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ONE-POT FABRICATION OF A BETAXOLOL HYDROCHLORIDE-LOADED NANOSTRUCTURED LIPID CARRIER THERMO-RESPONSIVE OCULAR NANOGEL FOR ENHANCED CORNEAL PERMEATION AND RETENTION

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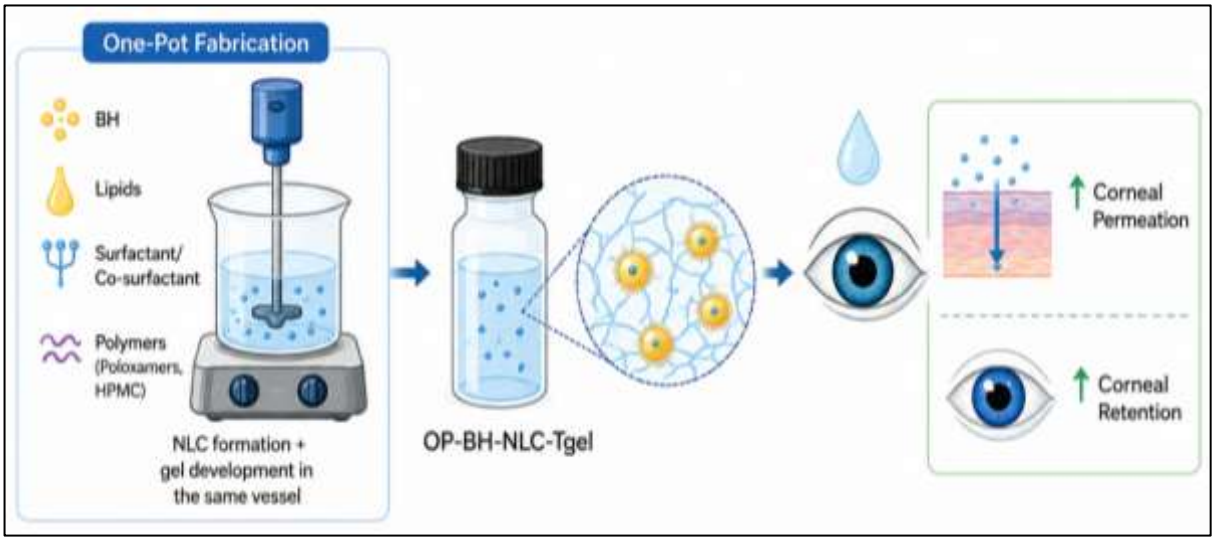
ABSTRACT

Topical treatment of glaucoma is limited by rapid precorneal clearance and poor ocular retention of conventional eye drops. The present study aimed to develop a betaxolol hydrochloride (BH)-loaded nanostructured lipid carrier (NLC) thermo-responsive ocular nanogel using a one-pot fabrication approach integrating NLC formation and gel development within the same processing vessel. The optimized OP-BH-NLC-Tgel formulation exhibited a particle size of approximately 178.27 ± 5.56 nm, low polydispersity, high entrapment efficiency (~96%), and a thermo-responsive gelation temperature of approximately 32–33 °C. The formulation showed pseudoplastic flow and controlled drug release, with only 18% BH release at 30 min compared with 46.2% from Betoptic® 0.25%. Ex vivo evaluation demonstrated significantly enhanced corneal drug permeation compared with Betoptic® 0.25% ($p < 0.05$) and 2.48-fold greater corneal BH retention. Histopathological examination revealed preservation of corneal architecture without apparent tissue damage following formulation exposure. Furthermore, the optimized formulation exhibited satisfactory preliminary physicochemical stability under room-temperature conditions (25–30 °C) for 6 months, with only minor changes in the evaluated parameters. Overall, the one-pot fabrication approach successfully generated a stable thermo-responsive BH-NLC nanogel with controlled drug release and enhanced corneal permeation and retention. The developed system represents a promising topical ocular delivery platform for improving the residence and delivery of BH in glaucoma management.

GRAPHICAL ABSTRACT



KEYWORDS: Betaxolol hydrochloride, Nanostructured lipid carriers, One-pot fabrication, Thermo-responsive nanogel, Ocular drug delivery, Glaucoma, Corneal permeation

INTRODUCTION

Topical ocular drug delivery remains challenging because rapid tear turnover, blinking, nasolacrimal drainage, and the corneal barrier substantially limit drug residence and transport following instillation. These clearance mechanisms can reduce the fraction of administered drug available at the ocular surface and often necessitate repeated dosing, particularly

in chronic disorders such as glaucoma. Consequently, the development of ophthalmic formulations capable of increasing precorneal residence while maintaining drug availability for transcorneal transport remains an important formulation objective [1-3].

Nanostructured lipid carriers (NLCs), composed of a mixture of solid and liquid lipids, have emerged as promising platforms for ocular drug delivery because their less ordered lipid matrix can accommodate drug molecules and provide opportunities for controlled drug release [4]. Their nanoscale dimensions and lipidic composition may promote intimate interaction with the corneal surface and facilitate drug partitioning across ocular barriers. However, NLCs administered as aqueous nano dispersions remain susceptible to precorneal elimination. Thus, although NLCs can address drug incorporation, release, and transport, prolonging their residence at the ocular surface remains an additional formulation requirement [5].

Thermo-responsive in-situ gels offer a complementary strategy by converting from a low-viscosity formulation before administration into a more viscous gel following exposure to ocular-surface temperature [6, 7]. Poloxamer 407 (P407) is particularly attractive for this purpose because of its temperature-dependent micellization and reversible sol–gel transition. Poloxamer 188 (P188) and viscosity-modifying polymers such as hydroxypropyl methylcellulose (HPMC) can further modulate gelation behaviour and gel strength [6–8]. Thermo-responsive ocular systems are therefore capable of combining convenient instillation with prolonged residence and sustained drug release. Recent reviews have highlighted P407/P188-based thermosensitive systems as an established approach for improving ocular retention and controlling drug release [7].

Combining an NLC with a thermo-responsive in-situ gel provides a complementary delivery architecture in which the lipid nanocarrier contributes to drug incorporation, nanoscale drug delivery and release modulation, while the thermo-responsive polymeric matrix provides prolonged precorneal residence through temperature-triggered sol–gel transition [8, 9]. Recent studies have demonstrated the potential of NLC-loaded in-situ gels for ophthalmic delivery, with such hybrid systems showing sustained drug release, improved ocular retention and favorable rheological characteristics compared with conventional formulations [10]. In particular, recent work on NLC-loaded ocular in-situ gels has highlighted the ability of combining lipid nanocarriers with a polymeric gel matrix to simultaneously address nanocarrier-mediated drug delivery and the limited residence time associated with conventional ophthalmic preparations [8].

Thermosensitive in-situ gels have received increasing attention because they can be administered as low-viscosity liquids and subsequently undergo gelation at ocular-surface temperature. Poloxamer 407 is one of the most widely investigated thermo-responsive polymers, while Poloxamer 188 and cellulose derivatives can be incorporated to modulate gelation temperature, gel strength, viscosity and retention characteristics [9, 10]. A recent 2026 review emphasized that sol–gel transition temperature and time, rheological behaviour, clarity, gelling capacity and ocular tolerability are critical quality attributes in the development of thermosensitive ophthalmic systems [9]. Thus, the successful integration of an NLC with a thermo-responsive matrix requires simultaneous control of both nanocarrier characteristics and gel-network behaviour.

Most reported NLC–in-situ-gel systems have employed a sequential manufacturing approach, in which the NLC is initially prepared and characterized before being incorporated into a separately prepared polymeric gel system [4, 8]. For example, recent ophthalmic studies have prepared optimized NLCs as an intermediate formulation and subsequently incorporated them into an in-situ gel containing polymeric gelling agents [8]. Although this approach enables independent optimization of the nanocarrier and gel phases, it introduces additional processing operations involving transfer, dilution, cooling and mixing. Such additional operations may influence the final dispersion characteristics and can increase the number of processing steps between preparation of the nanocarrier and the final dosage form.

From a formulation-development perspective, the manufacturing pathway itself can influence the characteristics of nanocarrier-based systems, because nanoparticle formation is governed by the interplay among composition, temperature, mixing, energy input and the physicochemical environment during particle formation. Consequently, incorporation of a preformed NLC into a polymeric network is not necessarily equivalent to generating the nanocarrier within the formulation environment in which the final gel is formed. Recent advances in lipid-based ocular nanocarriers have increasingly emphasized the importance of formulation architecture, interfacial behaviour, ocular bio-interactions and manufacturing considerations in determining the performance of these systems [4, 5].

A one-pot fabrication strategy provides a process-oriented alternative in which NLC formation and development of the thermo-responsive gel matrix are integrated within a common manufacturing sequence. Such integration has the potential to reduce intermediate handling and transfer operations and may provide a more direct route to the final dosage form. However, simplification of the manufacturing sequence cannot itself be considered evidence of improved formulation performance. The influence of one-pot processing must therefore be established experimentally by assessing whether the integrated process can maintain the critical quality attributes of the lipid nanocarrier while simultaneously producing the required thermo-responsive and rheological characteristics of the final ocular formulation [11, 12].

