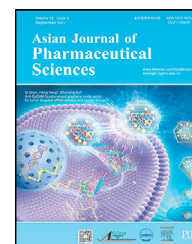


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Research Article

Overcoming corneal barriers: Posaconazole loaded cationic surfactant vesicles for enhanced ocular permeability and anti-fungal efficacy

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ABSTRACT

Posaconazole (PCZ) is a broad-spectrum anti-fungal drug approved by FDA and currently used off-label for the treatment of fungal keratitis (FK). Although ocular route serves as the most bioavailable route for treating FK, delivery of PCZ to the eye remains a challenge due to poor permeation through the cornea and rapid elimination from the eye. Here we outline a comprehensive formulation development process, beginning with *in silico* studies, progressing through *in vitro* evaluations, and ultimately achieving therapeutic benefits *in vivo*. We report modified niosome-like surfactant vesicles, hereafter termed as NioTherms, formulated using a novel and simple heat-mix method, encapsulating PCZ for ocular administration in the form of an *in situ* gel. Excipient screening performed using *in silico* simulations highly correlate with *in vitro* studies ($R^2=0.77$), guiding optimization by Quality by Design (QbD) approach for encapsulating PCZ in NioTherms resulting in particles with an average size of 180.7 ± 2.3 nm, zeta potential of $+27.5 \pm 2.2$ mV and entrapment efficiency of $87.6 \% \pm 1.7 \%$. A 2-fold increase in both mucin binding and cellular uptake indicates a functional role of positive surface charge in enhancing mucoadhesive properties of PCZ-NioTherms. In an *in vivo* murine ocular keratitis model, we demonstrate a 2-fold enhancement in trans-corneal permeability of PCZ-NioTherms and a 3-fold reduction in fungal burden compared to the control standard of care, the PCZ solution. Owing to a facile formulation process, we anticipate that PCZ-NioTherms would serve as a clinically translatable and patient compliant therapeutic intervention for treating FK.

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1. Introduction

Fungal keratitis (FK), or keratomycosis, is a severe and vision-threatening corneal infection characterized by ocular pain, redness, inflammation, and impaired vision [1]. Globally, over 1.05 million cases of culture-proven FK are reported annually, with Asia bearing the highest burden [2]. Although mortality is low, the risk of permanent vision loss is high, especially when timely diagnosis and treatment are delayed. The best example of this can be cited back to the corona virus pandemic where almost 60 % people affected due to ocular mucormycosis lost vision [3]. The treatment of FK is challenging, requiring a personalized approach. Primary treatment with topical antifungals like natamycin, amphotericin B and voriconazole has poor ocular penetration, limiting their efficacy for deep infections. Systemic antifungals and surgical interventions may be needed for severe cases, but pose toxicity risks. Drug resistance, low bioavailability and ocular toxicity further complicate treatment, highlighting the need for advanced targeted therapies to improve outcomes [4–6].

Posaconazole (PCZ), a broad-spectrum azole antifungal, shows superior efficacy against resistant fungal pathogens, making it a promising option for treating FK [7,8]. Although not commercially available as an ophthalmic preparation, PCZ is used off-label as a compounded eye drop from its oral form, Noxafil®, when other treatments fail or specific fungi are susceptible [9,10]. However, the current formulation lacks optimal efficacy due to poor permeation and rapid elimination from the eye, highlighting the need for a dedicated ocular preparation, preferably a nano formulation, to improve corneal permeation and enhance therapeutic outcomes. The potential of ocular nano formulations has been extensively explored, with several formulations already approved and others progressing through clinical trials [11]. Surfactant bilayered vesicles have emerged as a well-established nanosystem for delivering therapeutic agents via the ocular route. These vesicles, composed of surfactants, offer a flexible yet structurally stable architecture, which enhances permeation through the corneal barrier, improving drug delivery efficacy [12]. Additionally, there are a number of reports in the literature illustrating surface modifications to impart stability and mucoadhesive or surface retentive properties [13,14]. These properties help nanoparticles to interact with the anionic ocular surface increasing the precorneal retention and thus enabling higher flux of the encapsulated drug [15].

In this work, we therefore report a PCZ loaded ocular mucoadhesive formulation composed of cationic surfactant vesicles by a novel heat-mixing method. For the first time, we showcase ability of the introduced method to encapsulate PCZ into surfactant bilayered vesicles, further formulated as an *in situ* gel, making it a patient compliant dosage form. These vesicles are spontaneously formed upon the simple mixing without the use of sophisticated equipment to form a niosome-like system, hereafter referred as NioTherms. The heat-mixing approach eliminates the need for organic solvents traditionally used in niosome preparation, thus presenting a more environment friendly alternative. Therefore, this method opens the door for

an alternative preparation method for formulating cationic surfactant vesicles, making it a platform technology. Our research highlights the integration of *in silico* techniques to assess drug-excipient interactions during preformulation, the optimization of PCZ-NioTherms employing a quality by design (QbD) framework, and comprehensive *in vitro* and *in vivo* characterization of the formulated system. We have identified the ability of PCZ NioTherms to improve retention and permeation through the corneal surface and significantly reduce ocular fungal burden in an *in vivo* murine model. Thus, PCZ NioTherms showcase immense potential as a therapeutic intervention for the treatment of FK.

2. Materials and methods

2.1. Materials

PCZ was obtained as a gift sample from Bajaj Healthcare Ltd. (Thane, India). Transcutol P was provided as a gift sample from Gattefosse (Mumbai, India). Tween 80, Tween 85, Span 20, Span 60, dicetyl phosphate (DCP), Type III porcine mucin, sodium lauryl sulphate (SLS) and rhodamine B (RhB) were obtained from Sigma-Aldrich (Mumbai, India). Cremophor EL, 2-pyrrolidone and poloxamer 407 were procured from BASF (Mumbai, India). Polyethylene glycol 400 (PEG), propylene glycol (PG), cholesterol and dimethyl sulfoxide (DMSO) were purchased from SDFCL (India). Stearylamine was purchased from SRL laboratories. Lactose, dextrose, sucrose, mannitol and trehalose were obtained from Signet Chemical Corporation (India). Sabouraud dextrose agar (SDA) media and broth were purchased from Hi-Media (India).

2.2. Screening of surfactants and solubilizers

In silico, solubility analysis is an invaluable predictive tool that employs computational modelling and simulations to anticipate the solubility behavior of molecules by significantly minimizing the need for extensive experimental endeavours. To evaluate the miscibility of PCZ with surfactants and solubilizers, we utilized the Forcite and Blends modules within Material Studio (version 2017, Accelrys, Inc., San Diego, CA, USA) [16,17]. Blends module combines a modified Flory–Huggins model with molecular simulation techniques to efficiently calculate the compatibility of binary mixtures. This method determines the mixing, binding and interactions between specific molecules. The miscibility behaviour of the binary mixtures was analyzed in terms of mixing energy (E_{mix}) and interaction parameter (χ).

The *in silico* investigations involved initial preparation of chemical structures, followed by cleaning and energy minimization, subsequent geometry optimization, and determination of interaction parameter. The chemical structures of PCZ, surfactants and solubilizers were sourced from the PubChem database and imported as 3D atomistic documents into the software interface. All structures were cleaned to obtain their lowest energy confirmations which were subjected to geometry optimization using the classical simulation tool Forcite, with Smart algorithm and COMPASS II forcefield. To calculate E_{mix} between each pair of drug

and surfactant or solubilizer, drug was designated as the 'base,' and surfactants and solubilizers were designated as the 'screen.' Calculations were executed utilizing the same forcefield as employed for geometry optimization. Subsequently, Blends job was initiated, and the resultant output was analyzed to ascertain the values of E_{mix} and χ . The E_{mix} quantifies the variation in free energy observed when comparing the mixture's state to the combined free energies of its individual components (base and screen). This computation is performed through the following equation: $E_{\text{mix}} = 1/2 Z (E_{\text{bs}} + E_{\text{sb}} - E_{\text{bb}} - E_{\text{ss}})$. Where, E_{bb} and E_{ss} represent the binding energies between base and screen molecules when they exist independently, and E_{bs} and E_{sb} represent the binding energies between base and screen molecules. Given that the interactions E_{bb} and E_{ss} are typically negative under equilibrium conditions, the sign of E_{mix} is contingent upon the relative ratio of the absolute values of E_{bs} , E_{sb} , E_{bb} and E_{ss} . A lower E_{mix} value is indicative of miscibility, wherein more negative values correspond to higher miscibility. χ , describing the mutual drug–excipient interaction is calculated as: $\chi = E_{\text{mix}}/R \times T$. Where R and T are the molar gas constant ($\text{kg}\cdot\text{m}^2/\text{K}\cdot\text{s}^2$) and the temperature (K), respectively.

