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Corneal targeted nanoparticles for sustained natamycin delivery and their PK/PD indices: An approach to reduce dose and dosing frequency

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ABSTRACT

Natamycin is the only approved medication for the treatment of mycotic keratitis. Current dosage regimen include one drop of natamycin suspension (5% w/v) instilled in the conjunctival sac at hourly or two hourly intervals for several days which has poor patient compliance. The purpose of the present study was to design a corneal targeted nanoformulation in order to reduce dose and dosing frequency of natamycin and evaluate its pharmacokinetic/pharmacodynamic indices in comparison with clinical marketed preparation. The nanoparticles prepared by nanoprecipitation method were in nanometer size range with high entrapment efficiency and positive surface charge. *In-vitro* release studies indicated prolonged release of natamycin up to 8 h. *In-vitro* antifungal activity was comparable with marketed preparation. The performance of nanoformulations was evaluated in rabbit eyes. The concentration of natamycin in tear fluid was determined by using LC–MS/MS. The pharmacokinetic parameters such as area under the curve, $t_{1/2}$ and mean residence time were significantly higher and clearance was significantly lower for nanoformulations with that of marketed preparation. The optimized dosing schedule to maintain natamycin concentration above tenfold of MIC_{90} was one instillation in every 5 h. Moreover, 1/5th dose reduction of nanoformulation was also effective.

1. Introduction

Mycotic keratitis (MK) is a corneal fungal infection characterized by decreased vision, photophobia, feathery-edged infiltrates and satellite lesions across the cornea. It can lead to blindness and loss of the affected eye if not diagnosed early and treated rapidly (Chang and Chodosh, 2011; Chowdhary and Singh, 2005; Gopinathan et al., 2002; Liu et al., 2013; Shokohi et al., 2006; Xie et al., 2006). The predisposing factors involve ocular trauma associated with vegetable matter in rural areas and contact lens use in developed countries. Other risk factors include disproportionate use of broad spectrum antibiotics, steroid drops, increased number of eye surgeries (*e.g.* penetrating keratoplasty or laser assisted in situ keratomileusis (LASIK)) that lead to compromised corneal surface (Patel and Hammersmith, 2008; Rosa et al., 1994; Yildiz et al., 2010). Across the world *Aspergillus*, *Candida* and *Fusarium* species have been known to cause MK, out of which the most commonly implicated pathogen isolated from MK is filamentous *Aspergillus* (Tanure et al., 2000). Natamycin (pimaricin), the most valuable antifungal agent till date, has been considered as the drug of choice for filamentous MK. It is the only commercially available US-FDA approved agent for the treatment of MK. Natamycin 5% w/v suspension prescribed as one drop instilled in the conjunctival sac at hourly or two hourly intervals for several days (USFDA, 2008). Current therapy with natamycin appears unsatisfactory for several reasons such as high dosing frequency, long treatment duration (4–6 weeks), short residence time at the ocular mucosa due to quick clearance by the naso-pharyngeal drainage. This prolonged dosing schedule is difficult to

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attain resulting in suboptimal concentration at the corneal site, resulted in treatment failure and increase resistance to MK (Johns and O'Day, 1988; Thomas, 2003; Xie et al., 2001).

Poor efficacy of ophthalmic medications could be due to barely penetration through ocular barriers, blinking reflex, tear turnover, binding to the proteins and non reproductive absorption through the conjunctiva. As a result, only 2% or less of a topically applied natamycin dose can be absorbed into the anterior segment of the eye (Johns and O'Day, 1988). Hence, there is a clinical need to design a corneal targeted prolonged release delivery system with widely spaced dosing schedule to improve patient compliance and efficiency of therapy. However, there were a few reports in the literature on ocular delivery of natamycin using novel approaches (Bhatta et al., 2012; Phan et al., 2014).

To surmount aforementioned problems with marketed preparation, we aimed to develop poly-D-glucosamine (PDG) functionalized polycaprolactone (PCL) nanoparticles (NPs) for prolonged natamycin delivery. PCL is a biodegradable polymer that had been reported to have controlled release property, good drug loading capacity, (Losa et al., 1993; Marchal-Heussler et al., 1992) and excellent stability (Nagarwal et al., 2010). Moreover, it is inexpensive and can be produced in standardized bulk volumes, soluble in a range of solvents, has a low melting point (~60 °C) making it favorable excipients for the delivery of natamycin. However, the major limitation of PCL NPs is low retention time and rapid clearance on the eye surface owing to their negative surface charge.

Poly-D-glucosamine (PDG), a natural cationic polysaccharide, employed as a coating material to impart mucoadhesive properties. It has been reported that PDG had a higher binding affinity to the mucosal surface as a result of interaction of positively charged amino groups with the anionic mucosal sialic acid residues in the corneal and conjunctival surfaces and therefore increases the ocular retention time (Ludwig, 2005; Paolicelli et al., 2009). Moreover, PDG is reported to have fungicidal activity (Allan and Hadwiger, 1979), penetration enhancing properties (de la Fuente et al., 2010), ability to enhance paracellular transport of drugs (Schipper et al., 1997; van der Merwe et al., 2004) and compatibility with ocular milieu (Enriquez de Salamanca et al., 2006; Felt et al., 1999; Lin et al., 2010).

Thus, in the present work, natamycin encapsulated PDG coated PCL NPs (PDG–PCL NPs) were developed for sustained ocular delivery. Positive charged NPs likely to have higher affinity to the corneal surface as compared with negative charged NPs. The applicability of these NPs as ocular delivery vehicles was investigated by comparing the pharmacokinetic (PK), pharmacodynamic (PD) parameters with marketed preparation. In addition, the ocular pharmacokinetic profiles and rational PK/PD based dosage regimen in preclinical *NZ* rabbit model was also reported.

