of contact with the cornea be increased.

### **Fungal keratitis**

Fungal keratitis (keratomycosis) is a fungal infection of the cornea the incidence being between 6%-20% of all microbial keratitis cases, depending on the geographic location. It primarily affects the corneal epithelium and stroma, although the endothelium and anterior chamber of the eye may get involved in more severe cases. Fungal keratitis is common in tropical countries compared to the temperate regions. Its incidence in developed countries is reported to be increasing due to the widespread use of contact lenses. Infections due to filamentous fungi such as *Fusarium* and *Aspergillus* are found to be more common in tropical regions while temperate regions show a higher incidence of yeast infections such as *Candida* [8].

The main outcomes desired in an ophthalmic therapy for fungal keratitis is the resolution of the infection as rapidly as possible to give way to good visual outcome and to eliminate the need for therapeutic keratoplasty or permanent loss of vision[8]. Fungal keratitis is a challenging ophthalmologic condition that requires a high level of suspicion and aggressive treatment to prevent untoward outcomes [9].

Natamycin (NT) has been one of the mainstays in the treatment of fungal keratitis [10]. NT suspension is currently the only FDA approved ophthalmic antifungal formulation.

Natamycin is stable at pH 5-9. At pH levels higher or lower, they are found to undergo saponification and/or bond cleavage which leads to instability and hence loss of antifungal activity. NT is also susceptible to photo-oxidation and hence should be stored in the dark [10].

NT shows antifungal activity against both filamentous as well as non-filamentous fungal species, with potent activity against the filamentous species compared to other antifungal agents. It is found to be potent against *Aspergillus* and *Fusarium* species apart from *Candida* species. NT has low retention at the ocular surface which requires it to be frequently administered. Despite these limitations, NT has shown efficacy in treating superficial ophthalmic fungal infections mainly due to its trans-corneal penetration capability [10].

Currently, a formulation of NT (Natacyn<sup>®</sup> Alcon Laboratories, Fort Worth, TX) is available commercially for the treatment of fungal keratitis. It is available as an aqueous suspension containing 5% NT (50 mg/mL); pH adjusted to 5-7.5 to prevent chemical instability of NT. Benzalkonium chloride is added to the suspension to prevent bacterial growth [10]. Its higher penetration across intact cornea upon topical administration and its broad spectrum of activity makes natamycin the forerunner in the treatment of fungal keratitis. NT eye-drops have, thus, been the primary option for superficial corneal infections. Other antifungals such as Amphotericin *B. echinocandin*, and azoles are also used off label as a second-line of treatment [10].

Nanosuspensions (NS) are sub-micron colloidal dispersions of pure particles of drug, stabilized by surfactants. The particle size of the drug in the dispersed phase ranges from 100 to 1000 nm [11]. Surfactants act as stabilizing agents by lowering the surface tension between the dispersed

drug molecules and the dispersion liquid, allowing the formulation to stabilize [12]. Nanosuspensions contain particles of 100% pure drug devoid of any carriers or vehicles [13]. Hence a high drug loading in nanosuspensions could result in highly efficient drug transportation into the cells leading to high therapeutic concentrations, which in turn would maximize the pharmacological effects. However, surfactant-stabilized suspensions must possess sufficiently high energy barriers to prevent the suspended particles from coming close together and agglomerating.

PEGylated natamycin NLCs (nanostructured lipid carriers) with 0.3% drug load [14] and ion sensitive *in situ* gels of natamycin bilosomes [15] have been previously reported. The optimized PEGylated NLC (0.3%) had a particle size with a narrow PDI, high NT entrapment and drug content. In the *in vitro* studies, the NLC formulation also showed an improved transcorneal permeation and flux compared to the Natacyn<sup>®</sup> (5%) the marketed suspension, as well as concentrations statistically similar to Natacyn<sup>®</sup> in the inner ocular tissues [14].

The optimized NT bilosomes (NB) showed a 6-9 fold enhancement of transcorneal flux and ocular penetration. The *in situ* gel with 0.3% gellan gum was found to be cytocompatible with the desired viscoelastic and adhesive properties [15].

*In situ* forming gels are liquids at the time of instillation into the eye, but then undergoes rapid gelation in the cul-de-sac of the eye to form viscoelastic gels in response to environmental changes [16]. *In situ* gels tend to increase precorneal residence time of the drug in the cul-de-sac, leading to sustained release, enhanced bioavailability and reduced dosing frequency. The main reason for the popularity of *in situ* gels is the ease of administering accurate quantities compared to pre-gelled formulation [17]. Electrolyte sensitive *in situ* gel systems undergo a gel-sol

transformation when in contact with mono or divalent cations in the tear fluid, particularly  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ , thus forming a gel on the ocular surface [17]. Naturally occurring polysaccharides have been used as a matrix to obtain sustained drug delivery [18]. Gellan gum is an anionic polysaccharide which undergoes a sol-gel transition due to the temperature and ionic conditions present in the tear fluid, forming an ordered state of gellan chains responsible for the gelling effect [18].

A majority of the drug instilled into the eye, is drained out via reflexive blinking or through lacrimation. Thus, the desired formulation characteristics include prolonged pre-corneal residence time, non-irritating characteristics and suitable rheological properties [19]. From the perspective of patient compliance, a dosage form capable of maintaining contact with the cornea for extended periods of time, which would reduce the frequency of dosing, is desired [20].

In the current study, we attempted to develop a NT-NS and corresponding *in situ* gel (NT-NS-GG) with gellan gum as the gelling agent. Particle size, polydispersity index, assays and zeta potential of the NT-NS were evaluated. The optimized NT-NS was evaluated for stability studies at 25°C and 4°C for 30 days. For the NT-NS-GG, characteristics such as gelling time, gel retention time, viscosity of the formulation with and without the addition of simulated tear fluid were investigated. The optimized NT NS and NT-NS-GG were evaluated for *in vitro* release studies and transcorneal penetration studies.