Importantly, although NLC-loaded in-situ gels have received increasing attention in ocular drug delivery, recent studies have primarily focused on formulation composition, optimization, and characterization of the final NLC–gel system,

rather than investigating fabrication sequence as an independent process-related variable [8, 10]. For example, Sabale et al. developed a loratadine NLC-loaded ocular in-situ gel by first preparing and optimizing the NLC and subsequently incorporating the optimized NLC into a polymeric in-situ gel system [8]. Similarly, Singh et al. reported a vancomycin hydrochloride-loaded NLC incorporated into a thermo-responsive in-situ gel, with formulation variables optimized using a Box–Behnken design and the resulting system evaluated for physicochemical and permeation characteristics [10]. These studies establish the feasibility and potential advantages of NLC–gel combinations for ocular delivery; however, they also illustrate the predominantly sequential formulation paradigm, in which the nanocarrier and gel phases are developed as separate formulation components. Consequently, whether integration of nanocarrier formation and thermo-responsive gel development within a single manufacturing sequence can maintain the critical quality attributes and functional performance of the final ocular system remain insufficiently investigated. Betaxolol hydrochloride (BH) was selected as the model drug in the present study because its conventional topical administration is subject to the general limitations of precorneal clearance and limited ocular residence [13, 14]. Moreover, thermosensitive and nanocarrier-based approaches have previously demonstrated potential for improving the ocular delivery and pharmacodynamic performance of BH [13]. The present work therefore extends that platform by investigating a different manufacturing strategy rather than redeveloping the NLC composition.

Accordingly, the present study aimed to develop and evaluate a one-pot BH-loaded NLC thermo-responsive nanogel (OP-BH-NLC-Tgel) in which nanocarrier formation and thermo-responsive gel development were integrated into a common manufacturing sequence. The study specifically investigated whether this process-integrated approach could produce suitable nanoparticle characteristics, thermo-responsive gelation, rheological behaviour, controlled drug release, trans-corneal permeation and short-term stability. The novelty of the present work lies in evaluating fabrication sequence as a process-related variable and establishing a one-pot manufacturing strategy for an NLC-based thermo-responsive ocular nanogel, rather than simply incorporating a preformed NLC into a separately prepared gel matrix.

2. MATERIALS AND METHODS

2.1 Materials

Betaxolol hydrochloride (BH; assay 99%, pharmacopeial grade) was kindly supplied by Indoco Remedies Ltd., Patalganga, India. Glyceryl monostearate (GMS), Tween 20 (T20), and Transcutol® P (TC) were used as the solid lipid, surfactant, and co-surfactant, respectively. Tocopheryl acetate (TA; 65% assay) was procured from Medley Pharmaceuticals Ltd., Mumbai, India. Poloxamer 407 (P407), Poloxamer 188 (P188), and Carbopol 940 were obtained from Sigma-Aldrich, USA, while hydroxypropyl methylcellulose K4M (HPMC K4M) was also procured from Sigma-Aldrich, USA. P407, P188, and HPMC K4M were used for development of the thermo-responsive in-situ gel system. EDTA was used as a chelating agent. Sodium chloride, sodium bicarbonate, calcium chloride dihydrate, sodium hydroxide, and other chemicals and solvents used for formulation development and evaluation were of analytical grade. All materials were used as received without further purification. Simulated tear fluid (STF) was prepared using sodium chloride (0.67 g), sodium bicarbonate (0.20 g), and calcium chloride dihydrate (0.008 g) dissolved in 100 mL of deionized water.

2.2 Screening and Selection of Excipients

The excipients for development of the one-pot BH-NLC thermo-responsive nanogel (OP-BH-NLC-Tgel) were screened individually according to their intended functional role in the formulation. The screening comprised selection of the solid lipid, liquid lipid, surfactant, co-surfactant, and thermo-responsive polymeric components. The solid lipid was selected by visual examination of drug incorporation into the molten lipid, whereas the liquid lipid, surfactant, and co-surfactant were screened quantitatively for their ability to solubilize betaxolol hydrochloride (BH) using UV spectrophotometry [15]. The selected excipients were subsequently incorporated into the one-pot NLC– thermo-responsive gel system.

Screening of solid lipids: Candidate solid lipids were screened initially for their ability to incorporate BH into the molten lipid phase. An excess amount of BH was added separately to each candidate solid lipid and the mixtures were heated to a temperature above the respective melting point of the lipid to obtain the molten state. Each mixture was maintained under continuous mixing until the drug was uniformly dispersed in the molten lipid. The molten systems were then visually examined for the presence or absence of undissolved drug particles, drug crystallization, precipitation, or formation of an opaque or non-uniform dispersion. A clear and homogeneous molten system without visible drug crystals was considered indicative of adequate drug incorporation into the lipid phase. The solid lipid exhibiting the most satisfactory visual incorporation of BH and suitable handling characteristics for NLC preparation was selected for subsequent formulation development. Visual assessment of drug solubilization in molten solid lipids is an established preliminary approach for screening lipid excipients for lipid nanoparticle development [16].

Screening of liquid lipids & surfactants: Candidate liquid lipids were evaluated quantitatively for BH solubilization using the validated UV spectrophotometric method. A known excess amount of BH was added to a predetermined quantity of each liquid lipid and the mixtures were subjected to equilibration under controlled conditions with intermittent mixing to facilitate drug–lipid interaction. Following equilibration, the samples were centrifuged to separate undissolved drug from the lipid phase. The clear supernatant was carefully separated and appropriately diluted with a suitable solvent to bring the drug concentration within the validated linearity range of the analytical method. The absorbance of each diluted sample was measured at 272 nm using the validated UV spectrophotometric method, and the corresponding BH concentration was calculated from the previously established calibration equation. The solubility of

BH in each liquid lipid was expressed as the amount of drug solubilized per unit quantity or volume of lipid. The liquid lipid exhibiting the highest suitable BH solubilization capacity, together with acceptable physical compatibility with the selected solid lipid, was selected for subsequent NLC fabrication. Solubility-based screening of liquid lipids is routinely employed to identify lipid components capable of providing adequate drug incorporation and retention within NLC matrices. A similar procedure was adopted to screen suitable surfactants and co-surfactants for BH. Systematic screening of surfactants based on BH solubilization and their ability to produce stable lipid dispersions has been described as an important preliminary step in NLC formulation development [17, 18].

2.3 Screening of thermo-responsive polymers: Following selection of the lipid and surfactant/co-surfactant components, the thermo-responsive polymeric phase was established by sequential manual screening of Poloxamer 407 (P407), Poloxamer 188 (P188), and hydroxypropyl methylcellulose K4M (HPMC K4M) [19]. P407 was screened initially at concentrations of 17, 18, 19, 20, 21, and 22% w/v while maintaining P188 and HPMC K4M at 0.35 and 0.50% w/v, respectively. Each polymeric composition was incorporated into the corresponding formulation and evaluated for physical appearance, homogeneity, pH, particle size, polydispersity index (PDI), entrapment efficiency (EE), gelation temperature, gelation time, and viscosity. The P407 concentration providing an appropriate balance between low viscosity and flowability before administration and reproducible gelation near physiological ocular temperature was selected for subsequent screening. Concentration-dependent changes in P407 are known to markedly influence the sol–gel transition temperature and rheological behavior of thermo-responsive ophthalmic systems. The selected P407 concentration was subsequently maintained constant while P188 was screened at 0.20, 0.30, 0.40, and 0.50% w/v, with HPMC K4M maintained at 0.50% w/v. The resulting formulations were evaluated for appearance, homogeneity, pH, particle size, PDI, EE, gelation temperature, gelation time, and viscosity. P188 was incorporated to modulate the gelation characteristics of P407 and to obtain a thermo-responsive system with a sol–gel transition suitable for ocular administration. Previous studies have demonstrated that incorporation of P188 can modify the gelation temperature and rheological characteristics of P407-based ophthalmic systems. Finally, HPMC K4M was screened at concentrations of 0.25, 0.50, and 0.75% w/v while maintaining the selected concentrations of P407 and P188. The resulting formulations were evaluated for appearance, pH, viscosity, gelation temperature, gelation time, rheological behavior, and gel integrity following dilution with simulated tear fluid (STF). HPMC K4M was included as a viscosity- enhancing polymer to improve the mechanical strength and rheological characteristics of the thermo-responsive system. The concentration providing adequate viscosity and gel strength after gelation without compromising ease of administration at room temperature was selected. The use of HPMC in combination with P407/P188 for ophthalmic in- situ gels has previously been reported to modify viscosity, gelation behavior, rheological properties, and ocular performance [20]. At each stage of thermo-responsive polymer screening, only the concentration of the polymer under investigation was varied, while the concentrations of previously selected polymers and all other formulation components were maintained constant. The final polymeric composition was selected based on the combined assessment of physicochemical characteristics, gelation temperature, gelation time, viscosity, rheological behavior, and resistance to STF dilution. The selected excipient composition was subsequently used for one-pot fabrication of OP-BH-NLC-Tgel. The Composition and sequential screening design for OP-BH-NLC-Tgel is given Table 1.