The *in silico* results were then validated by determining solubility of PCZ in solubilizers and entrapment efficiency using several surfactants experimentally. The surfactant screening involved evaluating surfactants from different chemical classes, such as Spans, Tweens and Cremophor, for their capacity to encapsulate PCZ and to form stable vesicles. These surfactants were tested in combination with cholesterol at a molar ratio of 1:1. A fixed amount of PCZ was loaded into the formulations following the method outlined in section 2.3. The resulting NioTherms were then purified and analyzed for entrapment efficiency. The surfactant demonstrating the highest encapsulation efficiency was selected for further formulation development. Additionally, saturation solubility was assessed across various solubilizers to identify the one offering the highest solubility.

2.3. Fabrication of PCZ-NioTherms

The surfactant bilayered vesicles were fabricated using a novel heat mixing method. Briefly, PCZ was solubilized in Transcutol P as a solubilizer at 70 °C to 80 °C with gentle shaking. Tween 80 was then incorporated into the drug solution, followed by thorough homogenization. Cholesterol was added as a rigidity-enhancing agent, and stearylamine was included as a stabilizing charge modifier, leading to the formation of a non-aqueous phase mixture. Separately, double-distilled water was heated to the same temperature to create the aqueous phase. The aqueous and non-aqueous phases were rapidly combined with vigorous shaking, then stirred on a magnetic stirrer at 500 rpm for 15 min to promote the spontaneous formation of bilayered cationic surfactant vesicles, termed as NioTherms. The formulation was prepared under aseptic conditions within a sterile environment.

2.4. Optimization of PCZ-NioTherms

Optimization of PCZ-NioTherms was conducted using a Design of Experiments (DOE) approach, enabling a systematic

evaluation of the individual and interactive effects of various formulation parameters using Design-Expert® software (version 11). The literature survey identified surfactant concentration, cholesterol content and drug loading as independent variables, while particle size, polydispersity index (PDI) and entrapment efficiency served as dependent outcomes. Preliminary trials were conducted to determine the influence of independent variables and establish their upper and lower limits. Based on these parameters, 27 experimental batches were designed using the Box-Behnken Design (BBD) and executed to obtain the targeted responses. The resulting data were statistically analyzed using appropriate models, followed by numerical and graphical optimization to refine the formulation. The findings were interpreted using 3D response surface plots and single factor interaction plots generated by the software and validated against (%) Bias criteria as reported in literature [18,19].

2.5. Formulation of PCZ-NioTherm loaded in situ gel

The optimized PCZ-NioTherms were subjected to a freeze-drying process to enhance their stability and shelf life. Cryoprotectants (Sucrose, dextrose, lactose, mannitol, and trehalose) were screened and the selected cryoprotectant was added during freeze-drying to preserve the integrity of NioTherm vesicles. After freeze-drying, the NioTherms were reconstituted with a polymeric solution composed of Poloxamer 407 (15 %) to get a temperature triggered *in situ* gelling system [20]. The resulting gel was evaluated for gelling time, adhesiveness and gel strength using Texture Analyser, Brookfield Engineering Laboratories Ltd., as per the previously established protocols [21].

2.6. Evaluation of PCZ-NioTherms

2.6.1. Particle size, surface charge and morphology

The particle size and surface charge of the particles were analyzed by diluting the samples by ten folds using dynamic light scattering (DLS) using Zetasizer Nano ZSP (Malvern, USA) (For detailed method refer supplementary material). To further confirm the formation of bilayered unilamellar vesicles, morphology of the particles was examined using transmission electron microscopy (TEM) (Tecnai 12 G2, Philips, Netherland) which offers direct insight into their shape and bilayer integrity. Small-angle neutron scattering (SANS) analysis was employed to further confirm the structural integrity, the bilayer architecture and unilamellarity of the vesicles using SANS diffractometer at Guide Tube Laboratory, Dhruva Reactor, Bhabha Atomic Research Centre, Mumbai, India.

2.6.2. Purification and entrapment of PCZ-NioTherms

The purification of PCZ-NioTherm formulation was accomplished using a Sephadex G50 gel column via a minicolumn separation technique as reported in [22]. A minicolumn was prepared with the hydrated Sephadex gel, and 1 ml of the optimized NioTherm formulation was allowed to pass through the column, and fractions of the eluted formulation were collected by monitoring the turbidity

of the eluate. The collection process was continued until clear fractions were observed. These purified PCZ-NioTherms were then utilized for further characterization.

Entrapment efficiency was assessed using ultracentrifugation method, where NioTherms were centrifuged at 40,000 rpm for 30 min at 10 °C. HPLC (Waters Alliance, USA) equipped with a UV detector set at 254 nm was used to analyze the drug content in the formed pellet. The separation was achieved on a Inertsil C₁₈ ODS 2 column (4.5 mm × 15 cm, 5 µm packing), with PCZ eluted in a mobile phase of Acetonitrile: Water (60:40) at a flow rate of 1 ml/min. Entrapment efficiency was calculated using a formula: Entrapment efficiency (%) = Experimental drug content / Total drug content × 100 %. In addition, lyophilized PCZ-NioTherms were characterized using differential scanning calorimetry (DSC), X-ray diffraction (XRD) and fourier-transform infrared spectroscopy (FTIR) to confirm the complete encapsulation of the drug within the NioTherms.

2.6.3. *In vitro drug release*

A drug release study for PCZ loaded NioTherms was conducted to analyze physical stability of the formed system using a paddle apparatus following sample and separate method [23]. Briefly, NioTherms containing PCZ equivalent to 9 mg were dispersed in 900 ml dissolution media consisting of 0.3 % SLS in water for maintaining solubility and sink conditions due to low aqueous solubility of PCZ. The dissolution process was maintained at 37 °C with a paddle speed of 50 rpm over a 24-h period. Samples (1 ml aliquots) were collected at predetermined time intervals (0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h) and subjected to ultracentrifugation at 40,000 rpm for 30 min at 10 °C. The supernatant was analyzed via HPLC to determine the dissolved drug content, and the pellet was returned to the dissolution flask to prevent sample loss. After each sampling, the dissolution medium was replenished with an equal volume of fresh media to maintain sink conditions.

2.6.4. *Ex vivo corneal permeation*

Ex vivo models are the preferred choice for studying corneal permeation. Corneal permeation of the developed NioTherm in situ gel was studied on freshly excised goat cornea using Franz diffusion cell apparatus (Erweka, Germany). Briefly, cornea was separated from the eyeballs, followed by mounting it between the donor and receptor compartment of the Franz diffusion cell apparatus. The study was performed to compare the permeation behaviour of the developed formulation with the PCZ solution. The study was conducted at 37 °C using 0.3 % SLS in water as the diffusion media. The aliquots (1 ml) were withdrawn at predetermined time intervals (0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h) for a period of 24 h and were analyzed for the drug permeated through the cornea using HPLC.

2.6.5. *In vitro mucin binding and charge mediated adhesion*

The *in vitro* mucin binding potential of the positively charged/cationic PCZ-NioTherms was compared to that of uncharged/neutral and negatively charged/anionic PCZ-NioTherms with identical composition to assess the influence of surface charge on mucin interaction. Both formulations were incubated with deionized water and

an equal volume of 0.5 % Mucin III solution in phosphate buffer (pH 6.8). Zeta potential measurements were taken after 1-h incubation. Following this, the formulations were ultracentrifuged at 40,000 rpm for 30 min at 10 °C. The supernatant, containing unbound mucin, was analyzed using UV spectrophotometry (Jasco, Japan) to quantify the extent of mucin binding [24]. The experiment was conducted in triplicate (n=3). To further validate the binding of stearylamine and mucin at molecular level, *in silico* simulations were performed using AutoDock tool to simulate potential binding conformations and calculate their binding energies.

2.6.6. *In vitro fungal cell internalization*

A fungal uptake study was designed to understand the effectiveness of NioTherms as carriers for delivering compounds inside fungal cells. RhB loaded formulations were prepared by the method described in section 2.3 by adding 1 mg RhB dye in the non-aqueous phase instead of PCZ. Different formulations were prepared to evaluate impact of surface charge on uptake efficiency. DCP and stearylamine were added to obtain NioTherms with negative and positive charges respectively. A 1 ml suspension of mid-log phase *Candida albicans* MCC 1794 cells, with a concentration of 10⁶ CFU/ml was centrifuged at 3,000 rpm for 5 min to obtain a fungal cell pellet which was then washed with saline to remove any residual media and was then resuspended in 1 ml of the test formulations. The resulting suspension was incubated in dark for 30 min and centrifuged to get fungal cell pellet. The formed pellet was washed with saline to eliminate the traces of free RhB which was not taken up by the cells. This was then redispersed in saline and observed using confocal microscope (Leica microsystems CMS, Germany). The quantification of intensities of uncharged, anionic and cationic NioTherms was carried out using ImageJ software with DMSO treated cells as positive control.