## **CHAPTER II**

### **METHODOLOGY**

#### **Materials**

NT was purchased from Cayman Chemicals (Ann Arbor, MI). Polysorbate 80, poloxamer 188 was purchased from Acros Organics (Morris, NJ). Laboratory grade lecithins (Fair Lawn, New Jersey), Tyloxapol was purchased from Sigma-Aldrich Life Science (St Louis, MO). Gellan gum was obtained from Alfa Aesar (Ward Hill, MA). High performance liquid chromatography (HPLC) grade solvents and other chemicals (analytical grade) were supplied by Fisher Scientific (Hampton, NH). Slide-A-Lyzer™ MINI Dialysis Device, 10K was purchased from Thermo Fisher (Rockford, IL).

#### **Animal tissues**

Whole eyes of male albino New Zealand rabbits were acquired from Pel-Freez Biologicals (AR, USA).

#### **Preparation of natamycin nanosuspension (NT-NS) and *in-situ gels* (NT-NS-GG)**

##### **Selection of surfactants combination**

Two types of surfactant combinations were used: polysorbate 80 with poloxamer 188 and lecithin with poloxamer 188. Tyloxapol was used as a surface-active agent. PVP K 30 was used as a stabilizer.

##### **Method of Preparation of natamycin nanosuspension (NT-NS):**

NT loaded NS were prepared using homogenization method as per the compositions in Table 1 and 2. The surfactants along-with tyloxapol and poloxamer 188 were dissolved in sufficient volume of water with the aid of magnetic stirring. On forming a clear solution, NT was added to the above solution and stirred at 2000 rpm on a magnetic stirrer for 5 minutes at room temperature. This suspension was then homogenized using a T-25 digital Ultra-Turrax at 16,000 rpm for 5 minutes.

**Table 1: Composition of natamycin nanosuspension (NT-NS) with single surfactant**

<b>Ingredients</b>	<b>NT-Tylo</b>	<b>NT-HPMC</b>	<b>NT-Leci</b>
<b>Lecithin</b>	-	-	0.1%
<b>HPMC 4KM</b>	-	0.1%	-
<b>Tyloxapol</b>	0.1%	-	-
<b>Natamycin</b>	0.3%	0.3%	0.3%

**Table 2: Composition of natamycin nanosuspension (NT-NS)**

<b>Ingredients</b>	<b>LP1</b>	<b>LP2</b>	<b>TB5</b>	<b>TwB</b>	<b>TP1</b>	<b>TP2</b>	<b>TP3</b>	<b>TP4</b>	<b>F1</b>
<b>Natamycin</b>	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%	1%
<b>Lecithin</b>	0.5%	-	0.1%	-	-	-	-	-	-
<b>Polysorbate 80</b>	-	0.1%	-	0.1%	0.1%	0.5%	0.75%	1%	0.5%
<b>Poloxamer 188</b>	0.5%	0.5%	0.1%	0.2%	0.5%	0.5%	0.5%	0.75%	-
<b>Tyloxapol</b>	0.1%	0.1%	0.1%	0.1%	-	0.1%	0.1%	0.1%	0.3%
<b>PVP K 30</b>	-	-	-	-	-	-	-	-	1.5%
<b>Water</b>	Qs 10	Qs 10							

The natamycin nanosuspension *in-situ* gels (NT-NS-GG) were prepared using the NT-NS optimized formulation and with the addition of gellan gum to sufficient quantity of water and stirred continuously at 30°C till a clear solution was formed. The gellan gum was then added to the NT-NS with constant stirring at room temperature.

### **Characterization of NT-NS**

#### **Measurement of particle size, polydispersity index (PDI) and zeta potential (ZP)**

The particle size and PDI of the NT-NS formulation was determined by photon correlation spectroscopy at 25°C and with 173° backscatter detection, in disposable folded capillary clear cells. The samples for analysis were prepared as follows: the formulation was

vortexed for a couple of seconds, 10  $\mu\text{L}$  of the formulation was diluted to 1000  $\mu\text{L}$  using Milli-Q water filtered through a 0.2 $\mu$  syringe filter before addition. This sample was used to measure particle size and PDI.

Zeta potential (ZP) measurements were also carried out at 25°C using the laser Doppler velocimetry function of the same instrument.

### **Quantification of natamycin - HPLC conditions**

The natamycin content in the formulations were determined by the High-Performance Liquid Chromatography-Ultraviolet (HPLC-UV) technique. The mobile phase consisted of a mixture of phosphate buffer (0.2 M, pH 5.5) and ACN (acetonitrile) in the ratio of 70:30. The flow rate was maintained at 1 mL/min. A C<sub>18</sub> Phenomenex Luna® (5 $\mu$ , 250 x 4.6 mm) column was used. The temperature for the analyses was 25°C, 20 $\mu\text{l}$  of the sample was injected into the column and the UV detection wavelength was set to 304 nm at AUFS 1.00 [14]. The HPLC system consisted of a Waters 717 plus auto-sampler coupled with a Waters 2487 Dual  $\lambda$  Absorbance UV detector, a Waters 600 controller pump, and an Agilent 3395 Integrator.

### **Drug content of NT-NS and NT-NS-GG**

Briefly, the assay samples were prepared by diluting 100 $\mu\text{l}$  of the formulation to 10ml using methanol in calibrated volumetric flasks. Methanol was used since NT is soluble in methanol. The volumetric flask was then sonicated using a bath sonicator for 15 minutes. 1ml of this solution was then centrifuged at 13,000 rpm for 15 minutes (AccuSpin 17 R centrifuge, Fisher Scientific, Hampton, NH) and analyzed using the HPLC-UV method mentioned above.