2. Experimental

2.1. Materials

Natamycin and amphotericin B (internal standard, IS) pharmaceutical grade were received as generous gift samples from Cipla, India. PCL (mol. wt 14000), PDG (low mol.–wt 50–190 kDa, deacetylation degree 75–85%), pluronic F-68, mucin (from porcine stomach, type II), MOPS-buffer and methanol were purchased from Sigma, St. Louis, USA. Acetone was purchased from Merck specialties Pvt. Ltd India. Marketed natamycin ophthalmic suspension USP Natamet® was purchased from local pharmacy store. Dialysis membrane (mol. wt cut-off 12–14 kDa) was purchased from HiMedia Labs Pvt. Ltd Mumbai, India. Calibrated glass capillaries (microcaps) of 10 μL were obtained from Dummond Scientific Co., USA. Ultrapure water was obtained from a Milli Q PLUS PF water purification system. All other reagents were of analytical grade and purchased from standard chemical suppliers.

2.2. Methods

2.2.1. Preparation of NPs

The PCL NPs and PDG–PCL NPs were prepared by nanoprecipitation (solvent displacement) method (Bilensoy et al., 2009). In brief, 150 mg of PCL was dissolved in 10 mL of acetone with mild heating and 30 mg of natamycin was dissolved in 30 mL of methanol. Both the solutions were mixed and kept in bath sonicator for 15 min to allow complete mixing. A solution of PDG (1%, w/v) was prepared in 1% acetic acid and aliquots were added to water in order to obtain desired PDG concentration. The organic solvent mixture containing drug and polymer was added dropwise into 40 mL of water consisting of pluronic F-68 and PDG under moderate stirring followed by bath sonication for 15 min. PCL NPs were prepared by excluding PDG in the external phase. The resultant bulk suspension was centrifuged at 3000 rpm (REMI, India) for 20 min in order to separate the possible natamycin/polymer precipitated in the preparation process. Organic solvent was removed at 40 °C under vacuum using rotavapor (BUCHI, Switzerland). Blank NPs were also prepared in the same way devoid of natamycin.

2.3. Physico-chemical characterization

2.3.1. Particle size distribution and zeta potential

The NPs size distribution and zeta potential were determined ($n = 3$) using Malvern Nano-ZS (Malvern, UK) laser particle size analyzer. Prior to measurement, NPs were diluted with ultra pure water to minimize particle–particle interactions and multiple scattering. The samples were placed in a disposable polystyrene cuvette and particle size was measured at room temperature using a scattering angle of 173°. For zeta potential determination, samples were placed in a fixed-glass cell and measured at same operating conditions.

2.3.2. Fourier transform infra-red (FT-IR) spectroscopy

Drug-excipient and excipient–excipient interactions were obtained with a PerkinElmer spectrophotometer version 10.03.06. The spectrum was recorded for native natamycin, PCL, PDG, physical mixture, PCL NPs and PDG–PCL NPs. The samples were prepared as KBr discs. The scanning range was 4000–450 cm^{-1}.

2.3.3. Entrapment efficiency (EE) and drug loading (DL)

The amount of natamycin encapsulated in the PCL NPs and PDG–PCL NPs was determined by ultracentrifugation (Beckman Coulter, USA) of the bulk suspension at 25,000 rpm for 30 min and the supernatant was analyzed for free natamycin concentration using a previously described validated HPLC method (Bhatta et al., 2012). All the analysis was performed in triplicate.

The EE and DL were calculated using the following equations

$$EE\left(\%\frac{w}{w}\right) = \frac{\text{Total amount of natamycin added} - \textit{Amount of natamycin in supernatant}}{\text{Total amount of natamycin added}} \times 100$$

$$DL\left(\%\frac{w}{w}\right) = \frac{\text{Total amount of natamycin added} - \textit{Amount of natamycin in supernatant}}{\text{Total amount of formulation components}} \times 100$$

2.3.4. Scanning electron microscopy (SEM) analysis

The optimized batch of PCL NPs and PDG–PCL NPs were selected for SEM analysis to determine the surface morphology of NPs. Samples were mounted on microscope metal stubs and coated with Au–Pd (80:20) using a Polaron E5000 sputter coater. Samples were examined under a FEI Quanta 250 SEM using a SE detector at an accelerating voltage of 30 kV.

2.4. In-vitro studies

2.4.1. In-vitro release of natamycin from NPs and its kinetic evaluation

The *in-vitro* release of PCL NPs and PDG–PCL NPs were performed using dialysis bag. Freshly prepared nanoformulation (equivalent to 1 mg/mL of natamycin) was placed in a dialysis bag and suspended in a beaker containing 30 mL of simulated lachrymal fluid (SLF, pH 7.4) with tween 80 (1%, v/v). This assembly was kept in an oscillating thermostatic water bath (Julabo, Germany) maintained at 37 °C. At regular time intervals, 1 mL of natamycin releasing media was withdrawn and replaced with an equal volume of fresh SLF containing tween 80 (1%, v/v) to maintain sink conditions. The release study of Natamet® was also evaluated under the same conditions. All the samples were filtered through 0.45 μm membrane filter and analyzed for natamycin content using validated HPLC method.

In order to establish the natamycin release mechanism and express the release kinetics in quantitative terms, *in-vitro* release data of PCL NPs and PDG–PCL NPs were fitted to different mathematical models such as zero order, first order, Higuchi and Korsmeyer–Peppas models. The linear regression was performed and the best fit model was established based on the regression coefficients (r^2), where the model with highest r^2 was selected as the best fit.

The following equations were used to fit different release models.

Zero order kinetics:

$$Q_t = Q_0 + K_0 t$$

where Q_t is the amount of drug dissolved at time t, Q_0 is the initial amount of drug in the solution and K_0 is the zero order release constant.

First order kinetics:

$$\ln Q_t = \ln Q_0 + K_1 t$$

where Q_t is the amount of drug dissolved at time t, Q_0 is the initial amount of drug in the solution and K_1 is the first order release constant.

Higuchi model:

$$Q_t = K_H \sqrt{t}$$

where Q_t is the amount of drug dissolved at time t, K_H is the Higuchi dissolution constant.

Korsmeyer–Peppas model:

$$Q_t / Q_\infty = K t^n$$

where Q_t is the amount of drug dissolved at time t, Q_∞ is the total amount of drug dissolved when the pharmaceutical dosage form is exhausted. K is a constant reflecting the structural and geometric characteristics of the delivery system and n is the release exponent, indicative of the mechanism of drug release (Costa and Sousa Lobo, 2001; Siepmann and Siepmann, 2013).