2.6.7. *In vitro time kill assay*

A time-kill assay was conducted to evaluate the antifungal efficacy of the developed formulations over time. Fresh *Candida albicans* MCC 1794 cell suspension was prepared by inoculating overnight-grown colonies into RPMI-1640 medium, buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) to pH 7.0. The concentration of the fungal suspension was adjusted to 1 × 10⁶ CFU/ml. The fungal suspension (9.5ml) was added to separate conical flasks and mixed with 0.5 ml of the test solution. Test samples included PCZ NioTherms (equivalent to 1.5 µg/ml of PCZ), blank NioTherms (placebo NioTherms), PCZ solution (equivalent to 1.5 µg/ml of PCZ), and sterile PBS (negative control). All test samples were prepared in a 5 % (w/v) dextrose infusion solution. The cultures were incubated at 35 °C for 24 h. Subsequently, 400 µl aliquot from each flask was removed at specified time intervals (4 h, 8 h, 12 h, 24 h) and centrifuged to form a fungal cell pellet. The pellet was washed with saline to remove any residual test sample, centrifuged again, resuspended in sterile saline, and serially diluted. A 20 µl aliquot from each dilution was plated on SDA plates to determine fungal colony counts after a 48-h incubation at 35 °C.

2.6.8. *In vitro* irritancy evaluation

A Hen's Egg Test on Chorioallantoic Membrane (HET-CAM) was employed as suggested by 'The Interagency Coordinating Committee on the Validation of Alternative Methods' (ICCVAM) guidelines. The potential ocular irritancy of test substances was assessed based on their toxicity to the blood capillaries of chorioallantoic membrane of a chicken embryo, that mimic conjunctival blood vessels [25,26]. Fertilized 8 d old chicken eggs were carefully opened near the air cell to expose chorioallantoic membrane. Test samples (0.3 ml each), including 0.1 N NaOH solution (positive control), 0.9 % NaCl solution (negative control), placebo NioTherms and PCZ NioTherms, were applied to the membrane. The subsequent effects, such as lysis, haemorrhage and coagulation, were observed over 5 min, and an irritation score (IS) was assigned to each test sample based on these observations.

2.6.9. Stability studies

The freeze dried PCZ-NioTherms were packed and sealed in glass vials and subjected to stability conditions of 2-8 °C and 25 ± 2 °C /65 % $\pm$ 5 % RH in refrigerator and stability chamber (Thermolab, India) to determine the stability of the developed NioTherms. The samples were collected at time intervals of 1, 3 and 6 months and were analyzed for their particle size and entrapment efficiency and compared with the initial readings (0 month) which are essential for ensuring stability of nanoformulations.

2.6.10. Ocular pharmacokinetics

In vivo ocular pharmacokinetic performance of PCZ loaded NioTherms was observed in Sprague Dawley (SD) rats weighing 190–200 g. This study was performed in accordance with the Committee for Control and Supervision of Experiments on Animals (CCSEA) guidelines and after obtaining permission from Institutional Animal Ethics Committee of the Institute of Chemical Technology, Mumbai. The animals were divided into four groups each containing 12 rats. Before dosing, rats were anaesthetized with an intraperitoneal injection consisting of xylazine-ketamine mixture containing xylazine (9 mg/kg) and ketamine (90 mg/kg). Group I received a ocular application of 50 μ l CZ-NioTherm (equivalent to 17.5 μ g PCZ) *in situ* gel, Group II received 50 μ l of placebo NioTherm gel, Group III received 50 μ l PCZ solution (equivalent to 17.5 μ g PCZ), and Group IV served as a control, receiving 50 μ l saline in the eye. At pre-determined time intervals (0.5 h, 1 h, 2 h and 4 h) three animals from each group were sacrificed ($n=3$) using excess carbon dioxide and the eyeballs were surgically removed. These eyeballs were then homogenized and the drug content present in the whole eye was determined by HPLC method as described in section 2.6.2, additionally incorporating voriconazole (10 μ g/ml) as an internal standard.

2.6.11. *In vivo* ocular mucoadhesion

The influence of surface charge on the adhesion of NioTherms to the corneal surface was assessed using Confocal Laser Scanning Microscopy (CLSM) in isolated rat eyeballs. Cationic, anionic and neutral formulations were prepared as outlined in section 2.3, with coumarin 6 incorporated as a fluorescent

marker for CLSM imaging. Prior to administration, rats were anesthetized via intraperitoneal injection consisting of xylazine-ketamine mixture (xylazine: 9 mg/kg, ketamine: 90 mg/kg). 50 μ l of each test formulation was instilled into the conjunctival sac. At specific time intervals (0.5 h, 1 h, 2 h, 4 h), the rats were euthanized, and the eyeballs were promptly excised. The eyes were then embedded in optimal cutting temperature compound frozen using liquid nitrogen. Sagittal sections (5 μ m) of the entire eye were obtained using a cryostat HM 525 NX (Thermo scientific) and examined under a confocal microscope. The intensity of coumarin 6 fluorescence was quantified using ImageJ software for further analysis [24].

2.6.12. Repeat dose ocular toxicity

The repeat-dose ocular toxicity of the formulations was evaluated in SD rats according to a 28-days dosing regimen, in accordance with the CCSEA guidelines following a protocol approved by the Institutional Animal Ethics Committee of the Institute of Chemical Technology, Mumbai. The rats were divided into four groups, with each group comprising 10 animals (5 males and 5 females). Prior to the study, the animals were acclimatized for 7 days and maintained on a standard diet. At the onset of the study, the animals were administered with normal saline (negative control, group I), 1 % SLS solution (positive control, group II), placebo NioTherms (group III) and *in situ* gelling PCZ-NioTherms containing PCZ at concentration of 250 μ g/ml (group IV). The animals were dosed daily for 28 days, after which they were euthanized, and their eyeballs were isolated for analysis. The toxicity profile was evaluated through visual inspections for signs of ocular irritation, and histopathological examination of the sagittal sections of the isolated eyeballs to identify any morphological changes.

2.6.13. *In vivo* efficacy

The *in vivo* antifungal efficacy of the formulation was assessed using a murine model, in accordance with the CCSEA guidelines following the protocol approved by the Institutional Animal Ethics Committee of the Institute of Chemical Technology, Mumbai. The study involved male Swiss albino mice, each weighing 25–28 g. Prior to the main experiment, pilot studies were conducted to determine the optimal fungal inoculum concentration and drug dose. In the pilot study, six Swiss albino mice were immunocompromised by intraperitoneal injection of cyclophosphamide (200 mg/kg) for two consecutive days. To prevent bacterial infections in the immunocompromised mice, ceftazidime (5 mg/kg/d) was administered intravenously on the day of infection. Under isoflurane anesthesia, the corneal epithelium was scarified using a sterile needle, and FK was induced by instilling inoculum of *Candida albicans* MCC 1794 at concentrations of 1×10^3 and 1×10^6 CFU/ml [27,28]. Four days post-inoculation, the severity of infection was evaluated based on parameters such as redness, corneal opacity, discharge and chemosis, and compared with a control group that received no inoculum. The infected eyes were treated with 150 and 250 μ g/ml of PCZ to determine the concentration that resulted in a 50 % reduction in fungal burden compared to the animals that received no treatment. For the main study, the selected fungal concentration was administered to all immunocompromised mice. These infected mice were

then divided into four treatment groups, each containing six animals: control, placebo NioTherms, PCZ NioTherm in situ gel and PCZ solution. Treatments were administered according to the dose determined in the pilot experiment and continued for 2 d. Following the treatment, mice were sacrificed by carbon dioxide asphyxiation, and their eyeballs were enucleated. The eyeball was homogenized, appropriately diluted and spread onto SDA plates, which were incubated at 35 °C for 48 h. The number of colony-forming units on each plate was then counted. The morphology of the eyes, both before and after treatment, was evaluated based on visual inspections, with scoring applied to assess the extent of recovery. Additionally, histopathological analysis of the isolated eyeballs was performed pre and post-treatment to assess the impact of the treatment on tissue structure and determine the therapeutic efficacy at a microscopic level.

2.7. Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Statistical comparisons across groups were evaluated using one-way analysis of variance (ANOVA), followed by an unpaired t-test to assess pairwise differences. A P-value of less than 0.05 was set as the threshold for statistical significance. Analysis was conducted using GraphPad Prism software (version 7, GraphPad Inc., San Diego, USA).