### **Differential Scanning Calorimetry (DSC)**

DSC of pure natamycin and NT-NS was obtained using TA instruments DSC 25 Discovery series, New Castle, DE) equipped with Trios manager software. The NT-NS was

filtered using a vacuum filter apparatus with a 0.2 $\mu$ m filter (Isopore™ Membrane Filters, Merck Millipore Ltd.). The deposit on the filter was allowed to dry overnight and then gently scrapped off of the filter. Approximately 5mg of solid sample was sealed in the T0 aluminum pans and loaded into the DSC. The samples were scanned from 40 to 200°C at a heating rate of 10°C/min

### **Terminal steam sterilization**

Natacyn® and the optimized formulation were subjected to terminal steam sterilization. 2 ml of Natacyn® and NT-NS were taken in glass vials and placed in the autoclave (Tuttnauer Autoclave-Steam Sterilizer 3850 EL). A sterilization cycle of 121°C for 15 minutes at 15 psi [21] was used. The formulations were examined for their physical appearances and compared to the formulations prior to sterilization.

### **Characterization of NT-NS *in situ* gel (NT-NS-GG)**

#### ***In vitro* gelling behavior**

The *in vitro* gelling capacity of NT-NS-GG was determined by the gel formation time (GFT) and the gel residence time (GRT). These two parameters were visually inspected [15]. Briefly, 50  $\mu$ L of the formulation was added to 2 mL of freshly prepared simulated tear fluid (STF); pH  $7 \pm 0.2$  at 34°C. The composition of the STF was as follows: 0.678% sodium chloride, 0.0084% calcium chloride, 0.138% potassium chloride, and 0.218% sodium bicarbonate in deionized water [22]. This vial was maintained at 34°C in a shaking water bath (Precision™, Fisher Scientific) at 1000rpm for 12 hours. The time taken for the formulation to gel was noted down as the GFT. The vial was visually inspected periodically and the time required to break the gel formed recorded as the GRT.

#### **Rheological studies:**

The viscosity of NT-NS-GG was measured using the Brookfield cone and plate viscometer (Model: LVDV-II + Pro Viscometer, Middleboro, MA). It was calibrated before measuring the viscosity of the *in-situ* gel. About 500  $\mu$ L of the *in-situ* gel formulation was added to the center of the spindle, and the gap was adjusted accordingly. The spindle used was 52. The cup was maintained at 25°C. A CPE-52 Cone/Spindle was operated at 1, 2 and 5 rpm and the viscosity recorded using the Rheocalc software. The *in-situ* gel was evaluated first by itself and then with the addition of STF, wherein the formulation and STF were added in the ratio of 50:7.

#### ***In vitro* release studies:**

*In vitro* release studies were carried out using a multi-station hot plate magnetic stirrer (RT 10 power, IKA® WERKE, Wilmington, NC). Briefly, 20 mL of isotonic phosphate buffer saline (IPBS) (pH 7.4), containing 2.5% randomly methylated  $\beta$ -cyclodextrin (RM $\beta$ CD), was taken in scintillation vials. The lower end of the 10K membrane (Slide-A-Lyzer™ MINI dialysis), was submerged into the receiving medium (IPBS+ RM $\beta$ CD). Once the setup reached a temperature of  $34 \pm 0.2^\circ\text{C}$ , 200 $\mu$ l of the formulation was placed onto the membrane. The setup was maintained at  $34 \pm 0.2^\circ\text{C}$ , with constant stirring for the entirety of the study. The control consisted of a coarse suspension of NT (3 mg/mL). Aliquots were withdrawn from the vials at predetermined time intervals and replaced with equal volume of IPBS with 2.5% RM $\beta$ CD. NT-NS-GG was added, along with STF, into the membrane in the ratio of 50:7 [17]. The amount of drug released was quantified using the HPLC method mentioned previously. The study was carried out for 24 hours.

#### **Transcorneal studies:**

Transcorneal studies were carried out using corneas separated from whole rabbits' eye, obtained from Pel-Freez Biologicals® (Inc Rogers, AR, USA) shipped overnight in Hanks balanced salt

solution over ice. The eyes were used immediately upon arrival [22]. The cornea was excised and washed with Dulbecco's phosphate buffer saline (DPBS) (pH 7.4) prior to being mounted on vertical Valia-Chien cells (PermeGear, Inc®). The cornea was placed with the endothelial side facing the receiver chamber while the epithelial side faced the donor compartment. 200µl each of NT-NS and NT-NS-GG were added to the individual donor chambers. DPBS (5ml) (pH 7.4) with 2.5% w/v of RMβCD medium was filled into each of the receiving chambers maintained at  $34 \pm 0.2^\circ\text{C}$  for the entirety of the study. Aliquots of 800 µL were withdrawn from the receiving chambers at predetermined time points and replenished by adding an equal volume of fresh medium. The study was carried out for a total time period of 3 hours.

**Data analysis of Transcorneal studies.**

The transcorneal permeation across the rabbit cornea were calculated as follows [15]. The cumulative amount of drug permeated M was calculated:

$$M_n = V_r C_{r(n)} + \sum_{x=1}^{x=n} V_{s(x-1)} C_{r(x-1)} \quad (2)$$

Where n indicates sampling point (n= 1,2,3...8 which corresponds to 15, 30, 45, 180 minutes respectively)  $V_r$  and  $V_s$  represent volume of the medium in the receiver chamber (mL) and volume of the sample collected at the time point n (ml).  $C_{r(n)}$  indicates the concentration of the drug in the receiver medium at the time point n (µg/ml). The rate of NT permeation across the rabbit cornea was determined from the slope of cumulative amount of NT permeated vs. time (t).

The flux was calculated as per equation 3:

$$\text{Flux } (J) = (dM/dt)/A \quad (3)$$

Where, M represent the cumulative amount of drug transported and A designates the surface area of the cornea utilized in the experiment ( $0.636 \text{ cm}^2$ )

The transcorneal permeability of NT is calculated from the ratio of steady-state flux ( $J$ ) and the amount of drug added to the donor chamber ( $C_d$ ) as follows

$$P_{app} = \frac{\text{Flux } (J)}{C_d \times 60} \quad (4)$$

### **Stability studies**

The optimized formulations were stored at 4°C, 25°C, and at 40°C. Samples were withdrawn and analyzed according to predetermined time points and changes in particle size, PDI and assay % were evaluated.