Table 1. Composition and sequential screening design for OP-BH-NLC-Tgel

Screening stage	Batch	P407 (% w/v)	P188 (% w/v)	HPMC K4M (% w/v)
Fixed formulation composition	BH: 25 mg; GMS: 100 mg; TA: 100 mg; Tween 20: 111.11 mg; Transcutol P: 88.89 mg; final volume: 20 mL			
P407 screening	P407-1	17	0.35	0.50
	P407-2	18	0.35	0.50
	P407-3	19	0.35	0.50
	P407-4	20	0.35	0.50
	P407-5	21	0.35	0.50
	P407-6	22	0.35	0.50
P188 screening	P188-1	Selected P407	0.20	0.50
	P188-2	Selected P407	0.30	0.50
	P188-3	Selected P407	0.40	0.50

	P188-4	Selected P407	0.50	0.50
HPMC K4M screening	HPMC-1	Selected P407	Selected P188	0.25
	HPMC-2	Selected P407	Selected P188	0.50
	HPMC-3	Selected P407	Selected P188	0.75

Note: "Selected P407" and "Selected P188" indicate the concentrations identified during the preceding screening stage and maintained constant during the subsequent stage.

2.4 One-pot fabrication of BH-loaded NLC-based thermo-responsive nanogel

The BH-loaded NLC-based thermo-responsive nanogel was fabricated using an integrated one-pot approach in which formation of the lipid nanocarrier and subsequent development of the thermo-responsive gel matrix were performed sequentially within the same processing vessel, without isolation or transfer of the intermediate NLC dispersion [10, 21]. Briefly, the required quantities of glyceryl monostearate (GMS) and tocopheryl acetate (TA) were accurately weighed and heated to 75 ± 2 °C using a thermostatically controlled water bath until a homogeneous molten lipid phase was obtained. Betaxolol hydrochloride (BH; 25 mg) was incorporated into the molten lipid phase and sonicated for 5 min to facilitate uniform drug distribution. In parallel, the required quantities of Tween 20 and Transcutol® P were incorporated into the aqueous phase and maintained at 75 ± 2 °C for temperature equilibration with the lipid phase. The hot lipid phase containing BH was gradually added to the aqueous phase under continuous mixing to form a coarse emulsion. The resulting emulsion was subjected to high-speed homogenization at 15,000 rpm for 60 min, while maintaining the processing temperature at 75 ± 2 °C. High-energy homogenization followed by probe sonication is an established approach for reducing the dimensions of lipid dispersions and producing nanosized lipid carriers [11]. The homogenized dispersion was subsequently subjected to probe sonication at 40% amplitude for 1 min in continuous mode to further reduce the particle size and generate the BH-loaded NLC dispersion. The NLC dispersion was retained in the same processing vessel and was not subjected to intermediate separation, centrifugation, drying, or transfer. Following sonication, the dispersion was gradually cooled under gentle continuous stirring to 4 ± 2 °C. Once the formulation reached the refrigerated temperature, P407 was incorporated gradually under gentle stirring, followed sequentially by P188 and HPMC K4M (0.5% w/v). The polymeric components were incorporated in the order P407 → P188 → HPMC K4M to establish the thermo-responsive polymeric network. EDTA was subsequently incorporated under gentle stirring, and mixing was continued until a homogeneous formulation was obtained. The formulation was maintained at 4 ± 2 °C for 12–16 h to facilitate complete hydration and equilibration of the polymeric components. The final volume was adjusted to 20 mL using cold distilled water, followed by gradual adjustment of the formulation pH to 6.5–7.0 using dilute sodium hydroxide solution, where required. The formulation was subsequently maintained at 4 ± 2 °C for 2 h for final equilibration before characterization. Throughout the fabrication process, temperature was monitored using a calibrated digital temperature probe, while gentle agitation was maintained during cooling, polymer incorporation, volume adjustment, and pH adjustment to minimize aggregation and air entrapment. The final formulation was visually inspected for homogeneity, phase separation, aggregation, and visible particulate matter and was designated as OP-BH-NLC-Tgel.

2.5 Physicochemical characterization of OP-BH-NLC-Tgel

The test formulations were subjected to comprehensive physicochemical characterization to assess the influence of the integrated fabrication process on the critical quality attributes of the nanocarrier and the final thermo-responsive formulation. Particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, and surface morphology were evaluated for the NLC component, while gelation temperature, rheological behavior, dilution stability, drug-release characteristics, ex-vivo corneal permeation, and stability were subsequently evaluated for the final nanogel. These parameters were selected because particle size and size distribution, surface charge, morphology, drug loading/entrapment, physical stability, and release behavior are recognized as important attributes for comprehensive characterization of lipid nanocarriers [22].

2.5.1 Particle size, polydispersity index and zeta potential

The mean hydrodynamic particle size and PDI of the NLC dispersion obtained during the one-pot fabrication process were determined by dynamic light scattering (DLS), while zeta potential was measured by electrophoretic light scattering using a Zetasizer (Malvern Instruments Ltd., UK) [23]. Prior to measurement, an aliquot of the formulation was appropriately diluted with filtered distilled water to avoid multiple scattering and placed in a disposable low-volume cuvette. The measurements were performed at 25 ± 1 °C after equilibration of the sample, and the mean of three independent measurements was recorded. Particle size was expressed as mean hydrodynamic diameter (nm), PDI as a dimensionless measure of particle-size distribution, and zeta potential as surface charge (mV). Measurements were performed in triplicate using freshly prepared samples. DLS-based particle-size distribution and zeta-potential measurements are routinely employed for characterization of NLC systems and provide important information regarding particle population and colloidal stability [22].

2.5.2 Entrapment efficiency

The entrapment efficiency (EE) of BH in test formulation was determined by an indirect method based on quantification of the unentrapped drug in the aqueous phase. A predetermined quantity of the freshly prepared formulation was subjected to an appropriate separation procedure to separate the NLC-associated drug from free BH. The concentration

of unentrapped BH in the separated aqueous phase was determined using the validated UV spectrophotometric method at 272 nm. The percentage entrapment efficiency was calculated using:

EE (%) =[W_t−W_f]/ W_t ×100…. equation 1

where W_t represents the total amount of BH initially added to the formulation and W_f represents the amount of unentrapped BH present in the aqueous phase. The analysis was performed in triplicate. Indirect determination of entrapment efficiency through measurement of the unencapsulated drug fraction is an established approach for lipid nanoparticle systems [24].

2.6 Thermo-responsive gelation behavior

The thermo-responsive behavior test formulations were evaluated by determining the sol–gel transition temperature (T_{gel}) using the tube-inversion method, which has been widely employed for characterization of poloxamer-based thermo-responsive gel systems. In this method, the gelation temperature is identified as the temperature at which the formulation ceases to flow upon inversion of the sample container [25]. Briefly, 2 mL of the formulation was transferred into a clean glass test tube and equilibrated at the initial test temperature. The sample was gradually heated from 15 to 40 °C, and the formulation was allowed to equilibrate for 5 min at each temperature before the tube was inverted to assess its flow behavior. The temperature at which the formulation showed no visible flow upon inversion was recorded as the $T_{gel.}$ The determination was performed in triplicate, and the results were expressed as mean ± standard deviation. The selected temperature range was intended to encompass the transition from storage/handling conditions to the physiological ocular temperature. The effect of dilution with simulated tear fluid (STF) on the thermo-responsive behavior was also evaluated by mixing the formulation with STF at the predetermined formulation-to- STF ratio (25:7) and determining the T_{gel} using the same procedure. This assessment was included because dilution by tear fluid following ocular administration can alter the effective polymer concentration and consequently influence the sol–gel transition behavior [26].

2.7 Rheological characterization

The rheological behavior of the one-pot fabricated BH-NLC thermo-responsive nanogel (OP-BH-NLC-Tgel) was evaluated using a rotational rheometer (MCR 302, Anton Paar GmbH, Graz, Austria) equipped with an appropriate cone-and-plate or parallel-plate measuring system. Rheological characterization was performed to determine the flow behavior, viscosity, consistency, and temperature-dependent viscoelastic properties of the formulation, as these parameters are important for assessing ease of instillation at room temperature and gel strength and retention following exposure to physiological ocular temperature [27]. Prior to measurement, the required quantity of formulation was carefully loaded onto the measuring geometry, avoiding the incorporation of air bubbles, and allowed to equilibrate at the specified measurement temperature before data acquisition. The flow behavior of the formulation was evaluated by recording shear stress and apparent viscosity as a function of increasing shear rate at 25 °C and 37 °C, representing conditions before and after ocular administration, respectively. The obtained flow curves were fitted to the power-law model,

$$\tau = K\gamma^n \ldots. \text{ equation 2}$$

where τ represents shear stress, γ represents shear rate, K represents the consistency index, and n represents the flow behavior index.

A value of $n < 1$ was considered indicative of pseudoplastic or shear-thinning behavior, which is desirable for ophthalmic in-situ gels because the formulation can exhibit relatively higher viscosity under low-shear conditions while becoming less viscous during instillation and blinking [28]. The rheological measurements were performed in triplicate, and the results were expressed as mean ± standard deviation. The rheological characteristics of OP-BH-NLC-Tgel were subsequently compared with those reported for the previously developed formulation to determine whether the one-pot fabrication strategy preserved the desired flow and viscoelastic characteristics of the thermo-responsive nanogel.