3. Results and discussion

3.1. Screening of surfactants and solubilizers

The screening of surfactants and solubilizers, aided by *in silico* computational studies, were validated using wet lab techniques for selection of the most suitable excipients. Utilizing Forcite and Blends modules, molecular interactions were simulated with a focus on two critical parameters: E_{mix} and Chi, both serving as indicators of molecular compatibility. Notably, lower values of Chi and E_{mix} indicated stronger interactions between PCZ and the selected excipients [16,17], which correlated positively with experimental solubility and entrapment efficiency parameters. Transcutol P and Tween 80 emerged as the most effective excipients based on the criteria of solubility of PCZ in solubilizers, entrapment efficiency and particle size of formulated PCZ-NioTherms in surfactants. These excipients exhibited the lowest Chi and E_{mix} values (Fig. 1A, B, D and E), indicating stronger molecular interactions with PCZ. Experimentally, Transcutol P demonstrated the highest solubility of PCZ (48.73 mg/ml) (Fig. 1C), while Tween 80 achieved the maximum entrapment efficiency (68.22 %) and favourable particle size (280 nm) (Fig. 1F) in the formulated PCZ-NioTherms. The structural characteristics of Tween 80, particularly its long polyoxyethylene chains, facilitated robust hydrophobic interactions with PCZ, while its high hydrophilic-lipophilic balance (HLB) contributed to reduced surface tension at the drug-aqueous interface, further enhancing drug entrapment [18]. In contrast, Span surfactants, characterized by lower HLB values and more rigid structures, were less effective in facilitating optimal drug encapsulation. The advantageous combination of Tween 80's

structural flexibility and elevated HLB resulted in superior performance than the comparatively rigid Span surfactants, which proved inadequate for encapsulating hydrophobic drugs. Further, we could establish a reasonable statistical correlation [19] between Chi parameter and E_{mix} were in good agreement with the experimental solubility as statistically correlated ($R^2=0.744$, Chi parameter verses solubility, $R^2=0.750$, E_{mix} verses solubility) (Fig. 1G and H). Similarly, *in silico* findings in terms of Chi parameter and entrapment efficiency ($R^2=0.752$) and E_{mix} and entrapment efficiency ($R^2=0.777$) in the *in silico* predictions and experimental outcomes (Fig. 1I and J), indicating a good alignment between the *in vitro* and *in silico* results. These finding highlights the utility and reliability of *in silico* tools not only in predicting molecular interactions but also in optimizing drug formulation strategies. By elucidating the relationship between computational models and empirical data, the study underscores the potential of *in silico* approaches to enhance the selection of excipients, ultimately contributing to improved drug delivery systems for hydrophobic agents such as PCZ.

3.2. Fabrication of PCZ-NioTherms

PCZ-loaded NioTherms were fabricated using a novel heat mix method, which leverages the inherent self-assembling properties of surfactant molecules when subjected to energy inputs, such as heat and mechanical mixing (Fig. 1K) [20–22]. This approach eliminates the need of using traditional organic solvents, commonly used in vesicle formation, thereby offering a greener and potentially safer alternative for development of the NioTherm platform. Instead, solubilizer was added as a component of the system which plays a crucial role by modulating the thermodynamic parameters of the system, specifically enthalpy and entropy. The solubilizer works by reducing the interfacial and free energies within the surfactant mixture, which facilitates the spontaneous organization of surfactant molecules into a bilayered vesicular structure. This self-assembly process is driven by the optimization of surfactant packing, leading to the formation of stable, unilamellar vesicles that effectively encapsulated the therapeutic cargo-in this case, PCZ.

The elimination of organic solvents and the introduction of a solubilizer not only facilitated the fabrication process but also ensured the overall stability and uniformity by forming unilamellar NioTherms. This method ensured efficient vesicle formation with acceptable entrapment efficiency and stability, making it a promising strategy for the delivery of hydrophobic drugs like PCZ. This formulation approach represents a significant advancement in the field of surfactant-based drug delivery systems, combining simplicity and efficiency in the formulation process.

3.3. Optimization of PCZ-NioTherms

Statistical analysis using mathematical models indicated that the reduced quadratic model was significant for the assessment of entrapment efficiency and particle size whereas reduced linear model was significant for assessment of zeta potential. The effect of independent variables on entrapment efficiency is depicted in Fig. 2A–D. Examination

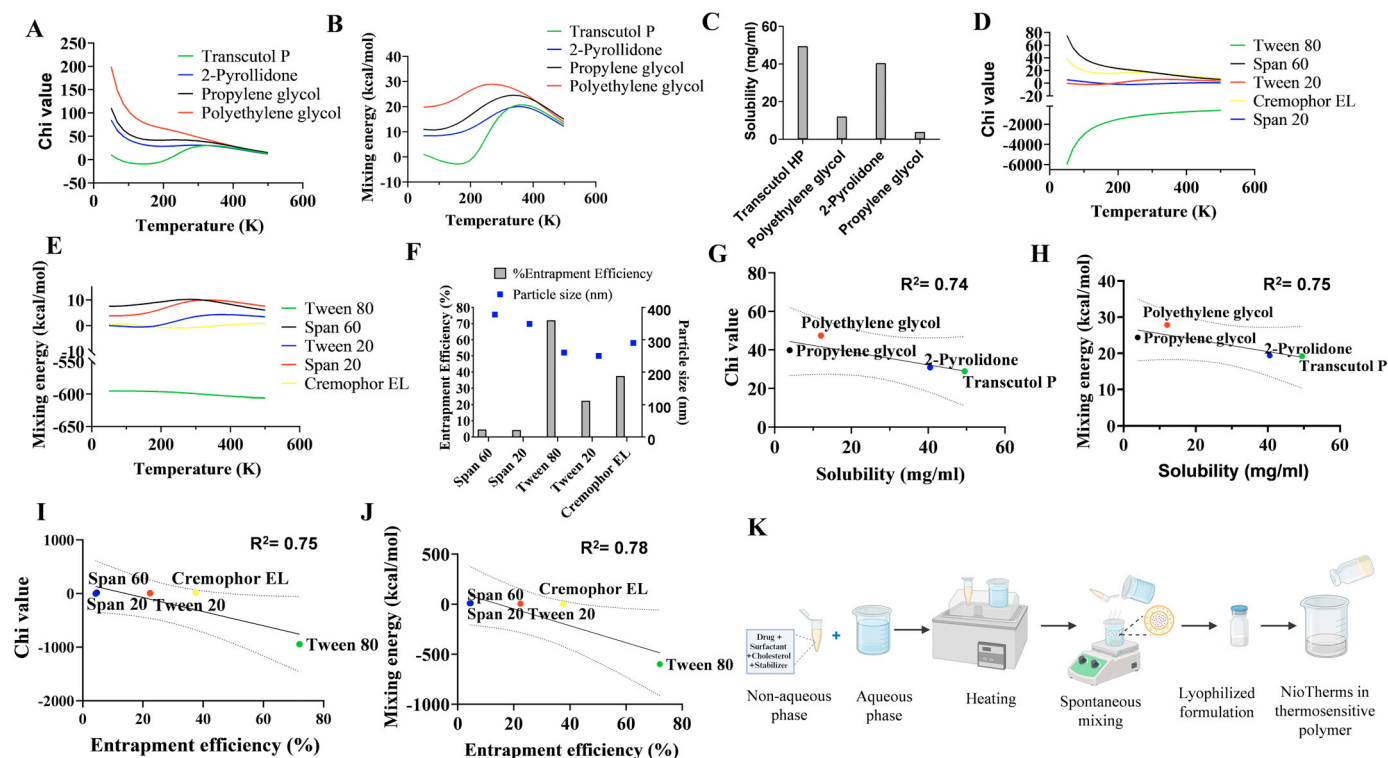


Fig. 1 – Formulation development and excipient selection using in silico and experimental techniques. (A) Chi parameters of solubilizers; **(B)** E_{mix} of solubilizers; **(C)** Experimental solubility of PCZ in solubilizers; **(D)** Chi of surfactants; **(E)** E_{mix} of surfactants; **(F)** Experimental entrapment efficiency and particle size data of PCZ-Niotherms using different surfactants; **(G)** Statistical correlation between Chi parameter and experimental solubility of PCZ in solubilizers ($R^2=0.744$) **(H)** Statistical correlation between E_{mix} and experimental solubility of PCZ in solubilizers ($R^2=0.75$); **(I)** Statistical correlation between Chi parameter and experimental entrapment efficiency of PCZ in surfactants ($R^2=0.752$); **(J)** Statistical correlation between E_{mix} and experimental entrapment efficiency of PCZ in surfactants ($R^2=0.777$), **(K)** Formulation scheme for PCZ-Niotherms. Data were obtained as a result of statistical correlation calculated using Pearson Correlation coefficient, expressed as R^2 value.