2.4.2. In-vitro antifungal activity

2.4.2.1. Minimum inhibitory concentration (MIC). The MIC_{90} was determined according to the broth dilution method adopted from CLSI (CLSI, 2006). *C. albicans* (Patient isolated) and *A. fumigatus* (Patient isolated) were used to evaluate the antifungal activity. Pure natamycin, PCL NPs, PDG–PCL NPs and Natamet® were tested at concentrations starting from 50 mg/L in 96-well plates by serial dilutions. Cell suspensions of *C. albicans and A. fumigatus* were prepared in the RPMI 1640 medium and adjusted to give a final inoculums concentration of $1.0 \times 10^3 - 5.0 \times 10^3$ and $0.4 \times 10^4 - 5.0 \times 10^4$ CFU/mL respectively. The microplates were incubated at 35 °C for 24 h (*C. albicans*) or 72 h (*A. fumigatus*). In order to minimize the errors in the experiment solvent control, organism control and medium control were also performed simultaneously. Blank NPs were used as control. MICs of the test solution were determined by visual assessment of fungal growth inhibition.

2.4.2.2. Zone of inhibition. An alternative method to assess the antifungal activity is zone of inhibition. The assay was performed in the sterilized petri plates containing sabouraud dextrose agar (20 mL) seeded with the test microorganism (0.2 mL). The disks were prepared by spiking appropriate volumes of PDG–PCL NPs and PCL NPs onto sterile blank disks (HiMedia Labs.) to make 0.005 and 0.010 mg of natamycin per disk. Along with NPs formulation, natamycin dissolved in DMSO and Natamet® were also checked for susceptibility toward *C. albicans* and *A. fumigatus*. These disks were dried in oven at 35 °C for 5 min. The dried disks were placed on solidified agar layer with the help of sterile forceps and kept at 4 °C in refrigerator for 15 min. The plates were incubated at 35 °C for 24 h (*C. albicans*) or 72 h (*A. fumigatus*). The diameter of zone of inhibition was observed using a zone finder. All the experiments were performed in triplicate (Bhatta et al., 2012).

2.4.3. Assessment of the mucoadhesive property

Mucoadhesive properties of PCL NPs and PDG–PCL NPs were evaluated by using two different techniques. The first method was based on the measurement of turbidity of the mucin dispersion after incubation with NPs. A solution of type-II porcine mucin (0.1%, w/v) was freshly prepared and mixed with equal volume of NPs suspension under moderate stirring. This mixture was incubated in an agitating water bath at 37 °C. The turbidity of the mixture at certain time intervals was measured at 650 nm (Thermo Scientific UV–visible spectrophotometer) and compared with turbidity of native mucin dispersion. The second method was based on changes in zeta potential of the NPs after incubation with mucin dispersion. The behavior of NPs was also examined in a mucin free aqueous dispersion under the same conditions.

2.4.4. Stability in different storage conditions

PCL NPs and PDG–PCL NPs were kept at 6 ± 2 °C and 25 ± 4 °C for a period of 180 days in amber-coloured glass vials to avoid photodegradation. Samples were withdrawn at different time intervals for particle size, zeta potential and EE measurements.

2.5. In-vivo studies

All the animal experiments were conducted in accordance with current legislation on animal experiments as per Institutional

Table 1
Effect of various natamycin-polymer ratios on particle size, zeta potential and entrapment efficiency of PCL NPs and PDG–PCL NPs (Mean ± SD, $n = 3$).

Formulation	Natamycin to polymer ratio	PDG (% w/v)	Particle size (d.nm)	Poly dispersity index	Zeta potential (mV)	EE (%)
PCL NPs	1:5	0	178.8 ± 1.70	0.204 ± 0.01	−21.3 ± 1.23	61.83 ± 0.07
PDG–PCL NPs	1:5	0.025	217.0 ± 2.00	0.210 ± 0.01	+43.5 ± 1.55	77.85 ± 1.08
PDG–PCL NPs	1:10	0.025	247.2 ± 1.16	0.234 ± 0.02	+42.8 ± 2.06	50.75 ± 1.66

Animal Ethics Committee at CSIR-Central Drug Research Institute (IAEC approval no IAEC/2013/71). Male *New Zealand* rabbits weigh between 2.0–2.5 kg were used in these studies. All rabbits were healthy and free of clinically observable signs of ocular abnormalities. Based on *in-vitro* studies, PDG–PCL NPs (both NPs 1% and NPs 5% dose strength) were selected for *in-vivo* PK studies.

2.5.1. Ocular tolerance

Ocular irritancy of the NPs was carried out according to modified Draize test (Huhtala et al., 2014). Animals were housed in an individual cage under standard conditions with *ad libitum* access to food and water. The test protocol was carried out on six rabbits, which involved a single unilateral application of 20 μL of the PDG–PCL NPs in lower conjunctival sac of the eye. The contra lateral eye was used as a control and received no treatment. The ocular tissue conditions were observed at 1, 12, 24, 36, 48, 60 and 72 h post dose. Redness, swelling and discharges of the conjunctiva were graded on a scale from 0 to 3, 0 to 4 and 0 to 3, respectively. Iris hyperaemia and corneal opacity were graded on a scale from 0 to 2 and 0 to 4, respectively. According to the Draize test, the ocular irritation scores for each rabbit were calculated by adding the irritation scores for the cornea, iris and conjunctiva. The eye irritation score was obtained by dividing the total score for all rabbits by the number of rabbits. Eye irritation was classified according to four grades: practically non-irritating, score 0–3; slightly irritating, score 4–8; moderately irritating, score 9–12; and severely irritating (or corrosive), score 13–16 (Wilhelmus, 2001).

2.5.2. Ocular pharmacokinetics

The precorneal retention profile of NPs (1% and 5% w/v containing natamycin) was determined and compared with Natamet®. Briefly, the rabbits were given an instillation of 20 μL of samples into the lower conjunctival sac of the left eye. After instillation, the eyelids were kept closed for 5 s to prevent the loss of the instilled suspension. Tear samples (10 μL) were collected by using calibrated glass capillary at 5, 15, 30, 60, 120, 180, 240 and 300 min post dose and stored in micro-centrifuge tubes at −80 °C until analysis. The amount of natamycin in the tear samples was analyzed by using in-house developed and validated LC–MS/MS method (Bhatta et al., 2011).