2.8 Effect of simulated tear fluid dilution on formulation behavior

The effect of STF dilution on the physicochemical and rheological behavior of OP-BH-NLC-Tgel was evaluated to simulate the changes occurring following ocular instillation and subsequent mixing of the formulation with the precorneal tear film. STF was prepared using sodium chloride (0.67 g), sodium bicarbonate (0.20 g), and calcium chloride dihydrate (0.008 g) dissolved in deionized water to a final volume of 100 mL, as described previously. An appropriate quantity of OP-BH-NLC-Tgel was mixed with STF at the predetermined formulation: STF ratio of 40:10 (v/v) under gentle stirring to obtain the diluted formulation. The mixture was allowed to equilibrate at 34–37 °C for 10 min before evaluation. The effect of dilution was assessed by determining the gelation temperature and rheological behavior of the diluted formulation using the same procedures described for the undiluted formulation. The gelation temperature was determined by the tube-inversion method, whereas the rheological behavior was evaluated at the selected temperatures representing pre- and post-ocular administration conditions. The percentage retention of the relevant parameter after STF dilution was calculated relative to the corresponding value obtained for the undiluted formulation. STF dilution studies are particularly relevant for ophthalmic in-situ systems because dilution with tear fluid can modify polymer concentration, ionic environment, viscosity, and gel strength after administration [29]. The ability of OP-BH-NLC-Tgel to retain an appropriate thermo-responsive transition and rheological profile following STF dilution was therefore considered an important indicator of its suitability for ocular administration. The results obtained after dilution were compared with those of the undiluted formulation and with the corresponding values reported for the previously developed formulation, thereby allowing assessment of whether the one-pot fabrication strategy preserved the functional behavior of the thermo-

responsive system.

2.9 In-vitro drug release study

The in-vitro release of BH test formulation was evaluated using the dialysis membrane diffusion method in simulated tear fluid (STF; pH 7.4). Dialysis membrane methods are widely employed for evaluating drug release from nanosized lipid and polymeric carriers because the semipermeable membrane retains the nanocarrier while permitting diffusion of the released drug into the external receptor medium [30]. Prior to the experiment, the dialysis membrane was appropriately pretreated and equilibrated with STF to minimize membrane-related effects on drug diffusion. A predetermined quantity of OP-BH-NLC-Tgel equivalent to 0.5 mg of BH was accurately transferred into the hydrated dialysis membrane, and both ends of the membrane were securely sealed. The formulation-containing dialysis membrane was immersed in 50 mL STF (pH 7.4) maintained at 37 ± 0.5 °C under continuous agitation at 100 rpm. Maintenance of physiological temperature and adequate agitation is important during dialysis-based release testing because temperature affects drug diffusion, whereas agitation minimizes the unstirred boundary layer surrounding the membrane and promotes uniform distribution of the released drug in the receptor phase [31]. The release study was continued for 12 h, and aliquots of the receptor medium were withdrawn at predetermined time intervals of 0.5, 1, 2, 3, 4, 6, 8, 10, 12 & 24 h. An equal volume of fresh STF maintained at 37 ± 0.5 °C was immediately replenished after each sampling to maintain a constant receptor volume and the concentration gradient required for drug diffusion. Maintenance of an appropriate donor-to-receptor volume ratio and sink conditions is particularly important in dialysis methods because insufficient receptor volume can result in accumulation of drug in the external medium and consequently alter the measured release profile [30]. The withdrawn samples were suitably diluted with STF, where required, and the concentration of BH was determined spectrophotometrically at 272 nm using the previously validated UV spectrophotometric method. The cumulative amount of drug released was calculated with correction for the volume of receptor medium removed during successive sampling, and the results were expressed as cumulative percentage drug release as a function of time. The release experiment was performed in triplicate, and the results were reported as mean ± standard deviation. The dialysis membrane molecular-weight cut-off was selected to permit free diffusion of BH while retaining the NLCs within the donor compartment, as appropriate MWCO selection is a critical determinant of the validity of dialysis-based release measurements [31]. The release profile of OP-BH-NLC-Tgel was subsequently compared with Betoptic marketed eye drop formulation.

2.10 Release kinetics

The cumulative percentage drug-release data obtained from OP-BH-NLC-Tgel were fitted to zero-order, first-order, Higuchi, and Korsmeyer–Peppas mathematical models to elucidate the release kinetics and probable mechanism of BH liberation from the formulation. The zero-order model was evaluated by plotting cumulative percentage drug release against time, whereas the first-order model was assessed by plotting the logarithm of the percentage of drug remaining against time. The Higuchi model was evaluated by plotting cumulative percentage drug release against the square root of time. The Korsmeyer–Peppas model was applied using the equation 3.

$$M_t/M_\infty = kt^n \ldots \text{equation 3}$$

where M_t/M_∞ represents the fraction of drug released at time t, k is the kinetic constant, and n is the release exponent. The model providing the highest coefficient of determination (R^2) was considered the best-fit model, while the value of n was used to interpret the predominant drug-release mechanism. Mathematical modelling of release profiles is routinely used to distinguish diffusion, dissolution, swelling, and erosion-controlled release behavior from nanocarrier and polymeric delivery systems [32]. The kinetic parameters obtained for the one-pot formulation were compared with those of the previously developed formulation to determine whether the one-pot fabrication strategy altered the underlying mechanism of BH release.

2.11 Ex-vivo transcorneal permeation study

The ex-vivo transcorneal permeation of BH from OP-BH-NLC-Tgel was evaluated using freshly excised goat corneas mounted on vertical Franz diffusion cells under the same experimental conditions employed for the previously developed formulation, thereby enabling direct comparison of the permeation performance of the two fabrication approaches [29, 33]. Fresh goat corneas were obtained from a local slaughterhouse and transported to the laboratory in cold normal saline at 4 °C. The cornea was carefully excised together with approximately 2–4 mm of surrounding scleral tissue and thoroughly washed with cold normal saline to remove adhering tissues and contaminants. Corneas exhibiting visible damage, opacity, or structural defects were discarded. The isolated cornea was mounted between the donor and receptor compartments of a Franz diffusion cell with the epithelial surface facing the donor compartment and the endothelial surface toward the receptor compartment. The effective corneal diffusion area was maintained at 0.64 cm^2. The receptor compartment was filled with freshly prepared simulated tear fluid (STF, pH 7.4) and maintained at the same temperature and under the same continuous stirring conditions used in the previously developed formulation. An accurately measured quantity of OP-BH-NLC-Tgel equivalent to the predetermined BH dose was carefully placed in the donor compartment so that the formulation remained in uniform contact with the corneal surface. The donor compartment was appropriately covered to minimize evaporation during the experiment. At predetermined time intervals, aliquots of the receptor medium were withdrawn through the sampling port and immediately replaced with an equal volume of fresh, pre-equilibrated STF to maintain a constant receptor volume and sink conditions. The withdrawn samples were suitably diluted, where required, and analyzed for BH content using the previously validated UV spectrophotometric method at 272 nm [34]. The cumulative amount of BH permeated through the cornea per unit area

was plotted against time, and the steady-state flux (Jss) was calculated from the slope of the linear portion of the permeation profile. The apparent permeability coefficient (P_{app}) was calculated using equation 4.

$$P_{app} = J_{ss}/C_0 \ldots. \text{ equation 4}$$

where C_0 represents the initial BH concentration in the donor compartment. The permeation enhancement ratio (ER) was calculated by dividing the P_{app} of OP-BH-NLC-Tgel by that of the corresponding reference formulation. These parameters are commonly used for quantitative assessment of transcorneal drug transport in Franz diffusion-cell studies. The experiment was performed using independent corneas, and the results were expressed as mean ± standard deviation. The cumulative amount permeated, steady-state flux, permeability coefficient, and enhancement ratio obtained for OP-BH-NLC-Tgel were compared directly with the corresponding values of the previously developed formulation, while maintaining identical permeation conditions to determine whether the one-pot fabrication strategy preserved or improved transcorneal delivery [29].

2.12 Ex vivo corneal drug retention study

Following completion of the 24-h ex vivo transcorneal permeation study, the corneas were carefully removed from the Franz diffusion cells and gently rinsed with simulated tear fluid (STF; pH 7.4) to remove any formulation remaining on the corneal surface. The corneas were then homogenized in STF, followed by extraction of BH using methanol. The resulting homogenate was centrifuged to separate the tissue debris, and the clear supernatant was collected and suitably processed for quantitative estimation of BH using the previously validated UV spectrophotometric method at 272 nm. The amount of BH retained within the corneal tissue was calculated and expressed as µg/cm². The corneal retention of BH from OP-BH-NLC-Tgel was compared with that from Betoptic® 0.25% [35, 36].