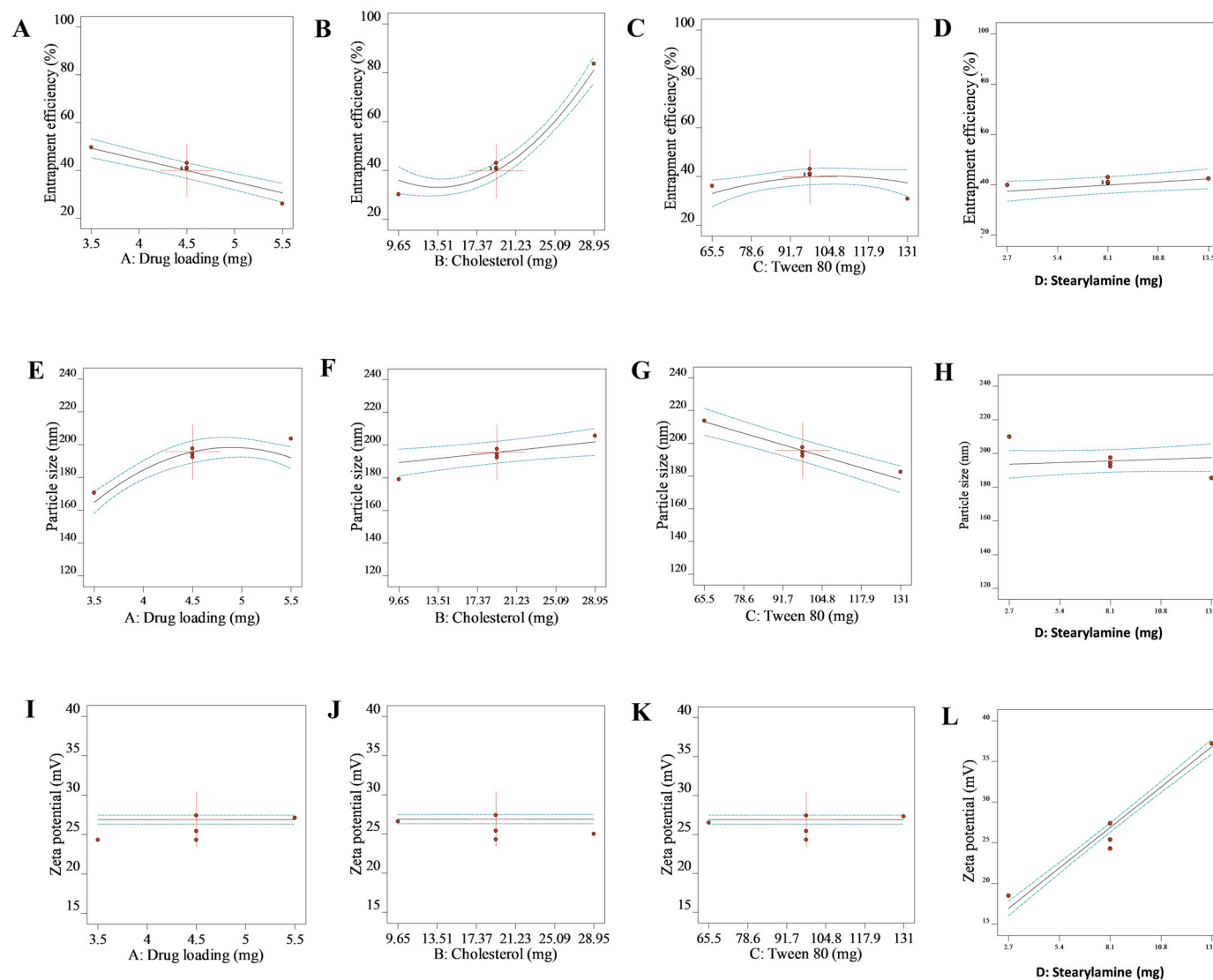


Fig. 2 – 2D plots depicting the effects of different dependent variables on responses. (A–D) Effect on entrapment efficiency; (E–H) Effect on particle size; (I–L) Effect on zeta potential. (ReferFig. S1–S3 for 3D response surface plots).

of response plots revealed that the amount of cholesterol, stearylamine and PCZ had a statistically significant impact ($P < 0.05$) on the entrapment efficiency of PCZ-NioTherms. Specifically, an increase in drug content led to a slight decrease in entrapment efficiency (Fig. 2A), likely due to leakage from the flexible surfactant bilayer. Conversely, increasing the amount of cholesterol enhanced entrapment efficiency (Fig. 2B), suggesting that the increased rigidity of the bilayered membrane due to cholesterol addition reduced membrane fluidity, thereby minimizing drug leakage from the vesicles [23]. Additionally, stearylamine being a charged lipid, served a dual purpose: it imparted positive surface charges while also complementing cholesterol in enhancing vesicular membrane rigidity by expanding the hydrophobic leaflet region, thereby improving entrapment efficiency to some extent (Fig. 2D) [24].

The effect of independent variables on particle size is depicted in Fig. 2E–H. In terms of particle size, the amount of drug, cholesterol and Tween 80 were identified as influential factors. As hydrophobic components, both the drug and cholesterol tend to reside in the bilayer compartment of the vesicle, leading to significant increase ($P < 0.05$) in the overall particle size with higher amounts of these components (Fig. 2E and F) [23,25,26]. In contrast, Tween 80, a surfactant with a long aliphatic chain, reduces surface tension, thereby decreasing particle size (Fig. 2G) [18,27].

The effect of independent variables on zeta potential is depicted in Fig. 2I–L. Zeta potential, a critical parameter for assessing the stability of colloidal systems, was significantly affected by the amount of stearylamine ($P < 0.05$). Stearylamine, being positively charged, imparts surface charges to the vesicles, leading to a linear increase in zeta potential (Fig. 2L). Higher zeta potential values indicate greater electrostatic repulsion between particles, which can enhance the stability of the colloidal system by preventing aggregation [28].

Based on the interactions and effects of independent variables as discussed, numerical and graphical optimization was performed following ANOVA, which suggested 10 potential solutions within the defined design limits. Validation of these solutions yielded a bias of 4.5 %, confirming the model's applicability [29]. The optimized formulation derived from the DOE consisted of PCZ (4mg), cholesterol (28.9 mg), Tween 80 (99 mg) and stearylamine (8.22 mg). This formulation was subsequently prepared and utilized for further characterization.

3.4. Formulation of PCZ-NioTherm loaded in situ gel

The optimized NioTherms were freeze dried and reconstituted in thermosensitive polymeric solution, which rapidly gels in situ to provide a sustained release of the encapsulated drug at the site of application. Poloxamer 407, a thermosensitive polymer, was selected for its ability to undergo a sol-gel transition at body temperature. The cryoprotectants were screened as depicted in Fig. 3C. Mannitol (5 % w/v) was found to be the most suitable cryoprotectant since it did not show a significant impact on particle size after reconstitution. The in situ gel showed an optimum gelling

time of 40 s, adhesiveness value of 3.2 mJ and gel strength of 2N, which is an indicative of a gel like texture property (Refer supplementary materials Table S3 for rheological evaluation of in situ gel)

3.5. Evaluation of PCZ-NioTherms

3.5.1. Particle size, surface charge, and morphology

DLS analysis revealed that the hydrodynamic diameter of PCZ-NioTherms followed a Gaussian distribution, with an average particle size of 180.7 ± 2.3 nm and a low PDI of 0.29 (particle size and size distribution data given in Fig. S4), essential for consistent and predictable in vivo performance. The zeta potential of PCZ-NioTherms was found to be 27.5 ± 2.2 mV indicative of good colloidal stability. Further, TEM images confirmed the spherical morphology with an average particle size of approximately 190 nm (Fig. 3A) and were in agreement with the particle size obtained from DLS. Additionally, given the potential influence of surfactants that have a propensity to spontaneously form micelles, SANS analysis was conducted to ensure the absence of micellar or mixed micellar system along with vesicle formation [30]. The SANS curve fitting followed Q^{-2} behaviour in the low Q range as exhibited by typical vesicular systems, and confirmed the presence of large unilamellar vesicles with a bilayer thickness of 33.3 Å (Fig. 3B).

3.5.2. Purification and entrapment

The purified PCZ-NioTherms were analyzed for the content of entrapped PCZ. The entrapment efficiency was found to be $87.6 \% \pm 1.7 \%$. The PCZ-NioTherms were further subjected to DSC, FTIR and XRD to ensure absence of free PCZ. The DSC thermogram of PCZ-NioTherms lacked the characteristic endothermic peak (at 169 °C) seen in the pure drug and physical mixture, indicating the amorphization and complete encapsulation of PCZ within the NioTherm vesicles (Fig. 3D). Similarly, FTIR spectra examined interactions of PCZ with the excipients which further confirmed the drug encapsulation, since no characteristic peaks of PCZ were observed at 2800 cm^{-1} (Fig. 3F). The XRD analysis showed no crystalline peaks for PCZ, thereby validating its amorphous state following PCZ encapsulation (Fig. 3E). Overall, physicochemical characterization of PCZ-NioTherms confirmed the formulation integrity and robustness with no detectable free or unbound PCZ.

3.5.3. In vitro drug release

A drug release study of the developed PCZ-NioTherms was conducted over a 24-h period to evaluate the stability and retention of the encapsulated PCZ. Throughout the study duration, no visible signs of drug precipitation or crystallization were observed within the formulation in the release media. PCZ-NioTherms showed $\sim 35 \%$ drug release at the end of 24 h whereas a rapid release of 100 % was observed from the PCZ-dispersion (Fig. 3G). It is worth noting that this study primarily focused on assessing the stability and structural integrity of the NioTherm vesicles under the test conditions. The findings confirmed that the PCZ-NioTherms maintained their stability over 24 h, effectively retaining the drug without significant leakage.