2.6. PK/PD indices and design of dosage regimen

PK/PD indices have been recognized as key factor in selecting appropriate dosage regimen and access the *in-vivo* efficacy of antimicrobial drugs exhibiting concentration dependent killing such as

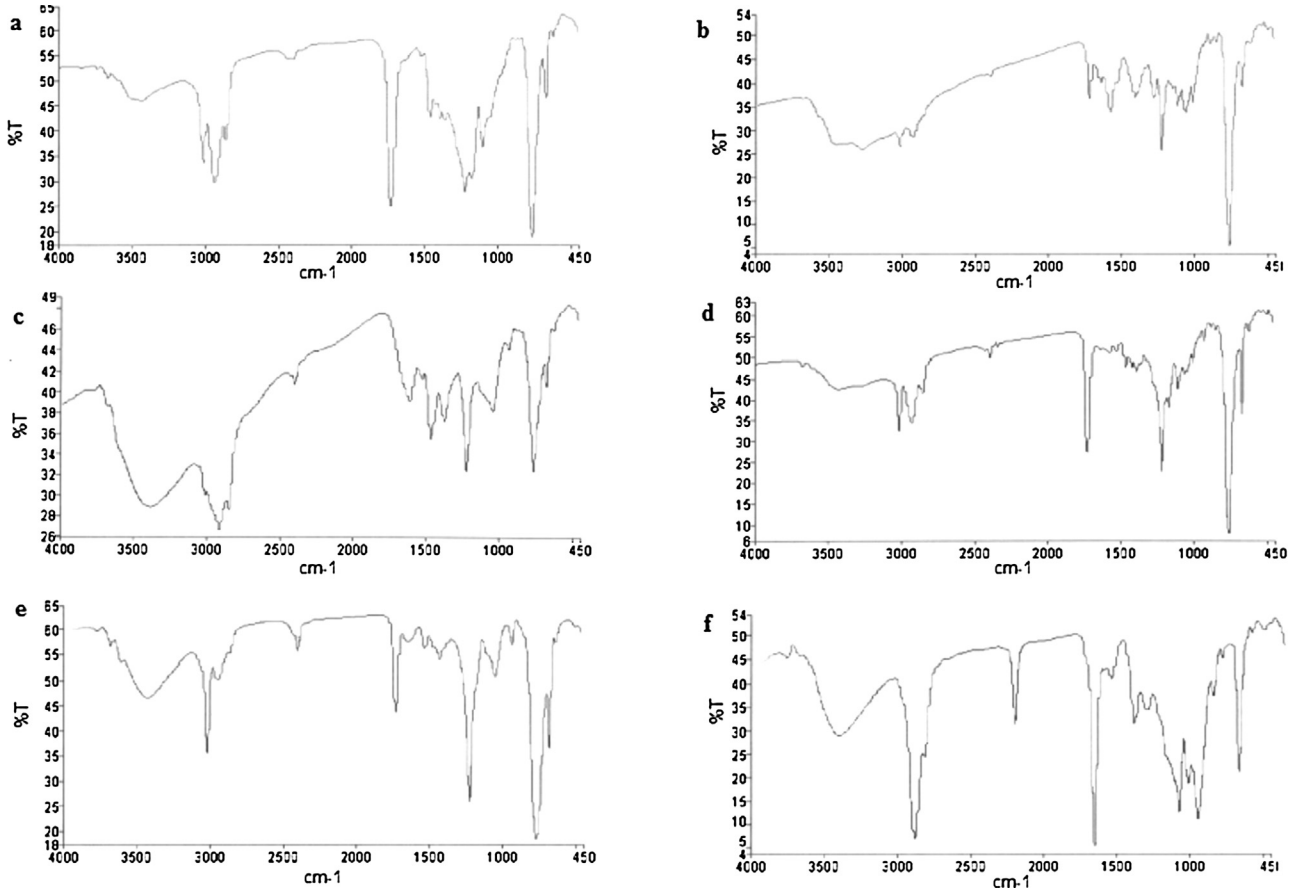

Fig. 1. FT-IR spectra of a) PCL, b) natamycin, c) PDG, d) physical mixture, e) PCL NPs and f) PDG–PCL NPs.

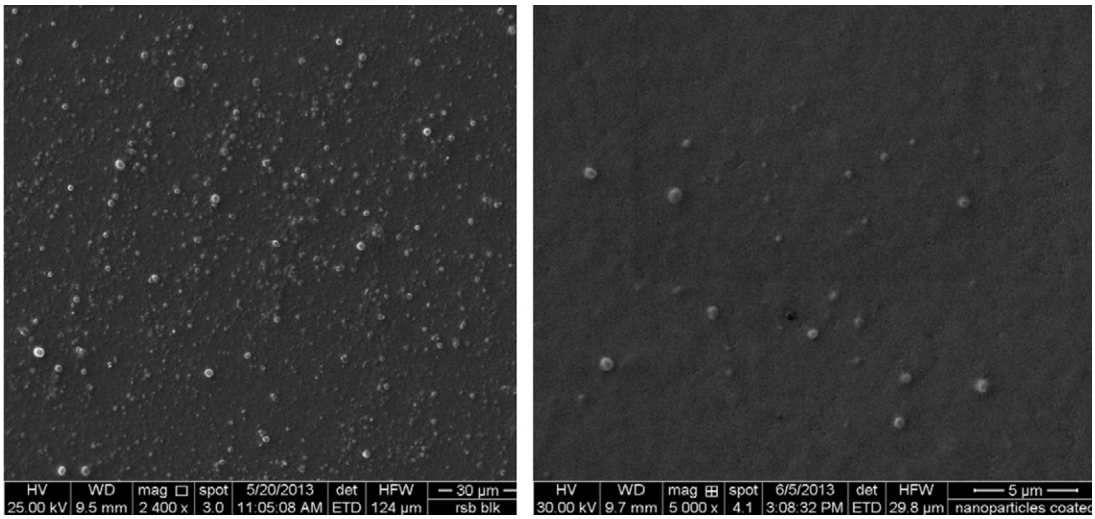

Fig. 2. Visualization of a) PCL NPs and b) PDG–PCL NPs by SEM.

natamycin (Liu et al., 2002; Schentag et al., 2001). Although the prime parameter MIC_{90} is used as a measure of the potency of an anti-microbial agent, its value cannot be relied upon for predicting *in-vivo* efficacy (Liu et al., 2002). Therefore, its relation to other PK parameters such as peak concentration (C_{max}) and AUC is the key to effectiveness. Based on the C_{max} and $AUC_{(0-t)}$ values obtained from the PDG–PCL NPs and Natamet® in tears following a single topical ocular administration to rabbits, the resulting PK/PD indices C/MIC_{90}, $AUC_{(0-t)}/MIC$ ratios, AUC above MIC and *T* above MIC were calculated using MIC_{90} values.