2.13 Histopathological Study

Histopathological evaluation was performed using excised goat cornea to assess possible structural alterations following exposure to OP-BH-NLC-Tgel. The corneas were treated with OP-BH-NLC-Tgel, 0.9% w/v NaCl (negative control), and KCl (positive control) under the same conditions employed for the previously developed formulation. Following treatment, the corneas were fixed in 10% v/v formalin, dehydrated using graded ethanol, embedded in paraffin wax, sectioned using a microtome, and stained with hematoxylin and eosin (H&E). The stained sections were examined microscopically for changes in corneal architecture and tissue integrity, and representative photomicrographs were obtained. Similar histopathological procedures have been reported for evaluating the ocular compatibility of nanocarrier-based in-situ gel formulations using excised goat cornea [37].

2.14 Stability study

A preliminary stability study of the optimized OP-BH-NLC-Tgel formulation was conducted under room-temperature conditions (25–30 °C) for 6 months. The formulation was filled into tightly closed containers and stored under ambient laboratory conditions. Samples were evaluated at the initial time point and after 3 and 6 months for appearance, pH, gelation temperature (Tgel), particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, and viscosity at 25 and 37 °C. The formulation was visually examined for changes in colour, homogeneity, precipitation, aggregation, or phase separation. The pH was determined using a calibrated digital pH meter, while Tgel was determined using the previously described tube-inversion method. Particle size, PDI, and zeta potential were determined by dynamic light scattering after suitable dilution of the formulation. Entrapment efficiency was determined using the previously described separation and drug-estimation procedure. Viscosity was measured at 25 and 37 °C using the previously described viscometric method. All measurements were performed in triplicate and expressed as mean ± standard deviation (SD). Formulation stability was assessed based on the extent of variation in the evaluated physicochemical and rheological characteristics during storage [23, 38].

3. RESULT

3.1 Screening and Selection of Excipients

The results of BH excipient screening are presented in Table 2. Among the tested solid lipids, GMS showed the highest and consistent BH incorporation (+++) at both 1:1 and 1:2 drug-to-lipid ratios and was therefore selected for further formulation development. Among the liquid lipids, TA exhibited the highest BH solubility (51.43 ± 1.63 mg/mL), followed by Miglyol 812 (37.76 ± 3.98 mg/mL). Therefore, TA was selected as the liquid lipid. For surfactants, Tween 20 showed substantially higher BH solubility (19.95 ± 1.64 mg/mL) than Tween 80 (7.33 ± 0.41 mg/mL), supporting its selection. Among the co-surfactants, Transcutol P demonstrated the highest BH solubility (16.71 ± 1.27 mg/mL), compared with PEG 400 (8.59 ± 1.23 mg/mL) and PEG 600 (4.76 ± 1.78 mg/mL), and was consequently selected. Based on the screening results, GMS, TA, Tween 20, and Transcutol P were selected as the solid lipid, liquid lipid, surfactant, and co-surfactant, respectively, for subsequent development of OP-BH-NLC-Tgel.

Table 2. Screening of BH solubility in candidate solid and liquid lipids, surfactants, and co-surfactants

Excipient category	Excipient	BH solubility / incorporation
Solid lipids		1:1(Drug: solid lipid)

	Cholesterol	+ +
	Tristearin	+ +
	GMS	+ + +
	Myristic acid	+ +
	Stearyl alcohol	+ + +
	Palmitic acid	+
Liquid lipids		BH solubility (mg/mL)
	Miglyol 812	37.76 ± 3.98
	Oleic acid	3.29 ± 1.09
	Palm oil	7.83 ± 0.73
	Castor oil	8.97 ± 0.78
	Tocopheryl acetate	51.43 ± 1.63
Surfactants		BH solubility (mg/mL)
	Tween 80	7.33 ± 0.41
	Tween 20	19.95 ± 1.64
Co-surfactants		BH solubility (mg/mL)
	Transcutol P	16.71 ± 1.27
	PEG 400	8.59 ± 1.23
	PEG 600	4.76 ± 1.78

Note: For solid lipids, BH incorporation was qualitatively graded according to visual appearance of the molten lipid–drug mixture: +, poor solubilization/opaque appearance; ++, partial solubilization/blurred appearance; and +++, complete or near-complete solubilization/transparent appearance. Quantitative solubility values for liquid lipids, surfactants, and co-surfactants are expressed as mean ± SD (n = 3).

3.2 Development and Selection of the Fixed NLC Composition

Following the selection of the individual excipients, preliminary BH-NLC formulations were prepared by systematically varying the amounts of GMS (85–125 mg), TA (85–125 mg), and the Tween 20: Transcutol P Smix ratio (1:1, 1.25:1, and 2:1), while maintaining the BH concentration at 25 mg and the final formulation volume at 20ml (Table 3). The resulting formulations were comparatively evaluated for particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, and physical appearance. Based on the overall formulation performance, NLC 5 exhibited a favorable combination of physicochemical characteristics, with a particle size of 178.27 nm, PDI of 0.265, and zeta potential of −37.5 mV, as shown in Figure 1. It also showed highest entrapment efficiency of 96.07 % among other nine formulations. Considering these attributes and its suitability for ocular delivery, NLC 5 was selected as the optimized preliminary formulation among the evaluated formulations. Accordingly, the composition of NLC 5, comprising 100 mg GMS, 100 mg TA, and a Tween 20: Transcutol P ratio of 1.25:1 (5:4), was selected as the fixed NLC composition [14]. This composition was subsequently maintained constant during the screening and optimization of the thermo-responsive polymeric phase.

Table 3. Preliminary formulation screening for selection of the fixed BH-NLC composition

F. No	BH (mg)	GMS (mg)	TA (mg)	Smix ratio	Particle size (nm)	PDI	EE (%)	Zeta Potential (mV)

NLC-1	25	85	85	1:1	272.62 ± 5.98	0.176 ± 0.01	83.43 ± 5.64	−17.4 ± 3.43
NLC-2	25	85	100	1.25:1	194.56 ± 6.67	0.183 ± 0.02	76.76 ± 4.67	−27.3 ± 3.93
NLC-3	25	85	125	2:1	512.29 ± 7.87	0.567 ± 0.02	80.13 ± 3.98	−23.7 ± 4.26
NLC-4	25	100	85	1.25:1	567.65 ± 8.65	0.123 ± 0.03	90.21 ± 6.54	−19.2 ± 3.97
NLC-5	25	100	100	1.25:1	178.27 ± 5.56	0.265 ± 0.02	96.07 ± 2.34	−37.5 ± 2.12
NLC-6	25	100	125	2:1	165.27 ± 4.42	0.341 ± 0.04	71.34 ± 5.89	−30.7 ± 3.41
NLC-7	25	125	85	2:1	109.27 ± 3.76	0.378 ± 0.01	63.74 ± 4.51	−29.8 ± 4.54
NLC-8	25	125	100	1.25:1	345.21 ± 4.01	0.298 ± 0.02	83.72 ± 6.67	−16.31 ± 5.56
NLC-9	25	125	125	1:1	564.27 ± 6.23	0.456 ± 0.04	63.48 ± 3.58	−34.9 ± 3.28

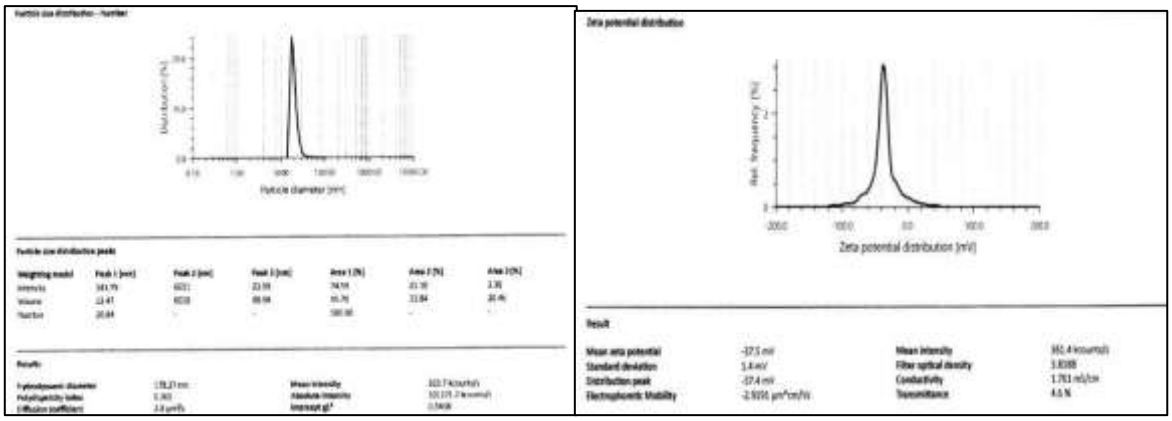


Figure 1. Particle size, PDI and Zetapotential of Optimized NLC formulation (NLC 5)

3.3 Screening and Selection of Thermo-responsive Excipients

Following selection of the fixed NLC composition, the thermo-responsive polymeric phase was optimized by sequential screening of P407, P188, and HPMC K4M. Throughout this study, the selected NLC composition was kept constant, and only the concentration of the polymer under investigation was varied. The screening results are summarized in Table 4.