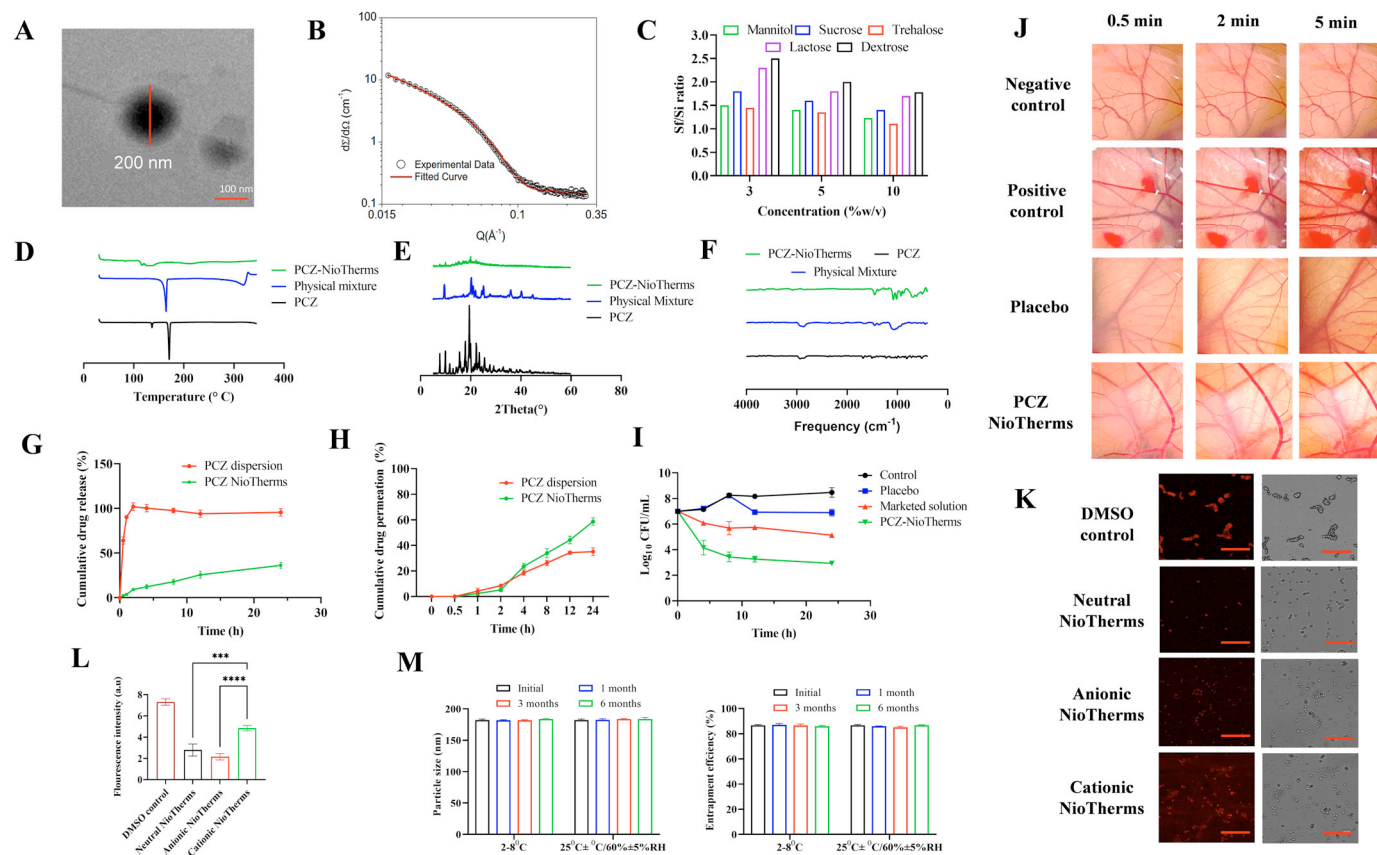


Fig. 3 – Evaluation of PCZ-Niotherms. (A) Representative TEM image depicting the spherical morphology and particle size (~ 180 nm) of PCZ-Niotherms; (B) SANS analysis confirming the vesicular structure of PCZ-Niotherms; (C) Cryoprotectant screening showing lowest S/Si ratio in 5 % (w/v) mannitol; (D-F) DSC, XRD and FTIR plots confirming encapsulation of PCZ in Niotherms by absence of characteristic drug peaks in the respective spectra; (G) *In vitro* drug release over a period of 24 h showing physical stability of PCZ-Niotherms (mean $\pm$ SD, $n=3$); (H) *Ex vivo* trans-corneal permeation through goat cornea ($n=3$); (I) *In vitro* time kill assay against *Candida albicans* MCC 1794 ($n=3$); (J) Representative images of *in vitro* irritation study using HET-CAM ($n=3$); (K) CLSM images of *Candida albicans* stained with RhB tagged Niotherms to indicate uptake of Niotherms by fungal cells (Scale bar: 50 μm); (L) Fluorescence intensity of uptaken Niotherms quantified using ImageJ ($n=3$); (M) Stability of lyophilized PCZ-Niotherms with respect to particle size and entrapment efficiency over a period of 6 months as per ICH guidelines ($n=3$). **** $P < 0.0001$, ** $P < 0.01$, ns: non-significant.

3.5.4. *Ex vivo* corneal permeation

To realize the kinetics of *in vitro* drug release in the previous section, permeation of PCZ from NioTherms was compared to that of a PCZ dispersion. After 24 h, the PCZ dispersion exhibited approximately 35 % transcorneal permeation, while PCZ-NioTherms showed a significantly higher permeation of around 60 % (Fig. 3H). This remarkable elevation in the permeation of PCZ across the corneal membrane may be attributed to the nano-size of the vesicles efficiently carrying hydrophobic PCZ across the corneal membrane. An initial lag was noted in the permeation of PCZ-NioTherms as compared to the PCZ dispersion since the formulation was administered in the form of *in situ* gel, increasing the retention time by sol-gel conversion post administration. We hypothesized that this process was complemented by electrostatic interactions between the mucin layer and *in situ* generated PCZ-NioTherm gel phase.

3.5.5. *In vitro* mucin binding and charge mediated adhesion

The role of mucin binding in improving retention of NioTherms was tested by measuring the change in nanoparticle surface charge after incubation with mucin solution. Mucin carries a net negative charge due to the presence of aspartate and glutamate residues on its surface. Following incubation with 0.5 % mucin, only positively charged NioTherms showed a net decrease in zeta potential asserting functional role of stearylamine in the formulation (Fig. 4A). To further validate this finding, the amount of free mucin post incubation was quantified using UV spectrophotometry. Compared to neutral and anionic NioTherms, a 2-fold reduction in total free mucin content was recorded and was indicative of a net neutralization of charges between mucin and stearylamine containing cationic NioTherms (Fig. 4B). These interactions between mucin and stearylamine were further validated using molecular modelling to evaluate the interaction between cationic head amino group of stearylamine and the carboxylic acid groups in mucin. A binding energy of -5.4 kcal/mol between stearylamine and mucin (PDB ID: 9GVQ) indicated a strong interaction. The observed interactions between the amine group of stearylamine and neighbouring carboxylic acid groups in mucin (TYR A:835, SER A:836, SER B:457, THR B:602, GLN B:603, THR B:491, and GLU B:474) were facilitated by electrostatic and hydrogen bond interactions (Table S5 and Fig. S5). These molecular modelling results closely align with the *in vitro* mucin binding data, as reflected by the changes in zeta potential shown in Fig. 4B. These findings suggest a strong positive correlation between cationic surface charge density and the extent of mucin interaction.

3.5.6. *In vitro* fungal cell internalization

Next, we studied the impact of surface charges on internalization of nanoparticles in fungal cells using confocal microscopy (Fig. 3K). The nanoparticle fluorescence intensity was quantified using ImageJ which showcased that both uncharged and negatively charged NioTherms showed lower fluorescence intensity in contrast to the cationic NioTherms which exhibited a 3-fold higher fluorescence intensity (Fig. 3L). Higher cellular uptake was attributed to enhanced

electrostatic interaction of positive surface charges with the negatively charged fungal cell wall. This suggests that the positive charge facilitated better membrane penetration, highlighting the importance of surface charges and the potential of cationic NioTherms as effective carriers for delivering PCZ to fungal cells by improving drug delivery efficiency [31].

3.5.7. *In vitro* Time kill assay

A Time kill assay was performed to better understand the *in vitro* time dependent efficacy of PCZ NioTherms. Both PCZ NioTherms and PCZ solution demonstrated a significant reduction ($P < 0.05$) in fungal burden as compared to the control. At later time points, marginal fungicidal activity was observed in the placebo group, likely due to the fungicidal effect of surfactants present in the formulation [32]. Nevertheless, PCZ NioTherms demonstrated a 2-fold greater reduction in fungal burden compared to the PCZ solution, likely attributed to the nanoscale size of the particles, which facilitated faster and more efficient uptake by fungal cells (Fig. 3I).