2.7. Statistical data analysis and dose simulation

Statistical data analysis was performed using the student *t*-test with $p < 0.05$ as the minimal level of significance. Ocular PK parameters were derived from tear concentration-time profile using Phoenix WinNonlin Version 6.3 (Pharsight Corporation, Mountain view, USA). Simulation at different dosing interval was evaluated by principle of superimposition using Microsoft excel software. The natamycin concentration at several time intervals was estimated graphically from tear concentration-time profile.

3. Results

3.1. Physico-chemical characterization

Table 1 illustrates the variations in particle size, zeta potential and EE of the NPs depending on the amounts of natamycin, PCL and PDG used. Among the prepared formulations, the ideal combination for ocular application was observed in case of PDG–PCL NPs containing natamycin to polymer weight ratio of 1:5 with respect to particle size and zeta potential. In case of PDG–PCL NPs, increase in particle size with positive zeta potential is attributed to the presence of PDG coat on PCL NPs surface. This observation is of great importance, since the magnitude of surface charge is a key factor in the stability of colloidal dispersions (Greenwood and Kendall, 1999).

3.2. FT-IR spectroscopy

The interaction between drug and excipients was evidenced by FT-IR spectra (Fig. 1). PCL exhibited carbonyl stretching (C=O) at 1727.13 cm^{-1}. The weak IR absorption band at 3442.68 cm^{-1} was due to hydroxyl groups of PCL. In the spectrum of natamycin, carbonyl stretching (C=O) was observed at 1714.71 cm^{-1} and the absorption peak at 3276.34 cm^{-1} assigned to NH_3^+ deformation. Peak at 1269.59 cm^{-1} is attributed to C—O—C (epoxy) stretching absorption. The spectrum of PDG showed absorption peak at 3395.07 cm^{-1} characteristic of the amino groups present in the structure. However, the intensity of the characteristic peak of PDG due to amino groups has been decreased in the spectrum of PDG–PCL NPs. This might be due to the electrostatic interaction of amino groups of PDG with the hydroxyl groups of PCL. Moreover, the IR spectrum of physical mixture and PDG–PCL NPS showed minor differences in the positions of the absorption bands of natamycin indicating the compatibility between natamycin and formulation excipients.

3.3. Surface morphology

SEM images of PCL NPs and PDG–PCL NPs were illustrated in Fig. 2. The PDG–PCL NPs exhibited a discrete spherical morphology suggesting possible stabilization of the formulation by virtue of the positive surface charge.

3.4. In-vitro studies

3.4.1. In-vitro natamycin release and kinetics

The natamycin release patterns of Natamet®, PCL NPs and PDG–PCL NPs were obtained by plotting the cumulative percentage of natamycin release against time. As depicted in the Fig. 3, almost 100% of natamycin was released within 2 h from conventional

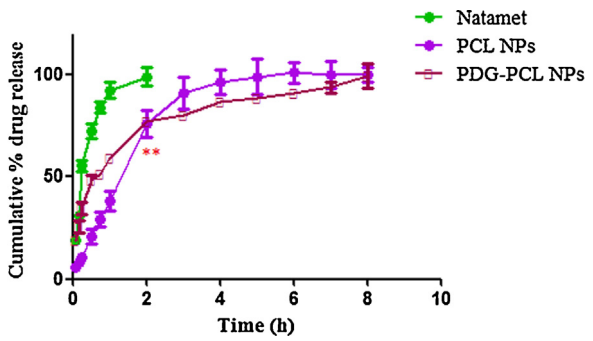

Fig. 3. *In-vitro* release profiles of the NPs formulation and Natamet® in SLF, pH 7.4. ** Significant difference between NPs and Natamet® at 2 h.

Table 2
In-vitro release kinetic parameters of natamycin loaded PCL NPs and PDG–PCL NPs.

Formulation	Zero order (r^2 value)	First order (r^2 value)	Higuchi (r^2 value)	Korsmeyer–Peppas		Best fit model
				r^2 value	n value	
PCL NPs	0.71	0.87	0.94	0.97	0.69	Korsmeyer– Peppas
PDG–PCL NPs	0.29	0.87	0.92	0.96	0.34	Korsmeyer– Peppas

Where r^2 is the regression coefficient and 'n' is the release exponent indicative of natamycin release mechanism. According to KP model, based on 'n' value, drug release from PCL NPs follow non-fickian diffusion (anomalous) whereas drug diffusion may occur partially by means of swollen matrix and water filled pores in the formulation in case of PDG–PCL NPs.

Natamet® and it was prolonged to 8 h in case of PCL NPs and PDG–PCL NPs. The release of natamycin from PCL NPs and PDG–PCL NPs was fitted best to Korsmeyer–Peppas (KP) model (Table 2).

3.4.2. In-vitro antifungal activity

The MIC_{90} values of NPs were compared with that of pure natamycin in DMSO as a reference sample. The MIC_{90} and zone of inhibition values were represented in Table 3. The results showed that the MICs of the PDG–PCL NPs were identical to that of the pure natamycin. The inhibition zone diameter using disk diffusion method was higher than that of the Natamet®, indicating the fungicidal action of the developed NPs. From the MIC values it is obvious that *Candida* was two times less susceptible to natamycin as compared with *Aspergillus* which was in consistent with literature (Johns and O'Day, 1988).