In the first stage, P407 was screened over the concentration range of 17–22% w/v. The formulations were compared in terms of visual appearance, gelation temperature, gelation time, and viscosity. Increasing P407 concentration produced a progressive change in the thermo-responsive and rheological characteristics of the formulations. Among the concentrations investigated, 18% w/v P407 provided the most appropriate combination of formulation appearance, gelation behavior, and viscosity and was therefore selected for subsequent P188 screening. In the second stage, P188 was screened at concentrations of 0.20–0.50% w/v while maintaining P407 at the selected concentration of 18% w/v. The incorporation of P188 influenced the gelation characteristics and viscosity of the P407-based system. Based on the comparative evaluation of visual appearance, gelation temperature, gelation time, and viscosity, 0.30% w/v P188 was selected as the most suitable concentration and was maintained for the subsequent HPMC K4M screening. In the final stage, HPMC K4M was screened at concentrations of 0.25, 0.50, and 0.75% w/v. Increasing HPMC K4M concentration resulted in an increase in formulation viscosity and influenced the gel characteristics. The concentration of 0.50% w/v HPMC K4M provided an appropriate balance between viscosity and gelation characteristics and was therefore selected. Accordingly, the final thermo-responsive polymeric composition consisted of 18% w/v P407, 0.30% w/v P188, and 0.50% w/v HPMC K4M. This optimized polymeric composition was subsequently used for the development and detailed characterization of the one-pot BH-NLC thermo-responsive nanogel (OP-BH-NLC-Tgel).

Table 4. Sequential screening and selection of thermo-responsive polymer concentrations for OP-BH-NLC-Tgel

Screening stage	Batch	P407 (% w/v)	P188 (% w/v)	HPMC K4M (% w/v)	Appearance	Tgel (°C)	Gelation time (s)	Viscosity at 25 °C (cP)	Viscosity at 37 °C (cP)

P407 screening	P407-1	17	0.35	0.50	Clear, homogeneous	36.0 ± 0.3	20 ± 1	35 ± 2	700 ± 25
	P407-2	18	0.35	0.50	Clear, homogeneous	34.0 ± 0.2	15 ± 1	45 ± 2	850 ± 30
	P407-3	19	0.35	0.50	Clear, moderately viscous	32.5 ± 0.3	12 ± 1	60 ± 3	1050 ± 35
	P407-4	20	0.35	0.50	Clear, viscous	31.5 ± 0.2	10 ± 1	75 ± 3	1250 ± 40
	P407-5	21	0.35	0.50	More viscous	30.5 ± 0.3	8 ± 1	95 ± 4	1450 ± 45
	P407-6	22	0.35	0.50	Highly viscous	29.5 ± 0.2	7 ± 1	120 ± 5	1650 ± 50
P188 screening	P188-1	18*	0.20	0.50	Clear, homogeneous	33.5 ± 0.2	14 ± 1	42 ± 2	800 ± 30
	P188-2	18*	0.30	0.50	Clear, homogeneous	33.0 ± 0.2	12 ± 1	48 ± 2	900 ± 30
	P188-3	18*	0.40	0.50	Clear, slightly viscous	33.5 ± 0.3	10 ± 1	58 ± 3	1050 ± 35
	P188-4	18*	0.50	0.50	Clear, more viscous	34.0 ± 0.2	9 ± 1	70 ± 3	1200 ± 40
HPMC K4M screening	HPMC-1	18*	0.30*	0.25	Clear, relatively fluid	34.0 ± 0.2	15 ± 1	35 ± 2	650 ± 25
	HPMC-2	18*	0.30*	0.50	Clear, homogeneous	33.0 ± 0.2	12 ± 1	50 ± 2	950 ± 30
	HPMC-3	18*	0.30*	0.75	Clear, more viscous	32.0 ± 0.3	9 ± 1	75 ± 3	1250 ± 40
Final formulation	OP-BH- NLC-Tgel	18	0.30	0.50	Clear, homogeneous	34.2 ± 0.2	12 ± 1	66.7 ± 2.8	1321.21 ± 38.6

*Concentration selected from the preceding screening stage and maintained constant during the subsequent screening stage.

The optimized OP-BH-NLC-Tgel containing 18% P407, 0.30% P188, and 0.50% HPMC K4M was evaluated for visual appearance, pH, gelation temperature, gelation time, and temperature-dependent viscosity (Table 4). The formulation appeared clear and homogeneous, indicating satisfactory physical compatibility of the incorporated NLC with the thermo-responsive polymeric system. The formulation exhibited a pH of 6.9 ± 0.07, which was considered suitable for ophthalmic administration. The optimized formulation demonstrated a gelation temperature of 34.2 °C with a gelation time of 12 ± 1 s. The observed transition temperature was close to the physiological temperature of the ocular surface and therefore indicated an appropriate thermo-responsive response. A similar P407/HPMC ophthalmic system containing 20% P407 and 0.5% HPMC exhibited a gelation temperature of 32.8 ± 0.3 °C and gelation time of 10.7 ± 0.3 s, supporting the suitability of the present formulation's thermo-responsive characteristics [28]. The viscosity of OP-BH- NLC-Tgel increased markedly with temperature, from 66.7 ± 2.8 cP at 25 °C to 1321.21 ± 38.6 cP at 37 °C. This substantial increase in viscosity upon exposure to physiological temperature confirmed the temperature-dependent sol– gel transition of the formulation. The observed behavior is consistent with P407-based thermo-responsive systems, in which increasing temperature promotes dehydration of the hydrophobic polypropylene oxide domains, micellar association, and increased micellar packing, resulting in a substantial increase in viscosity [28]. Notably, the viscosity at 37 °C (1321.21± 38.6 cP) was highly comparable to the 1392 ± 5.7 cP reported for a 20% P407/0.5% HPMC ophthalmic gel at the same temperature. The viscosity at 25 °C (66.7 ± 2.8 cP) was moderately higher than the

corresponding reported value of 56.1 ± 6.8 cP, which may be attributable to differences in polymer composition and the presence of the NLC components in the present formulation [28]. Overall, the optimized OP-BH-NLC-Tgel exhibited a clear appearance, ophthalmically acceptable pH, rapid gelation near ocular physiological temperature, and pronounced temperature-dependent viscosity enhancement, supporting its suitability as a thermo-responsive ocular delivery system.

3.4 Rheological and Flow Behaviour of Optimized OP-BH-NLC-Tgel

The rheological behaviour of the optimized OP-BH-NLC-Tgel was evaluated over a shear-rate range of 1–1000 s^{-1} (Figure 2). The formulation exhibited a progressive decrease in apparent viscosity from 1344 ± 35 cP at 1 s^{-1} to 63 ± 3 cP at 1000 s^{-1}, demonstrating pronounced shear-dependent flow behaviour. The experimental data were well described by the power-law model, yielding a flow behaviour index (n) of 0.549 and a consistency index (K) of 1.441 $Pa \cdot s^n$ (R^2 = 0.9979). The value of $n < 1$ confirms the pseudoplastic or shear-thinning nature of the formulation (Table 5). Such shear-thinning behaviour is advantageous for ocular administration because the formulation can maintain relatively higher viscosity under low-shear conditions, thereby contributing to prolonged precorneal residence, while its viscosity decreases under the higher shear conditions associated with administration and blinking, facilitating instillation and subsequent spreading over the ocular surface [39, 40].

Table 5. Rheological behaviour of optimized OP-BH-NLC-Tgel

Shear rate (s^{-1})	Apparent viscosity (cP)
1	1344 ± 35
2	997 ± 54
5	723 ± 23
10	540 ± 19
20	390 ± 18
50	266 ± 11
100	179 ± 9
200	136 ± 6
500	79 ± 4
1000	63 ± 3
Flow behavior index (n)	0.549
Consistency index (K)	1.441 $Pa \cdot s^n$
R^2	0.9979

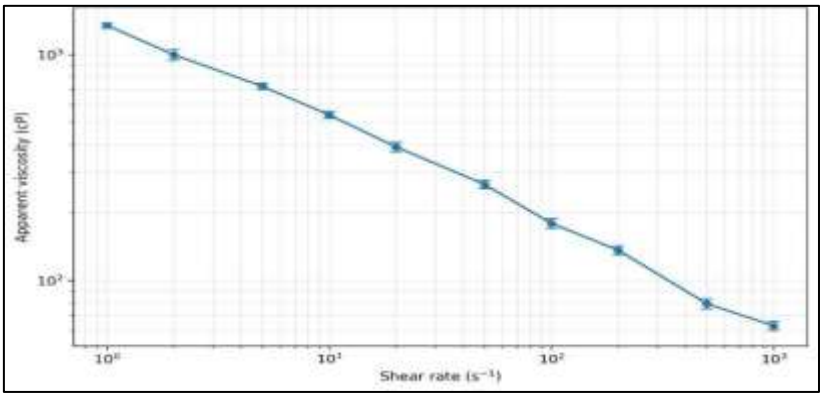


Figure 2. Rheological flow behaviour of optimized OP-BH-NLC-Tgel showing the decrease in apparent viscosity with increasing shear rate.