### **Statistical analysis:**

Results were presented in their mean values  $\pm$  standard deviation.

## **CHAPTER III**

### **RESULTS AND DISCUSSION**

#### **Quantification of natamycin**

NT was quantified using standard calibration plots ranging from 0.5-50 µg/mL. The standard curve was linear for the above range of concentration. The limit of detection and quantification were 0.05 and 0.1 µg/mL. The standard curves in all the assays, *in vitro* drug release and transcorneal permeation studies were linear with a coefficient of determination ( $r^2$ )  $\geq$  0.99.

#### **NT-NS formulation development and characterization**

Natamycin loaded nanosuspensions were prepared by homogenization method. The selected placebos were loaded with NT and analyzed for particle size. An almost 2-fold increase was observed in the particle size after probe sonication. Hence, probe sonication process was not used for further development of NT-NS.

The particle size of the formulation was measured prior to stirring at 2000 rpm with a magnetic stirrer and post homogenization using T-25 digital Ultra-Turrax for 5 minutes at 16,000 rpm. Table 3 summarizes the physico-chemical properties of the NT loaded nanosuspensions formulated after Ultra-Turrax processing.

**Table 3: Physio-chemical characteristics-Particle size, PDI, ZP and assay of natamycin nanosuspension (NT-NS) (mean  $\pm$  SD, n=3)**

<b>Formulation</b>	<b>Particle Size (nm)</b>	<b>PDI</b>	<b>ZP (mV)</b>
<b>NT-Tylo</b>	1237 $\pm$ 130.9	0.75 $\pm$ 0.03	-15
<b>NT-HPMC</b>	2585 $\pm$ 725.2	1	-10.3
<b>NT-Leci</b>	1046 $\pm$ 741.7	0.80 $\pm$ 0.169	-35.5
<b>LP1</b>	586 $\pm$ 61.3	0.44 $\pm$ 0.4	-38.9
<b>LP2</b>	708 $\pm$ 181.5	0.68 $\pm$ 0.0	-44.2
<b>TB5</b>	929 $\pm$ 84.1	0.63 $\pm$ 0.0	-45.6
<b>TwB</b>	3923 $\pm$ 1990	0.77 $\pm$ 0.1	-44.6
<b>TP1</b>	3834 $\pm$ 2157.9	1 $\pm$ 0	-37.3
<b>TP2</b>	1283 $\pm$ 200.0	0.85 $\pm$ 0.1	-33.8
<b>TP3</b>	2846 $\pm$ 241.1	1 $\pm$ 0	-40.2
<b>TP4</b>	790 $\pm$ 59.8	0.71 $\pm$ 0.1	-30.8
<b>F1</b>	859 $\pm$ 77.7	0.77 $\pm$ 0.0	-39.9

An increase in the percentage of NT caused an increase in the particle size of the formulation. Formulations containing single surfactants showed a high particle size (refer Table 3), hence it was hypothesized that a surfactant combination would be more successful in reducing the particle size. Formulations containing lecithin with poloxamer 188 and polysorbate 80 with poloxamer 188 were formulated keeping the drug load (0.3%) and tyloxapol (0.1%) content constant while varying the concentration of the surfactant. Lecithin with poloxamer 188 was found to be a better combination compared to polysorbate 80 with poloxamer 188 as it was more

effective at reducing the particle size. The results suggested that a higher level of surfactants was needed to prevent the suspended drug particles from agglomerating. Hence, the drug load of NT was kept at 0.3% during the preparation of NT-NS [22].

Probe sonication resulted in an increase in the particle size of the formulations. The probe sonicator uses high energy causing the reduction in particle size, however in case of nanosuspensions, the high energy produced resulted in the suspended nanoparticles getting charged and attracted towards each other, facilitating agglomeration.

A smaller particle size and a narrower PDI is desirable for the formulation of NS as larger particles may cause irritation in the eye upon instillation while a narrow PDI is indicative of possible long term stability owing to the decrease in Ostwald ripening [23].

Zeta potential indicates the surface charge of the particle which could be used to predict the degree of repulsion present between the suspended particles [23].

From the twelve formulations prepared, four were selected on the basis of their particle size and are listed in Table 4. The formulations selected were LP1, TB5, TP4 and LP2.

**Table 4: List of formulations selected**

<b>Parameter</b>	<b>TP4</b>	<b>TB5</b>	<b>LP2</b>	<b>LP1 *</b>
<b>Particle size (nm)</b>	790 ± 59.8	929 ± 84.1	708 ± 181.5	586 ± 61.3
<b>PDI</b>	0.71 ± 0.1	0.63 ± 0.0	0.68 ± 0.0	0.44 ± 0.4
<b>ZP (mV)</b>	-30.8	-45.6	-44.2	-38.97
<b>Assay (%)</b>	-	-	-	107 ± 0.6

*\*LP1 is the formulation selected for future studies.*

The selected formulations were stored at 4°C and at 25°C. They were evaluated for particle size, PDI, zeta potential at periodic intervals for 30 days (n=3). Room temperature samples of TB5, LP2, and TP4 showed signs of deposits within 30 days of storage. The formulation LP1 was chosen for further evaluation as it did not show any signs of deposits and possessed the lowest particle size amongst the NT loaded formulations.

### Stability studies

The particle size, PDI, ZP and assay of the NS was evaluated at regular time intervals for about 30 days as shown in Table 5. After 30 days of storage the formulation kept at 25°C showed an increase in the particle size, PDI and assay, and a decrease in the Zeta Potential (ZP). The particle size and ZP was reduced upon storage at 4°C while the PDI was fairly constant. Hence it could be inferred that storing the NS at 4°C was beneficial in retarding the particle size growth, which in turn kept the PDI uniformly distributed, conferring stability to the formulation.