3.4.3. Assessment of mucoadhesive property

As depicted in the Fig. 4, the turbidity of the mucin and PDG–PCL NPs dispersion was increased over the time and found to be higher than native mucin. This increase in turbidity reflects that PDG–PCL NPs could interact with the negatively charged mucin particles. In addition, the surface charge of the PDG–PCL NPs decreased significantly after incubation with mucin indicating the mucoadhesive capability of PDG–PCL NPs (Fig. 5).

3.4.4. Stability in different storage conditions

Stability results (Table 4) showed that PDG–PCL NPs exhibited preferable colloidal stability with no apparent aggregation or precipitation of particles. At 25 ± 4 °C, after 180 days, the particle size slightly increased and EE reduced to 74.09%. On the other hand, at 6 ± 2 °C, improved stability was observed. The increase in particle size was lower than that of 25 ± 4 °C and there were no considerable changes in zeta potential or EE. The PDG–PCL NPs were characterized by optimum natamycin retention properties, as natamycin leakage in six months was between 1% and 2% of the natamycin entrapped.

3.5. In-vivo studies

3.5.1. Ocular tolerance

A promising delivery system indented to use in the eye must not damage the ocular surface. The conjunctival discharge, swelling, iris hyperaemia and corneal opacity were scored to be zero in all observations indicating no signs of irritation or injurious effect to the eye. Therefore, the PDG–PCL NPs had a potential impact on the clinical aspects as a prolonged natamycin delivery vehicle.

3.5.2. Ocular pharmacokinetics and PK/PD indices

Ocular pharmacokinetics of NPs (1% and 5% w/v) were compared with Natamet® (Fig 6 and Table 5). The PDG–PCL NPs exhibited significant enhancement in $AUC_{(0-\infty)}$, MRT and decrease in clearance as compared with Natamet® indicating the prolonged exposure of natamycin at precorneal region by PDG–PCL NPs. The $AUC_{(0-\infty)}$ was increased significantly with NPs 5% (~6.38 fold) and NPs 1% (~1.21 fold). The much higher $AUC_{(0-\infty)}$ indicates an increase in natamycin bioavailability by PDG–PCL NPs. The MRT of the NPs 1% (~133 min) and NPs 5% (~144 min) was approximately 6 fold higher than Natamet® (~23 min). Moreover, clearance (CL) of PDG–PCL NPs (both NPs 1% and NPs 5%) was significantly lower than Natamet®.

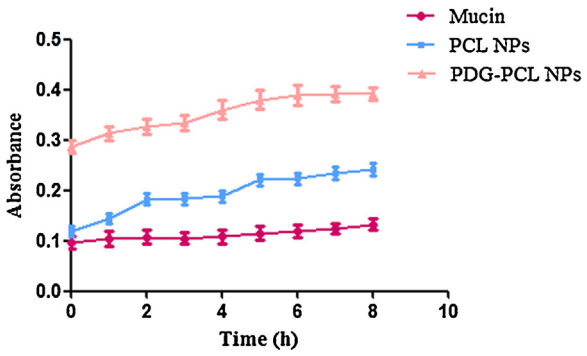

Fig. 4. Interaction between NPs and mucin dispersion by turbidimetric assay.

Table 3
In-vitro antifungal susceptibility by MIC_{90} and Zone of inhibition methods (Mean ± SD, $n = 3$).

Test item	MIC_{90} (mg/L)		Zone of inhibition (mm)			
	Candida albicans	*Aspergillus fumigatus*	*Candida albicans*		*Aspergillus fumigatus*	
			0.005 mg	0.010 mg	0.005 mg	0.010 mg
DMSO	–	–	–	–	–	–
Natamycin in DMSO	3.12	1.56	9.3 ± 0.65	12.1 ± 0.34	13 ± 0.37	15.34 ± 0.44
Natamet®	3.12	1.56	6.6 ± 0.34	9.87 ± 0.26	6.6 ± 0.42	9.19 ± 0.63
Blank NPs	–	–	–	0.15 ± 0.07	–	0.12 ± 0.04
PCL NPs	3.12	1.56	7.6 ± 0.29	11.2 ± 0.59*	6.9 ± 0.61	10.6 ± 0.46
PDG–PCL NPs	3.12	1.56	8.6 ± 0.83[a]	11.54 ± 0.81[a]	8.9 ± 0.35[a]	12.34 ± 0.87[a]

[a] Significant difference for PCL NPs and PDG–PCL NPs versus Natamet®.

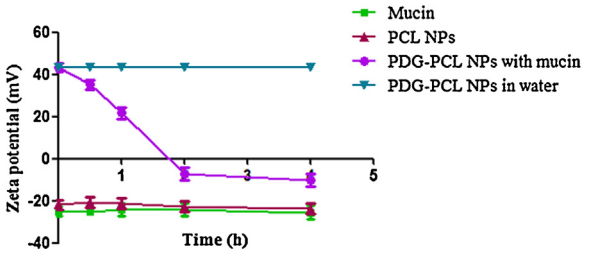

Fig. 5. Estimation of zeta potential values of the NPs during incubation with 0.1% mucin dispersion.

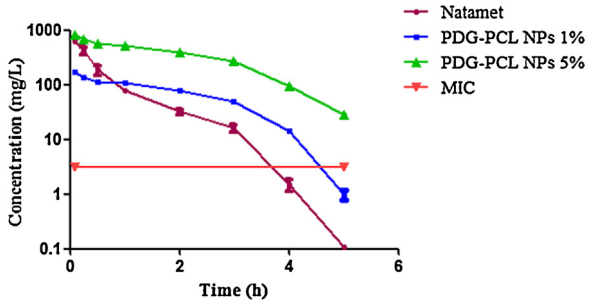

Fig. 6. Tear-concentration time profiles of Natamet® and PDG–PCL NPs.

The effective antimicrobial concentrations required to maintain the therapeutic efficacy should be above ten times of the MIC_{90}. Dose simulation, based on PK/PD indices was performed using MIC_{90} values 10 times higher (considering 31.25 mg/L) than the observed MIC_{90}. The reason behind this decision is that for effective antimicrobial activity, C_{max}/MIC and $AUC_{(0-t)}$/MIC should be higher than 10 and 125 respectively to avoid emergence of resistance (Lewis, 2007; Schentag et al., 2001).