3.5 Effect of simulated tear fluid dilution on formulation behavior

The effect of STF dilution on the thermo-responsive behavior of OP-BH-NLC-Tgel is presented in Table 6. The formulation exhibited only a minor change in gelation temperature following STF dilution, decreasing from 34.2 ± 0.2 °C to 33.7 ± 0.04 °C, corresponding to a temperature shift of only 0.5 °C. The maintenance of a gelation temperature close to physiological ocular temperature indicates that dilution by simulated tear fluid did not substantially interfere with the thermo-responsive behavior of the formulation. Such behavior is desirable for ophthalmic in-situ gels because the formulation should remain sufficiently fluid during administration and undergo rapid gelation following exposure to ocular temperature.

The gelation time decreased from 12 ± 1 s before dilution to 10 ± 1 s after STF dilution, demonstrating that the formulation retained rapid gel formation following exposure to the simulated tear environment. The relatively small change in gelation time further suggests that dilution did not adversely affect the ability of the P407-based polymeric system to undergo temperature-induced gelation. Rapid gelation following instillation is advantageous because it can minimize precorneal drainage before establishment of the gel network.

A decrease in viscosity was observed following STF dilution at both investigated temperatures. At 25 °C, viscosity decreased from 66.7 ± 2.8 to 58.9 ± 2.7 cP, corresponding to 88.3% retention of the initial viscosity. At 37 °C, the viscosity decreased from 1321.21 ± 38.6 to 1225 ± 28.9 cP, corresponding to 92.7% retention. The reduction in viscosity following STF dilution can be attributed primarily to dilution of the polymeric components and the consequent decrease in polymer concentration. However, the relatively high viscosity retention demonstrates that the formulation maintained substantial structural integrity even after simulated tear-fluid exposure [29].

Importantly, the formulation continued to demonstrate a pronounced temperature-dependent increase in viscosity after STF dilution. The viscosity increased from 58.9 ± 2.7 cP at 25 °C to 1225 ± 28.9 cP at 37 °C, representing an approximately 20.8-fold increase. This substantial increase confirms that the thermo-responsive characteristics of OP-BH-NLC-Tgel were preserved following STF dilution. The temperature-dependent increase in viscosity can be attributed to temperature-induced dehydration and micellization of P407, followed by increased micellar packing and formation of a three-dimensional gel network. The presence of HPMC K4M may additionally contribute to the viscosity and structural strength of the polymeric network.

Overall, the minimal 0.5 °C change in gelation temperature, rapid gelation within 10 ± 1 s, and retention of 88.3% and 92.7% viscosity at 25 and 37 °C, respectively, demonstrate that OP-BH-NLC-Tgel maintained its functional thermo-responsive behavior after STF dilution. These findings indicate that the one-pot fabricated formulation can withstand the dilution expected following ocular administration while retaining an appropriate sol–gel transition and viscosity profile. Thus, the STF dilution study provides additional evidence supporting the potential of OP-BH-NLC- Tgel for prolonged precorneal residence and ocular drug delivery.

Table 6. Effect of simulated tear fluid dilution on the gelation and viscosity characteristics of optimized OP-BH- NLC-Tgel

Parameter	Before STF dilution	After STF dilution
Gelation temperature (°C)	34.2 ± 0.2	33.7 ± 0.04
Gelation time (s)	12 ± 1	10 ± 1
Viscosity at 25 °C (cP)	66.7 ± 2.8	58.9 ± 2.7
Viscosity at 37 °C (cP)	1321.21 ± 38.6	1225 ± 28.9

3.6 In-vitro Drug Release

The in-vitro release profiles of BH from Betoptic® 0.25% and the optimized OP-BH-NLC-Tgel are presented in Figure 3. Betoptic® exhibited a comparatively rapid release, with 46.2 ± 2.24% of BH released within 0.5 h and 84.2 ± 5.67% released by 2 h. The cumulative release subsequently approached completion, reaching 93.8 ± 4.29% at 4 h and 99.7 ± 3.19% at 24 h. In contrast, OP-BH-NLC-Tgel demonstrated a distinctly slower and more sustained release profile. Only18.0 ± 1.5% of the drug was released during the first 0.5 h, increasing progressively to 42.0 ± 2.8% at 2 h, 56.0 ± 3.4% at 4 h, and 77.0 ± 4.5% at 12 h. At 24 h, the formulation released 89.0 ± 4.8% of the incorporated BH. Thus, compared with Betoptic®, the thermo-responsive nanogel substantially attenuated the initial release and maintained drug release over the entire 24-h study period. The slower release from OP-BH-NLC-Tgel can be attributed to the combined diffusional and matrix-retention effects of the lipid nanocarrier and thermo-responsive polymeric network. Entrapment of BH within the NLC lipid matrix can retard drug diffusion, while incorporation of the NLC into the P407/P188/HPMC-based gel further increases the diffusional path length and restricts drug mobility. The progressive release observed over 24 h therefore indicates that the formulation provides a substantially more sustained release pattern than the marketed aqueous formulation. Importantly, the release profile of OP-BH-NLC-Tgel was not characterized by an abrupt initial burst, with only approximately 18% drug release during the first 30 min, compared with 46.2% from Betoptic®. This difference is particularly relevant to ocular delivery, where rapid loss of drug from the precorneal environment can limit the duration of therapeutic exposure [41]. The sustained release observed from OP-BH-NLC-Tgel, together with its temperature-

triggered gelation and shear-thinning characteristics, supports its potential to maintain drug availability at the ocular surface for an extended period.

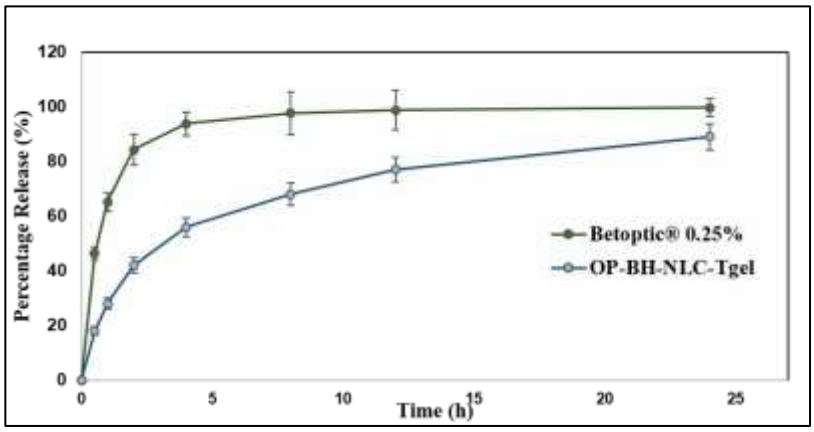


Figure 3. In-vitro release profile of BH from Betoptic® 0.25% and optimized OP-BH-NLC-Tgel

3.7 In-vitro Release Kinetics and Mechanism

The release data were further subjected to kinetic modelling using zero-order, first-order, Higuchi, and Korsmeyer–Peppas models to elucidate the release kinetics and probable mechanism of BH release. The corresponding coefficients of determination ($R^{2)}$ and the Korsmeyer–Peppas release exponent (n) are presented in Table 7. For Betoptic® 0.25%, the first-order model provided the highest coefficient of determination ($R^2 = 0.959$), compared with the zero-order ($R^2 = 0.756$), Higuchi ($R^2 = 0.909$), and Korsmeyer–Peppas ($R^2 = 0.905$) models. This indicated that BH release from the marketed formulation was predominantly concentration-dependent, consistent with its relatively rapid release profile observed during the initial hours of the study. In contrast, OP-BH-NLC-Tgel exhibited a markedly different kinetic behaviour. The Korsmeyer–Peppas model showed the highest goodness of fit ($R^2 = 0.998$), followed by the Higuchi model ($R^{2 =} 0.956$), first-order model ($R^{2 =} 0.926$), and zero-order model ($R^{2 =} 0.858$). The Korsmeyer–Peppas release exponent (n = 0.64) indicated anomalous (non-Fickian) transport, suggesting that BH release was governed by a combination of drug diffusion and polymeric matrix relaxation rather than by a single release process. The superior fit of the Korsmeyer–Peppas model is consistent with the composite nature of OP-BH-NLC-Tgel, in which the lipid nanocarrier provides an additional diffusional barrier while the P407/P188/HPMC K4M thermo-responsive matrix further modulates drug transport [42]. Overall, the kinetic analysis demonstrated a change in release behaviour from predominantly concentration-dependent release for Betoptic® to anomalous transport for OP-BH-NLC-Tgel. This difference supports the role of the NLC–thermogel architecture in modulating BH release and providing a sustained release profile over 24 h.