**Table 5: Stability studies of optimized NT-NS (LP1) formulation at 4°C and 25°C (mean ± SD n=3).**

Formulation	Day 1	Day 30	
	NT-NS	25°C	4°C
Particle size (nm)	586 ± 61.3	620 ± 45.2	522 ± 40.9
PDI	0.44 ± 0.4	0.52 ± 0.1	0.45 ± 0.4
ZP (mV)	-38.97	-32.13	-31.63
Assay (%)	107 ± 0.6	118 ± 0.3	92.7±3.7

The NT-NS formulation stored at 40°C degraded after 14 days of storage, the buff colored nanosuspension turned yellow.

Gellan gum is an anionic polysaccharide polymer used as an *in situ* gelling agent as it undergoes a sol-gel transformation when in contact with the cations present in the tear fluid [15]. *In situ* gelling properties are desired to prolong the residence time in the cul-de-sac [24]. Gellan gum has been used previously in the preparation of NT bilosomes, and was found to be compatible with natamycin [15]. On addition of gellan gum in NT Nanosuspension (0.3%), a thick gel was immediately formed which suggested that the addition of gellan gum at lower concentrations would be suitable. The formulation should possess optimum viscosity for it to be easily instilled into the eye but should contain a sufficient amount of gellan gum to form a gel rapidly on contact with the tear fluid. Table 6 represents the components present in the *in situ* gel formulation.

**Table 6: Composition of natamycin *in situ* gel (NT-NS-GG)**

<b>Ingredients</b>	<b>LP1 0.1% GG (%w/v)</b>	<b>LP1 0.2% GG (%w/v)</b>	<b>LP1 0.3% GG (%w/v)</b>
Natamycin	0.3	0.3	0.3
Lecithin	0.5	0.5	0.5
Poloxamer 188	0.5	0.5	0.5
Tyloxapol	0.1	0.1	0.1
Gellan Gum	0.1	0.2	0.3

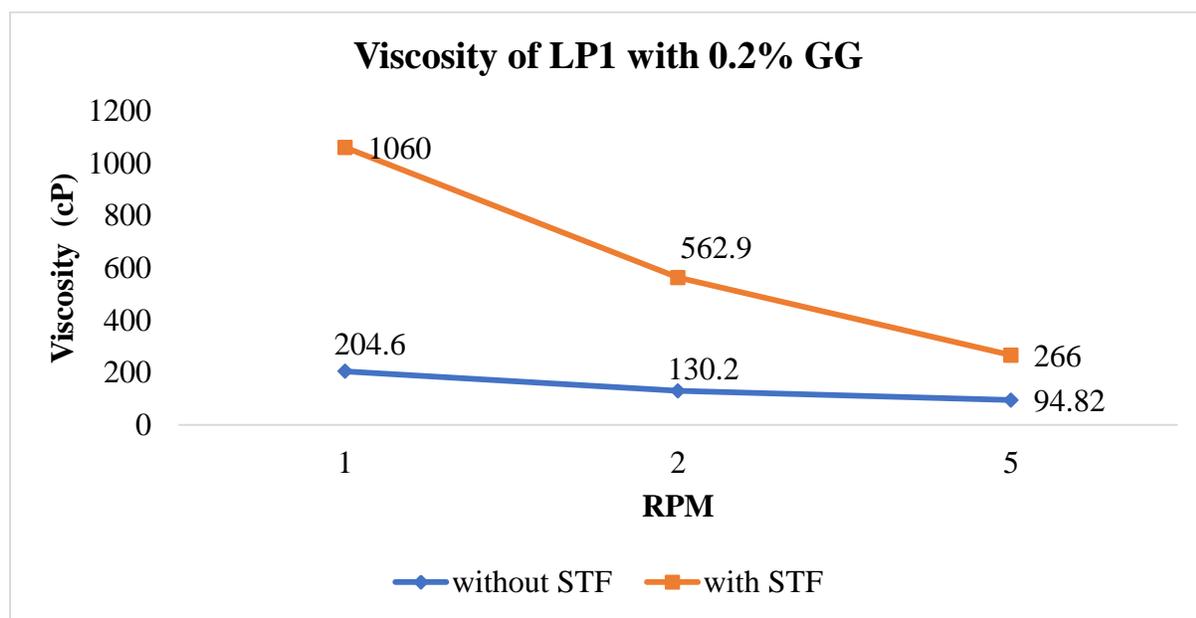
*LP1 indicates the optimized nanosuspension, and GG stands for in-situ gel prepared using gellan gum. Each formulation was prepared for 10ml.*

Gelling capacity refers to the time taken for the formation of gel and the time it takes for the gel to remain intact in the tear fluid [23]. Formulations LP1 0.1% GG and LP1 0.2% GG were evaluated for gel formation time (GFT) and gel retention time (GRT). Both the formulations gelled immediately on addition to STF at 34°C, however the gel formed by LP1 0.1% GG did

not maintain its integrity for 12 hours. LP1 0.2% GG was selected for further studies as it showed a satisfactory GFT and GRT. The outcomes of GFT, GRT and the viscosity of the selected formulation (LP1 0.2% GG) is represented in Table 7.

**Table 7: Rheological evaluation of natamycin *in situ* gel (NT-NS-GG) (n = 3)**

Formulation	Gellan gum (%)	Gel formation time (sec)	Gel retention time (h)	Viscosity (cP)	Viscosity with STF (cP)
LP1 0.1% GG	0.1%	< 5	< 12	-	-
LP1 0.2% GG	0.2%	< 5	> 12	130.2	562.9
LP1 0.3% GG	0.3%	Gelled in the vial	-	-	-