Using the principle of superimposition, tear concentration-time profiles for different formulations as a function of time were simulated up to 10 h (Fig. 7). Since rapid and aggressive treatment is required to prevent ocular mortality, subsequent dosing was based on the time where the concentration was maintained at ten times of the MIC_{90} (Bispo et al., 2010; Iyer et al., 2006). The dose frequency calculated for NP 1% was 3.5 h as compared with Natamet® instillation in every 2 h to maintain therapeutic concentration above 31.2 mg/L.

The ideal dosing interval which could maintain the concentration above 31.2 mg/L by Natamet®, NPs 1% and NPs 5% were 120 min, 210 min and 300 min respectively. The optimized dosing schedule was one instillation every 5 h (2 instillations per 10 h) for NPs 5% and one instillation every 3.5 h (3 instillations per 10 h) for NPs 1% as compared with one instillation per hour or two hour intervals of Natamet®. Detailed simulation data for different time schedules were given in Supplementary figures (S1–S4).

4. Discussion

Nanotechnology has a revolutionary impact in the field of ocular drug delivery, where the limitations of rapid clearance and poor bioavailability can be overcome by encapsulating the drugs in NPs (Nagarwal et al., 2010). Supplemented with small particle size, NPs may lessen ocular shear force during blinking elimination process, reduce discomfort and improve precorneal residence time by surface modifications thereby reducing the dose and dosing frequency, made them interesting candidates for ocular delivery (Gratieri et al., 2010; Kambhampati and Kannan, 2013).

Thus, present study was aimed to develop a nanoparticulate delivery system with a high payload of antifungal agent which is affordable, stable, reduces corneal toxicity and maintain therapeutic concentration at the corneal site. PCL, a hydrophobic biocompatible polymer has been extensively used in ocular applications (Barbault-Foucher et al., 2002; Marchal-Heussler et al., 1993). A hydrophobic drug, such as natamycin, is supposedly more compatible with the hydrophobic PCL carrier and may result in higher drug loading and sustain release. PDG, known as chitosan, is employed as a coating material because it has been reported to impart positive surface charge (Artursson et al., 1994). The combination of negatively charged PCL and positively charged PDG may result in stable, optimum positively charged NPs with high electrostatic affinity toward the negatively charged corneal surface, thus increase in precorneal retention time. PDG coating has been confirmed by the increase in particle size and surface charge of PDG–PCL NPs as compared with PCL NPs.

For a polymeric drug delivery system to be proposed as an ophthalmic drug carrier, it should have prolonged residence time at the precorneal region. The surface charge of the mucin might be altered by the adhesion of the PDG–PCL NPs if the NPs have mucoadhesive property. The optimized formulation has a mean particle size of 217 ± 2 nm with a high positive surface charge of +43.5 ± 1.55 mV. Mucoadhesive studies revealed that PDG–PCL NPs significantly decreased the surface charge of mucin indicating the mucoadhesive strength of PDG–PCL NPs. The electrostatic interaction is the most expectable mucoadhesive mechanism between primary amino functional groups of PDG and the sialic and sulphonic acid substructures of mucus (Andrews et al., 2009; Qaqish and Amiji, 1999). In addition, the free $-NH_2$ and $-OH$ groups may interact with mucin via hydrogen bonding. Whilst PDG may provide improved drug delivery via a mucoadhesive mechanism, it has also been shown to enhance drug absorption via the paracellular route through neutralisation of fixed anionic sites within the tight junctions between mucosal cells (Bravo-Osuna et al., 2007).

In-vitro drug release in simulated ocular conditions (SLF, pH 7.4 at 37 °C) showed a biphasic release process with an initial burst release followed by a much slower sustained release phase for upto 8 h. Burst release is due to the drug present at the outermost layer of the NPs and this could be beneficial in terms of sufficient drug levels in the cornea to kill the microbes immediately after instillation into the eye. Prolonged release of natamycin from NPs was ascribed to the sustained release properties of PCL. However, PDG coat on PCL NPs provided extra layer which not only

Table 4
Stability of PDG–PCL NPs in different storage conditions (Mean ± SD, $n = 3$).

Temperature	Particle size (d nm)		Zeta potential (mV)		EE (%)	
	0 days	180 days	0 days	180 days	0 days	180 days
25 ± 2 °C	218.16 ± 4.8[a]	221.85 ± 4.2[a]	42.96 ± 2.7[a]	42.19 ± 1.1[a]	75.26 ± 0.7[a]	72.09 ± 2.7[a]
6 ± 2 °C	216.73 ± 2.2[a]	219.66 ± 5.3[a]	43.36 ± 1.5[a]	42.59 ± 2.3[a]	75.13 ± 0.9[a]	74.06 ± 1.3[a]

[a] No significant difference was observed compared with 0 days (Table 1).

Table 5
Pharmacokinetic parameters of Natamet® and PDG–PCL NPs (Mean ± SD, $n = 3$).

Formulation	$AUC_{0-\infty}$ (min μg/mL)	$t_{1/2}$ (min)	C_{max} (μg/mL)	CL (mL/min)	MRT (min)	% RB
Natamet®	17989.61 ± 2791.41	16.27 ± 2.33	765.02 ± 11.18	0.057 ± 0.009	23.47 ± 3.36	100%
NPs 1% (w/v)	21792.48 ± 1387.42	92.71 ± 7.36[a]	163.14 ± 7.85[a]	0.009 ± 0.001[a]	133.74 ± 10.62[a]	605.70
NPs 5% (w/v)	114898.62 ± 3238.77[a]	100.46 ± 11.23[a]	792.66 ± 18.19	0.009 ± 0.001[a]	144.93 ± 21.22[a]	638.69
P value	<0.0001	<0.0001	<0.0001	<0.0008	<0.0001	

[a] Significant difference as compared with Natamet®

$$\text{Relative bioavailability}(\%RB) = \frac{AUC(NPs) \times Dose(Natamet^{I})}{AUC(Natamet^{I}) \times Dose(NPs)} \times 100.$$

contributes to mucoadhesion but also prolonged the release of natamycin from the PCL matrix.