3.5.8. *In vitro* irritancy evaluation

Unwarranted irritancy of the developed formulation was evaluated by closely monitoring key indicators such as haemorrhage, lysis, and coagulation on the chorioallantoic membrane of fertilized eggs [33,34]. Upon visual inspection, our lead formulation exhibited no signs of irritation (irritation score of 3) and was comparable to the placebo and negative control (0.9 % saline) groups [35]. On the contrary, the positive control (1 % NaOH solution) caused significant damage within 30 s of administration including immediate haemorrhage, lysis and coagulation (Fig. 3J). These findings confirmed the safety profile of the formulation in terms of irritation potential, supporting its suitability for further application.

3.5.9. Stability studies

PCZ-NioTherms exhibited stability, with no significant changes in particle size or entrapment efficiency observed over the six-month study period, indicating that the formulation remained stable under the conditions tested (Fig. 3M).

3.5.10. *In vivo* mucoadhesion

To supplement our *in vitro* results, the effect of surface charges on the mucoadhesive properties was studied on rat eyeballs *in vivo* using CLSM. The fluorescence of coumarin-6 tagged uncharged, anionic and cationic NioTherms on the corneal surface are shown by representative images in Fig. 4C. The fluorescence intensity of positively charged NioTherms was significantly higher as compared to the uncharged (1.5-fold) and negatively charged (2.1-fold) NioTherms during the initial time points (calculated using ImageJ). At the later time points, fluorescence intensity of the uncharged and negatively charged NioTherms drastically diminished whereas that of the positively charged NioTherms was retained (Fig. 4D) [36]. Such high fluorescence intensity, indicative of higher mucoadhesion, further validates our hypothesis of enhanced interactions and retention happening at the corneal surface.

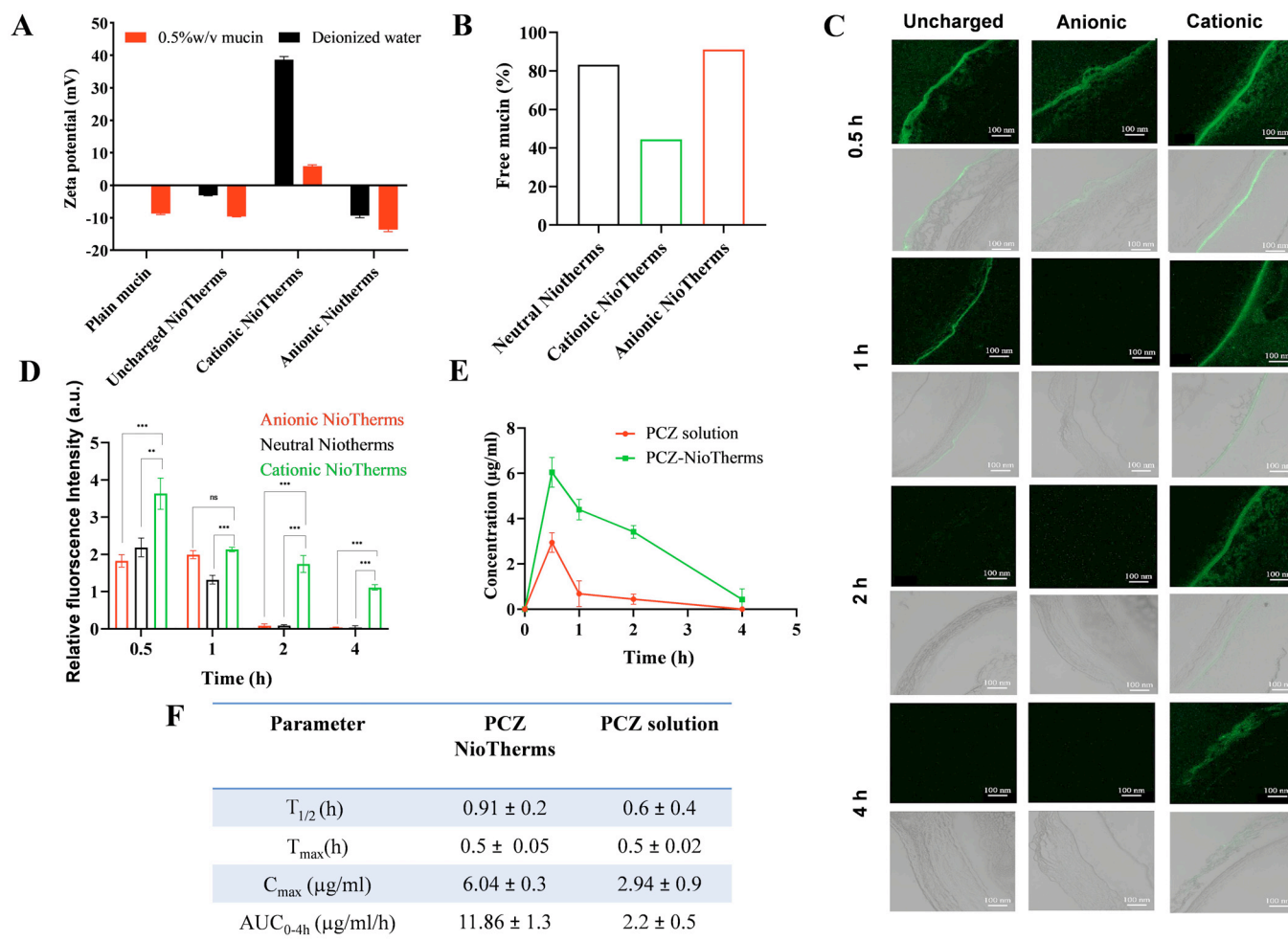


Fig. 4 – In vitro and in vivo ocular mucoadhesion of PCZ-NioTherms and ocular pharmacokinetics. (A) In vitro mucin binding assay by zeta potential measurements; (B) In vitro mucin binding assay by UV spectroscopy to analyse the unbound mucin; (C,D) Representative CLSM images of the sagittal cryo-sectioned rat eyeballs showing fluorescence intensity indicating adhesion of uncharged, anionic and cationic coumarin-6 loaded NioTherms at the corneal surface and their corresponding relative fluorescence intensity calculated using ImageJ software expressed as a bar graph; (E) Concentration versus time profile curve (ocular PK) of PCZ solution and PCZ-NioTherms over a period of 4 h determined in whole eyeball of SD rat; (F) Pharmacokinetic parameters of the ocular PK studies. Data are shown as (mean±SD, n=3). ** $P < 0.0001$, ** $P < 0.01$, ns: non-significant.**

3.5.11. Ocular pharmacokinetics

Since the cationic NioTherms depicted an enhanced *in vivo* mucoadhesion profile, we further evaluated the effect of higher retention of cationic PCZ-NioTherms by determining the concentration of PCZ in the whole eye bulb of rats. The concentration versus time profile of PCZ NioTherms and PCZ solution is shown in Fig. 4E. The concentration of PCZ NioTherms in the whole eye was found to be significantly higher ($P < 0.05$) than the PCZ solution during a period upto 2 h. The mean ocular pharmacokinetic parameters were analyzed with the help of PK Solver, version 2.0 and are listed in Fig. 4F. The results demonstrated a significant difference ($P < 0.05$) in the main PK parameters namely $T_{1/2}$, AUC_{0-4h} , C_{max} and T_{max} . It was found that the $T_{1/2}$, AUC_{0-4h} , C_{max} and T_{max} of PCZ-NioTherms were increased by 1.51-folds, 5.39-folds, 2-folds and 1.2-folds, respectively, as compared to the PCZ solution. These results can be attributed to the presence of positive

surface charges leading to enhanced retention as confirmed by mucoadhesion study, and nanosize of the vesicles leading to improved permeation across the corneal membrane, and therefore higher concentration in the rat eye balls. Herein, we demonstrate an extrapolation of the *ex vivo* permeation results in the *in vivo* model.

3.5.12. Repeat dose ocular toxicity

Histopathological evaluation of eye sections after 28 days of repeated dosing was conducted to assess any morphological changes indicative of irritation or toxicity to the eye tissue. Damage and loss of corneal epithelium was observed in the positive control group. In contrast, no significant morphological changes were observed in the eye tissues of animals treated with the developed placebo or PCZ NioTherm *in situ* gel, indicating the formulation's safety and non-toxic profile (Fig. 5H).