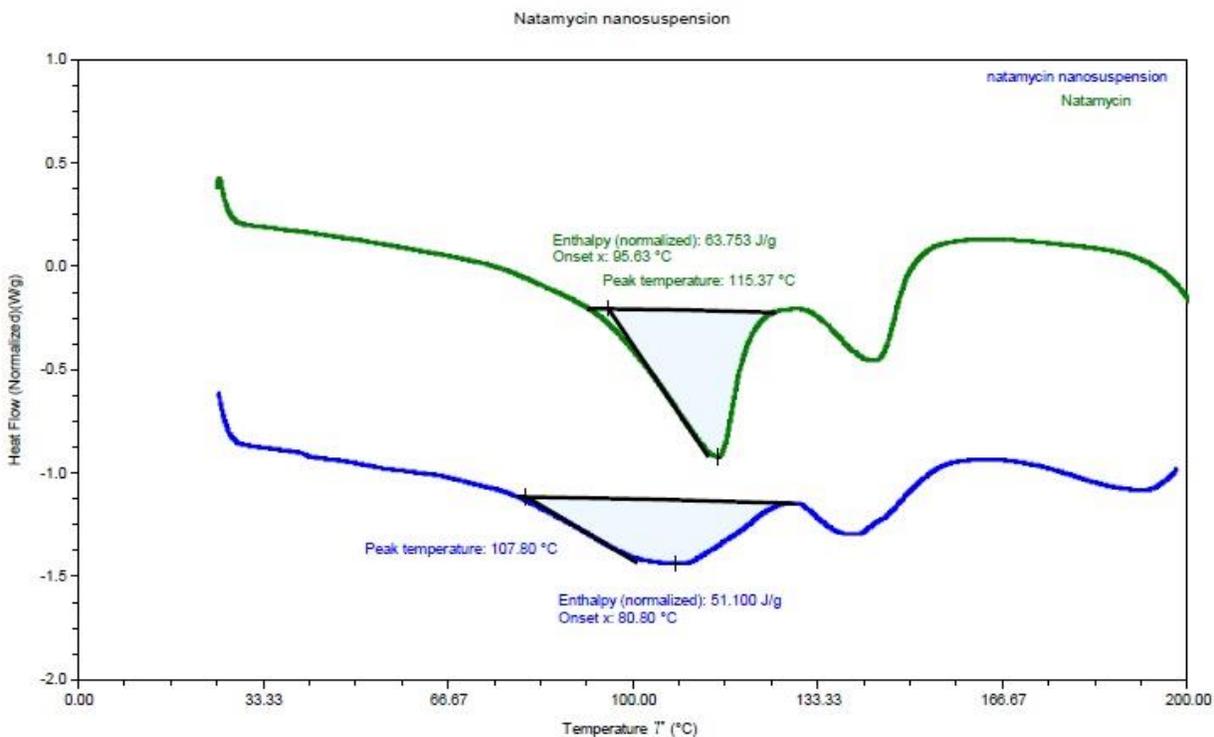
The spindle was operated at 1, 2 and 5 RPM respectively.

**Figure 1: Viscosity of optimized NT-NS-GG (LP1 0.2% GG) with and without simulated tear fluid (STF).**

Viscosity of the formulation is essential in determining the residence time of the drug into the eye [23]. Increase in the RPM (revolutions per minute) caused a linear increase in the shear rate. An increasing shear rate caused a decrease in the viscosity. Viscosity of LP1 0.2% GG was evaluated using the Brookfield Viscometer; the spindle was operated at 1, 2 and 5 RPM respectively. The addition of freshly prepared STF instantly increased the viscosity of the formulation as shown in Figure 1. The rapid increase in the viscosity of the formulation immediately on addition of freshly prepared STF is indicative of the fact that the gellan gum incorporated in the NT NS underwent a sol to gel transformation. This further confirmed the theory that the formulation was being activated in the presence of ions similar to those present in the eye.

#### **Differential scanning calorimetry (DSC)**

The sample of pure drug and LP1 was run from 25°C to 200°C with a gradual increase of 10°C/min.



TA Instruments Trios V4.1

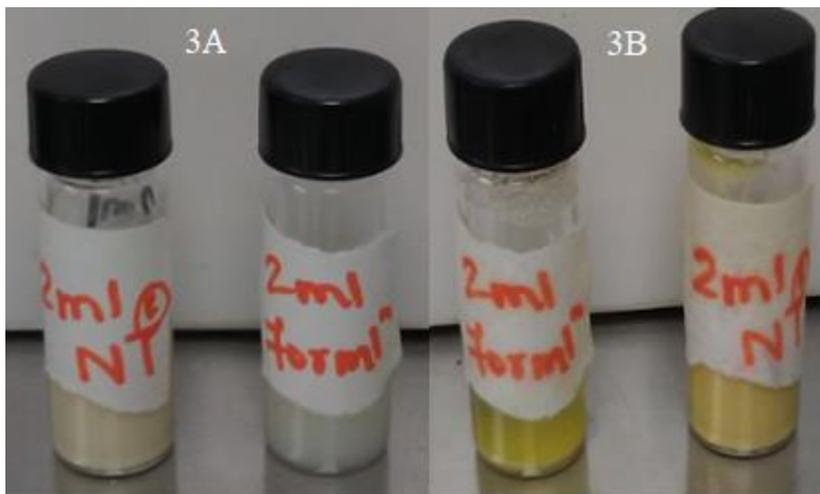
**Figure 2: DSC thermogram of pure NT and optimized NT-NS (LP1)**

The DSC thermogram of pure NT and of NT-NS is portrayed in Figure 2. The DSC study was carried out to determine any change in the NT which may have occurred during the formulation process of the NS. The DSC thermogram of pure NT showed an endothermic peak at 115°C corresponding to the melting point of pure NT. The thermogram of NT-NS showed a pattern similar to that of pure NT with the endothermic peak at around 107°C. It could be inferred from the DSC thermogram peaks that the formulation process used did not cause any changes in NT. The slight flattening of the peak in the DSC thermogram of the NS could be due to the presence of excipients in formulation or the presence of residual water molecules.

### **Terminal steam sterilization**

The NT-NS and Natamycin® showed immediate signs of degradation after autoclaving. Figure 3A and 3B depicts the NT-NS and Natamycin® prior to autoclave and post autoclave in

Figure 3. NT was proven to undergo degradation when autoclaved at 110°C for 20 minutes [25]. The buff colored formulations turned yellow-orange on autoclaving at 121°C for 15 minutes indicating signs of possible degradation. Analyzing the NS formulation and Natacyn<sup>®</sup> for assay confirmed that both samples were degraded.