MK is a corneal surface infection where the concentration of drug in the local precorneal region plays a crucial role in the management of the disease condition. Tears, as a homogeneous system, spread across the eye surface. Therefore, the concentration of drug in tear fluid directly reflects the amount of drug present at target site for therapeutic efficacy. Thus, estimation of drug concentration in tear fluid was ideal for therapeutic drug monitoring in case of MK. But the major limitation in ocular drug delivery is the short residence of the drug at precorneal region due to rapid tear clearance and eye blinking. As evident from tear concentration time profile, the much higher MRT of NPs indicates prolonged residence time of natamycin at precorneal region. In addition, the observed AUC was much greater (~6 fold) than Natamet®. As higher dose and frequent administration of polyene antibiotics may give rise to corneal irritation and systemic toxicity (due to naso-pharyngeal systemic absorption) on a longer period of time, (Kambhampati and Kannan, 2013) emphasis has been given on the possibility of natamycin dose reduction from 5% to 1%. With this idea we evaluated the pharmacokinetics of NPs with 1% dose strength.

Even with low dose strength of NPs 1%, higher ocular bioavailability and maintenance of drug concentration above MIC for prolong period was observed as compared with Natamet®. The observed $AUC_{(0-\infty)}$ was ~1.21 fold and MRT ~5.69 fold higher for NPs 1%. Thus 6 fold reduction in NPs 5% dose could achieve equivalent ocular bioavailability to marketed Natamet® (5% w/v) suggesting the possibility of dose reduction. Conversely, the reduced dose of natamycin by NPs 1% has resulted in lower C_{max} than Natamet®. Clearance of natamycin by PDG–PCL NPs was not changed significantly by dose difference and lower than Natamet®.

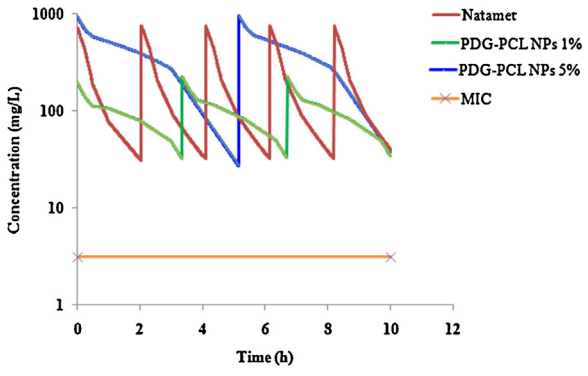

Fig. 7. Simulated ocular concentration-time profile of natamycin for 10 h at a dosing interval of 120 min for Natamet®, 210 min for PDG–PCL NPs 1% and 300 min for PDG–PCL NPs 5%.

This attributes to the mucoadhesive property of the PDG–PCL NPs with ocular surface. As PDG–PCL NPs have a tendency to release the entrapped drug slowly over prolonged period of time, positively charged PDG coating provides additional binding force with the negatively charged mucin surface, resulting in reduced clearance values which would improve the therapeutic performance of natamycin.

The MIC_{90} values were found to be 1.56 and 3.12 mg/L for *A. fumigatus* and *C. albicans* strains respectively. Even though *Candida* was less susceptible to natamycin, the MIC_{90} for *C. albicans* (3.12 mg/L), along with PK parameters, was taken into the account for PK/PD indices. The rationale behind this decision was to design dosage regimen effective for least susceptible *i.e.*, *C. albicans* (3.12 mg/L as MIC_{90}), as well as *A. fumigatus*. The minimum natamycin concentration for multiple dosing was maintained at tenfold of MIC of *C. albicans i.e.* 31.25 mg/L to have PK/PD indices $C_{max}/MIC > 10$ for clinical effectiveness.

The PK/PD indices of both NPs 5% and NPs 1% were compared with Natamet®. In case of NPs 5%, the parameters such as C_{max}/MIC_{90} and $AUC_{(0-10)}/MIC$ were greater than the standard values (10 and 125 respectively) indicating the therapeutic effectiveness of the nanoformulation. The parameters 'time (T) above MIC_{90}' and 'AUC above MIC_{90}' were higher for NPs 5%. Similarly, the PK/PD indices of NPs 1% were higher than Natamet®, indicating the better efficacy (Table 6). Although C_{max} by NPs 1% was less than NPs 5% and Natamet®, PK/PD indices indicated its sufficient efficacy as $C_{max}/MIC > 10$. The reduced exposure achieved by NPs 1% compared with NPs 5% may result in higher safety profile and reduced systemic exposure from naso-pharyngeal drug drainage.

According to simulation data, the ideal dosing interval for NPs 1% and NPs 5% was one instillation every 3.5 h (3 instillations per 10 h) and one instillation every 5 h (2 instillations per 10 h), respectively. The dosing frequency of NP 1% was higher than that of NP 5% dose strength. Based upon these considerations, the dosage regimen with NPs 5% will be required for the initial period of time when MK has a severe effect but in later stages it can be reduced to low dose (NPs 1%) for the effective management with optimum efficacy and safety. Thus, with significant increase in dosing interval and therapeutic efficiency, patient compliance can be improved by corneal targeted PDG–PCL NPs.

Table 6
Estimated PK/PD indices of Natamet® and PDG–PCL NPs.

PK/PD indices	Units	NPs (1% w/v)	NPs (5% w/v)	Natamet®
C_{max}/MIC	Unitless	52.28	254.05	245.19
$AUC_{(0-10)}/MIC$	min	17969.87	63507.37	33573.36
AUC above MIC	min μg/mL	18798.151	101224.26	20313.71
T above MIC	min	272	430	233

AUC above MIC and T above MIC were calculated based on time at which the concentration reaches MIC_{90} *i.e.*, 3.12 mg/L.

5. Conclusion

The NPs formed by interactions between PDG and PCL rendered sufficient EE as well as prominent surface charge dependent corneal adherent properties. PDG–PCL NPs were well tolerated with ocular milieu and able to maintain the natamycin concentration above MIC_{90} for prolonged period. Overall, bioavailability of natamycin was significantly increased by corneal targeted PDG–PCL NPs as compared with marketed preparation. Thus, by using PDG–PCL NPs it is possible to reduce the dose and dosing frequency of natamycin with improved patient compliance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2014.10.035.

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