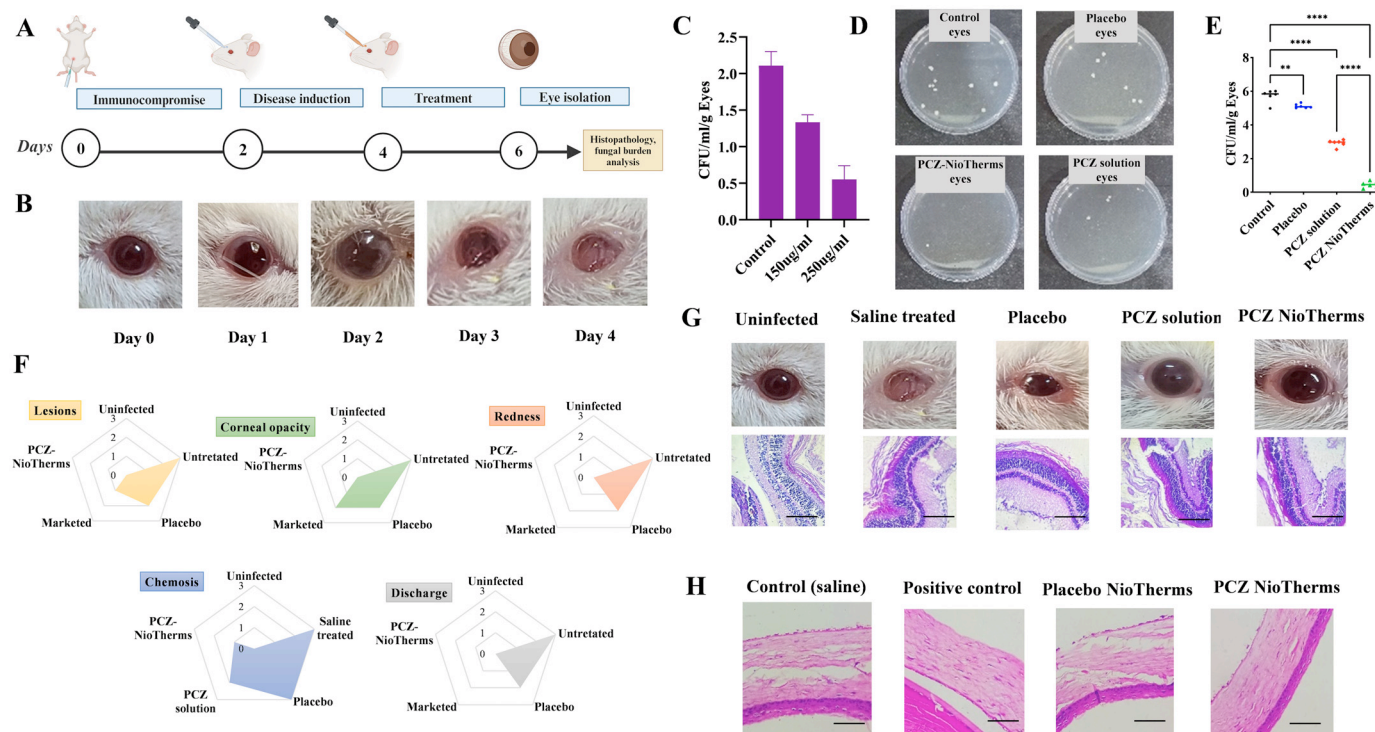


Fig. 5 – In vivo ocular antifungal efficacy in Swiss albino mice and toxicity study. (A) Diagrammatic representation of in vivo ocular antifungal efficacy study in Swiss albino mice; **(B)** Progression of ocular FK after inoculating the eyeballs with *Candida albicans* MCC 1794 suspension over a period of 4 d; **(C)** PCZ dose determination to obtain 50 % reduction in fungal burden in mice eye balls; **(D)** Representative SDA plates to depict the fungal burden post treatment in groups administered with control saline (top left), placebo NioTherms (top right), PCZ-solution (bottom left) and PCZ-NioTherms (bottom right); **(E)** Graph representing the fungal burden per gram of eye tissue in treatment groups (mean $\pm$ SD, n =6); **(F)** Radar plots representing lesions, corneal opacity, redness, chemosis and discharge as visual markers for intensity of infection in Swiss albino mice, where each apex of the plot represents either uninfected mice, saline treated mice, placebo treated mice, PCZ-solution treated mice or PCZ-NioTherms treated mice; **(G)** Representative photographs and their respective histopathological sections of eyeballs of uninfected mice, saline treated mice, placebo treated mice, PCZ-solution treated mice and PCZ-NioTherms treated mice; (Scale bar: 1 mm) **(H)** Histopathological sections of rat eyeballs stained with haematoxylin and eosin after 28 d repeated ocular administration of saline (Scale bar: 100 μ m), 1 % SLS (positive control), placebo NioTherms and PCZ-NioTherms respectively (left to right). (B) and (G) make use of similar control (uninfected mice) group for comparison purposes. ****P < 0.0001, **P < 0.01, ns: non-significant.

3.5.13. *In vivo* efficacy studies

The *in vivo* ocular pharmacodynamic activity was tested as per the designed protocol (Fig. 5A). The *in vivo* efficacy of the treatment was assessed by evaluating visual observations, fungal burden, and histopathological analysis of eye sections. Initially, the fungal concentration required to induce infection was determined by monitoring morphological changes, including lesion severity, corneal opacity, redness, chemosis and discharge [37]. Significant morphological changes, including corneal opacity and lesions particularly on Day 4 post-infection, were observed in the animals infected with a fungal inoculum of 10^6 CFU/ml indicating progressive destruction of corneal cells due to fungal colonization (Fig. 5B).

Subsequent treatment with PCZ solutions at concentrations of 150 and 250 $\mu\text{g/ml}$ revealed that 250 $\mu\text{g/ml}$ was necessary to achieve a 50 % reduction in fungal burden when compared to the animals without treatment, which was then used for further experiments (Fig. 5C).

After confirming the effectiveness of PCZ at 250 $\mu\text{g/ml}$, PCZ-NioTherm *in situ* gel and PCZ solution were administered at the same concentration to evaluate the potential of NioTherms as a drug delivery system. We observed a significant reduction ($P < 0.05$) in fungal load in the group treated with *in situ* gelling PCZ-NioTherm formulation (1 CFU/ml/g of eye tissue) compared to the control group (6 CFU/ml/g of eye tissue). The PCZ NioTherm *in situ* gel formulation outdid the PCZ solution by showing 3-folds higher fungal load reduction (Fig. 5D and E). This enhanced efficacy is attributed to the nanonization that facilitates permeation through the corneal surface. Interestingly, the interaction of cationic PCZ NioTherms with the mucin layer, as demonstrated in the *in vitro* studies, translated effectively to *in vivo* results in the animal model. This observation was substantiated in the animal model, where the cationic NioTherms demonstrated a similar action *in vivo*, leading to increased drug retention at the ocular surface leading to better activity. An extrapolation of *in vitro* fungal cell uptake to the *in vivo* experiments was strongly evidenced and correlated by a greater reduction in fungal burden and enhanced therapeutic outcomes. Additionally, the improved therapeutic effect of the developed system may be attributed to the thermoresponsive sol-gel transformation resulting in an increased residence time. The visual inspection was scored as per the clinical grading system for FK to better understand and quantify these visible changes on the corneal surface (Fig. 5F). Visual analysis of the eyes before and after treatment indicated that the morphology was significantly restored in the group treated with PCZ-NioTherms compared to the PCZ solution. Histological examination of infected eyes revealed presence of yeast on the corneal epithelium and stroma was observed in saline treated animals. Infected corneas typically contained inflammation, fungal involvement and destruction of stroma. The PCZ NioTherm treated eyes recovered the damaged tissues and improved the pathological lesions in comparison with the placebo NioTherms and PCZ solution (Fig. 5G). Overall, the *in vivo* results demonstrated the potential of cationic NioTherms as an effective carrier for ocular administration of anti-infective drugs like PCZ.

4. Conclusion

The application of nanoparticles via ocular route is well established, with several formulations already approved and others in clinical trials. However, a significant gap remains between research and market translatability, largely due to challenges in scaling up preparation methods. In this study, we developed a simple and green method for formulating a surfactant-based nanovesicular system with robust structural integrity, reproducibility, and possible scalability, offering a platform for formulation of such vesicles. A BCS II drug, PCZ was successfully encapsulated in the surfactant based NioTherms, and the challenges associated with the physiochemical properties of PCZ for ocular delivery were addressed. The results obtained from *in silico* and *in vitro* studies were in agreement and established a robust correlation between the computational and experimental methods. The QbD approach provided a better handle over formulation variables and allowed for the precise formulation optimization based on desired constraints. *In situ* sol-gel transition induced by cationic NioTherms demonstrated strong mucoadhesion, excellent corneal permeation and prolonged residence time on the ocular surface, enhanced interaction with the fungal cell wall and cellular internalization. We also present an excellent corroboration between the *in vitro* anti-fungal activities and the *in vivo* efficacy data by fungal burden reduction thereby achieving therapeutic benefit in ocular FK mice model. Overall, PCZ-NioTherm formulation system demonstrated a safe, non-irritant and non-toxic alternative to the existing therapeutic interventions for the treatment of FK.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ajps.2025.101087](https://doi.org/10.1016/j.ajps.2025.101087). The figures and tables with “S” before the serial number are included in the Supplementary material.

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