*NT<sup>®</sup> and Forml<sup>n</sup> refer to Natacyn<sup>®</sup> and NT-NS respectively.*

**Figure 3: Pre autoclave (3A) and post autoclave (3B) images of NT nanosuspension and Natacyn<sup>®</sup> (marketed formulation).**

Since the formulation could not withstand the autoclave conditions, it would be recommended that the NT-NS be formulated in an aseptic environment.

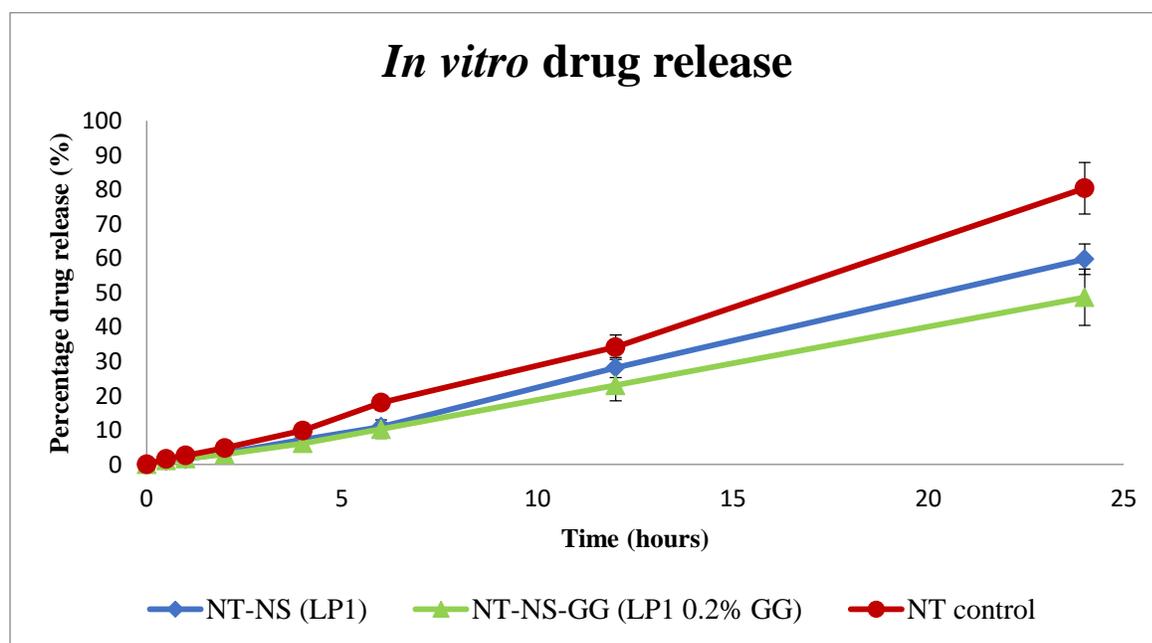
**In-vitro release study**

The study was carried out for 24 hours using 10K membrane. Table 8 shows the *in vitro* release study profile of the NS, the *in situ* gel and the control.

**Table 8: *In vitro* drug release profiles of natamycin nanosuspension (NT-NS), *in situ* gel (NT-NS-GG) and NT control (mean ± SD, n=3)**

Time (h)	<i>In vitro</i> drug release
----------	------------------------------

	NT-NS (LP1)	NT-NS-GG (LP1 0.2%GG)	Control (NT-C)
0	0	0	0
0.5	0.94 ± 0.0	1.12 ± 0.1	1.65 ± 0.0
1	1.56 ± 0.1	1.68 ± 0.3	2.59 ± 0.1
2	3.37 ± 0.3	2.94 ± 0.6	4.79 ± 0.5
4	7.21 ± 0.8	6.12 ± 1.5	9.86 ± 1.1
6	10.96 ± 1.2	10.26 ± 2.7	17.98 ± 0.4
12	28.17 ± 2.9	23.05 ± 4.5	34.12 ± 3.6
24	59.73 ± 4.4	48.65 ± 8.1	80.38 ± 7.4



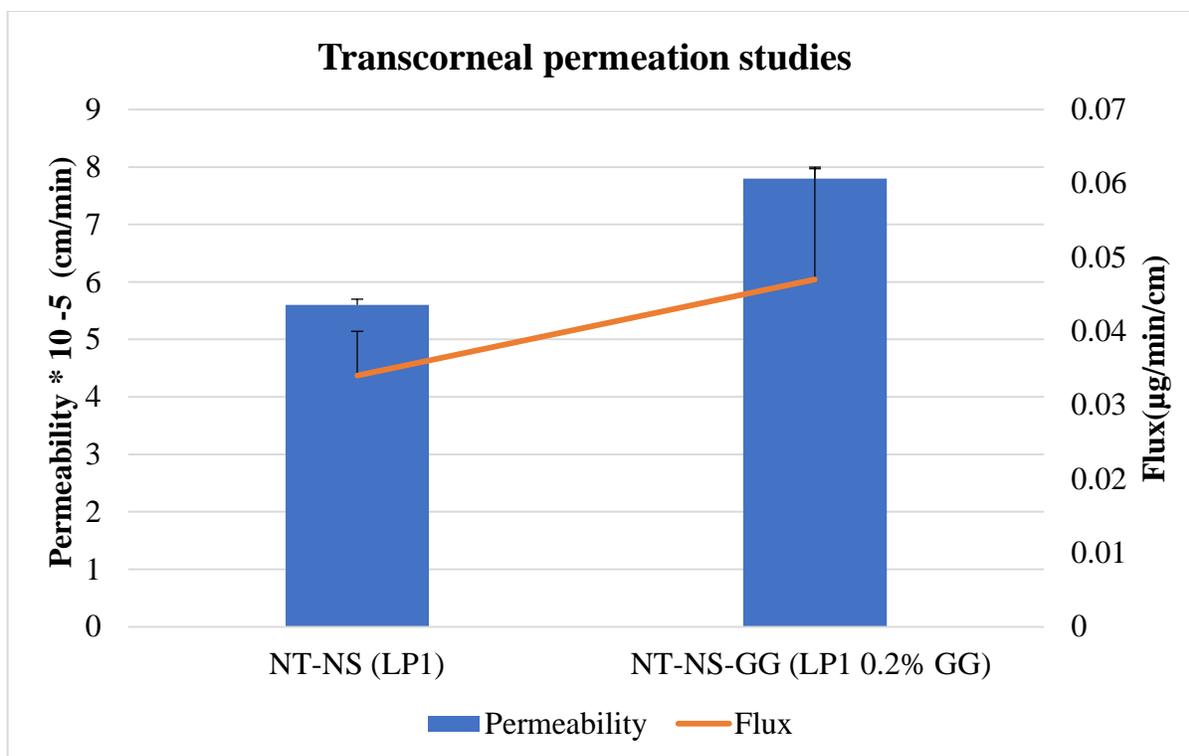
**Figure 4:** *In vitro* release study of optimized NT-NS (LP1), NT-NS-GG (LP1 0.2% GG) and NT control formulations (mean ± SD, n=3)

The drug release profile of the optimized NT-NS and its corresponding NT-NS-GG was compared to that of NT control formulation containing 0.3% NT similar to the drug load present in the optimized formulation. The drug release profile for the NT-NS (LP1) and NT-NS-GG (LP1 with 0.2% GG) is shown in Figure 4. LP1 0.2% GG showed a higher drug release (compared to LP1) in the initial hour of the study; however its release gradually diminished as the study progressed. Overall, the control formulation of NT showed the highest release ( $80.38 \pm 7.49\%$ ) followed by LP1 ( $59.73 \pm 4.45\%$ ) and lastly the LP1 0.2% GG ( $48.65 \pm 8.1\%$ ) in 24 hours. The slow release from the NT-NS *in situ* gel could be due to the transformation into gel form on addition of the STF - leading to a sustained release of drug from the gel matrix as the study progressed. This slow release could be beneficial in providing a rapid initial release followed by a sustained release of NT which could potentially lead to a reduced dosing frequency.

### Transcorneal studies

**Table 9: Transcorneal permeation studies of natamycin nanosuspension (NT-NS) and *in situ* gel (NT-NS-GG).**

	LP1 (NT-NS)	LP1 0.2% GG (NT-NS-GG)
Flux ( $\mu\text{g}/\text{min}/\text{cm}$ )	$0.034 \pm 0.0$	$0.047 \pm 0.0$
Permeability ( $\text{cm}/\text{min} \times 10^{-5}$ )	$5.6 \pm 0.1$	$7.8 \pm 0.2$



indicates statistically significant at level of  $p < 0.05$ , compared with NT-NS.

**Figure 5: *In vitro* transcorneal flux and apparent permeability coefficient of natamycin from optimized NT-NS (LP1), NT-NS-GG (LP1 0.2% GG) (mean ± SD; n=3).**

The transcorneal permeation profile is summarized in Table 10 and represented graphically in figure 5. At the end of 3 hours LP1 0.2% GG showed a higher mean permeability and flux compared to LP1. This could be due to the addition of gellan gum which may have enhanced the penetration of NT from the formulation. This was almost similar to the pattern observed in the *in vitro* release data conducted wherein LP1 0.2% GG showed a higher release rate compared to LP1 in the first hour of the study. It could be hypothesized that LP1 0.2% GG is somewhat more effective in delivering NT to the ocular tissues as compared to the NS.

## CHAPTER IV

### CONCLUSION

In this study, natamycin loaded nanosuspensions and its corresponding *in situ* gels were successfully formulated using lecithin and poloxamer 188 as the surfactants. The formulation had a better particle size with narrower PDI. The in-situ gel formulated, showed a higher permeation and flux in the transcorneal studies when compared to the natamycin nanosuspension, the *in vitro* release data showed a higher release from the nanosuspension compared to the *in situ* gel over a 24 hour time period. The *in situ* gel formulated was activated with the addition of simulated tear fluid, as was confirmed by the rheological studies.

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## VITA

### Experience

- *Internship*

June 2015--Vergo Pharma Research Laboratories Pvt. Ltd, Goa India

-Overview of the workings of a formulation development laboratory

-Standard Operating Procedures of all the departments.

-Analytical development laboratory: Exposure to working of the HPLC and interpretation of data.

Release study of tablets using dissolution apparatus, working of IR spectrophotometer.

-Calibration of pH meters, weighing balance and analysis of water quality in the manufacturing plant.

December 2012--Watson Pharma, Goa India

-A step by step demonstration of large scale manufacture of tablets by wet granulation technique.

-Working of equipment such as V cone blenders, Fluidized Bed Driers, granulators, automatic tablet compression and capsule filling machine demonstrated by the staff.

-Quality Control Department: Calibration of pH meters, working of HPLC.

-Microbiology testing laboratory

### Education

Master of Science in Pharmaceutical Science with emphasis in Pharmaceutics and Drug

Delivery-*May 2019*

The University of Mississippi, Oxford MS

Research advisor: Dr. Soumyajit Majumdar

Bachelors in Pharmaceutical Sciences- *July 2016*

PES Rajaram and Tarabai Bandekar College of Pharmacy, Goa India

### **Skills**

- Formulation development of nanoformulations (Nanostructured Lipid Carriers, Nanosuspensions and Solid Lipid Nanoparticles)
- Development of analytical method using HPLC-UV
- In vitro release study of nanosuspensions
- Transcorneal permeation studies using isolated rabbit cornea
- Measurement of viscosity using Brookfield cone and plate viscometer
- Measurement and interpretation of particle size, PDI and zeta potential using Zetasizer Nano ZS Zen3600 (Malvern Instruments)
- Proficient with Zotero software for reference and citation management