Table 7. Kinetic modelling of BH release from Betoptic® 0.25% and optimized OP-BH-NLC-Tgel

Release model	Betoptic® 0.25%	OP-BH-NLC-Tgel
Zero-order	0.756	0.858
First-order	0.959	0.926
Higuchi	0.909	0.956
Korsmeyer–Peppas	0.905	0.998
Korsmeyer–Peppas, n	0.54	0.64
Most likely best-fit model	First-order	Korsmeyer–Peppas

3.8 Ex-vivo transcorneal permeation study

The ex-vivo transcorneal permeation profiles of Betoptic® 0.25% and OP-BH-NLC-Tgel across freshly excised goat cornea are presented in Table 8 and Figure 4. Both formulations demonstrated a time-dependent increase in cumulative drug permeation throughout the 24 h study period; however, OP-BH-NLC-Tgel exhibited markedly greater transcorneal permeation than the marketed formulation. At 0.5 h, the cumulative amount of BH permeated from Betoptic® 0.25% and OP-BH-NLC-Tgel was 57.48 ± 6.07 and 93.16 ± 9.09 µg/cm², respectively. The permeation from OP-BH-NLC-Tgel remained higher at all subsequent sampling intervals, reaching 270.64 ± 16.87 µg/cm² at 2 h and 614.15 ± 32.45 µg/cm² at 6 h, compared with 135.45 ± 11.31 and 240.06 ± 18.78 µg/cm², respectively, for Betoptic® 0.25%. At 12 h, OP-BH-NLC-Tgel achieved a cumulative permeation of 876.34 ± 39.08 µg/cm², whereas Betoptic® 0.25% showed 366.56 ± 24.21 µg/cm². After 24 h, the cumulative permeation further increased to 998.97 ± 43.61 µg/cm² for OP-BH-

NLC-Tgel compared with 411.43 ± 28.98 µg/cm² for Betoptic® 0.25%. Thus, OP-BH-NLC-Tgel exhibited approximately 2.43-fold higher cumulative transcorneal permeation than Betoptic® 0.25% at the end of the study. The enhanced permeation may be attributed to the nanosized lipidic carrier, which provides a large interfacial surface area and facilitates intimate contact with the corneal surface, while the lipidic components and surfactant system may improve drug partitioning and transport across the corneal barrier. In addition, incorporation of the NLC into the thermo-responsive in-situ gel may prolong precorneal residence and maintain the formulation in close contact with the corneal surface, thereby contributing to the enhanced overall drug permeation [43, 44].

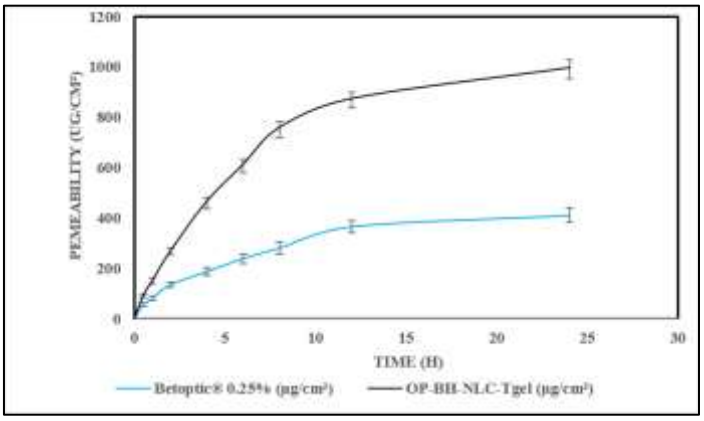


Figure 4. Ex-vivo transcorneal permeation of BH from Betoptic® 0.25% and OP-BH-NLC-Tgel

Table 8. Ex vivo transcorneal permeation parameters of BH from Betoptic® 0.25% and OP-BH-NLC-Tgel across goat cornea (Mean ± SD, n = 3).

Parameter	Betoptic® 0.25%	OP-BH-NLC-Tgel
Flux, Jss (µg/h·cm²)	25.86	65.36
Permeability coefficient, Kp (cm/h)	0.0259	0.0654
Final permeation at 24 h (µg/cm²)	411.43 ± 28.98	998.97 ± 43.61
Permeation enhancement ratio*	1.00	2.43

3.9 Ex vivo corneal drug retention

The amount of BH retained within the corneal tissue following completion of the 24-h ex vivo permeation study is presented in Table 9. Betoptic® 0.25% showed a corneal BH retention of 25.66 ± 2.76 µg/cm², whereas OP-BH-NLC-Tgel exhibited substantially higher retention of 63.54 ± 8.91 µg/cm². Thus, OP-BH-NLC-Tgel demonstrated a 2.48-fold enhancement in corneal BH retention compared with the marketed formulation. The enhanced corneal retention observed with OP-BH-NLC-Tgel may be attributed to the combined characteristics of the nanostructured lipid carrier and thermo-responsive gel matrix [45]. The nanosized lipidic system provides a large surface area and facilitates intimate interaction with the corneal surface, which may promote partitioning and deposition of BH within the corneal tissue. Furthermore, incorporation of the NLC into the thermo-responsive gel can increase the viscosity of the formulation following exposure to ocular temperature, thereby reducing rapid drainage and maintaining the formulation in close contact with the corneal surface. This prolonged contact may provide a sustained concentration gradient that favors drug uptake into the corneal tissue. The higher corneal retention of OP-BH-NLC-Tgel is consistent with the enhanced transcorneal permeation observed in the preceding study, where the formulation exhibited higher cumulative BH permeation, steady-state flux, and permeability coefficient than Betoptic® 0.25% [36]. Collectively, the enhanced corneal deposition and permeation indicate that the one-pot fabricated OP-BH-NLC-Tgel can improve the availability of BH at the corneal barrier and may contribute to prolonged ocular drug delivery.

Table 9. Ex vivo corneal retention of BH following the permeation study (Mean ± SD, n = 3).

Parameter	Betoptic® 0.25%	OP-BH-NLC-Tgel
BH retained in cornea (µg/cm²)	25.66 ± 2.76	63.54 ± 8.91
Retention enhancement ratio	1.00	2.48

3.10 Histopathological evaluation of corneal tissue

Representative H&E-stained histological sections of goat corneal tissue are shown in Figure 5. The KCl-treated positive-control cornea (Figure 5A) exhibited marked disruption of the normal corneal architecture, with an irregular and fragmented tissue surface and evident structural disorganization. These alterations confirmed the presence of tissue damage and supported the use of KCl-treated cornea as the positive-control group. In contrast, the 0.9% w/v NaCl-treated cornea (Figure 5B) showed a relatively intact and well-preserved corneal architecture, with an organized stromal matrix and a comparatively smooth tissue surface, confirming its suitability as the negative control. The cornea exposed to OP-BH-NLC-Tgel (Figure 5C) demonstrated largely preserved tissue architecture, with no obvious gross disruption of the corneal tissue or marked structural disorganization compared with the NaCl-treated control. The stromal region appeared comparatively organized, and no prominent tissue separation, extensive disruption, or apparent degenerative changes were observed in the examined section. Although minor surface irregularity was visible, it was not accompanied by an obvious loss of overall corneal architecture. Thus, the histological appearance of the formulation- treated cornea was broadly comparable to that of the negative control. These findings indicate that OP-BH-NLC-Tgel did not produce apparent histomorphological damage to the goat corneal tissue under the conditions of the ex vivo study. The preserved corneal architecture following formulation exposure provides preliminary evidence of local corneal tissue compatibility. This finding is particularly relevant for the developed thermo-responsive nanogel, which is designed to prolong formulation contact with the ocular surface and thereby potentially increase corneal exposure. Overall, the histopathological observations support the suitability of OP-BH-NLC-Tgel for further ocular evaluation [46].

Figure 5. Representative H&E-stained histological sections of goat corneal tissue: (A) KCl-treated positive control showing marked tissue disruption; (B) 0.9% w/v NaCl-treated negative control showing preserved corneal architecture; and (C) OP-BH-NLC-Tgel-treated cornea showing largely preserved tissue morphology without apparent treatment-associated structural damage.

3.11 Stability study

The optimized OP-BH-NLC-Tgel formulation was subjected to a preliminary stability study under room-temperature conditions (25–30 °C) for 6 months [47]. The formulation was evaluated initially and after 3 and 6 months for appearance, pH, Tgel, particle size, PDI, zeta potential, entrapment efficiency, and viscosity. The results are presented in Table 10 and Figure 6. The formulation remained clear and homogeneous throughout the 6-month storage period, with no visible precipitation, aggregation, or phase separation. The pH exhibited only a minor increase from 6.91 ± 0.07 initially to 6.94 ± 0.05 after 3 months and 6.97 ± 0.03 after 6 months. This limited variation indicates that the formulation maintained its physicochemical characteristics during storage. The Tgel remained practically unchanged, with values of 34.2 ± 0.2 °C, 34.3 ± 0.2 °C, and 34.3 ± 0.1 °C at the initial, 3-month, and 6-month time points, respectively. The negligible variation in Tgel demonstrates that the thermo-responsive behaviour of OP-BH-NLC-Tgel was maintained during storage. Preservation of Tgel is important for ensuring reproducible sol–gel transition following administration to the ocular surface. Particle size showed a slight increase from 178.27 ± 3.23 nm initially to 180.90 ± 4.06 nm at 3 months and 182.14 ± 3.97 nm at 6 months. The relatively small increase in particle size suggests the absence of substantial particle aggregation or growth during storage. In addition, the PDI changed from 0.265 ± 0.07 initially to 0.280 ± 0.03 at 3 months and 0.244 ± 0.02 at 6 months. The absence of a progressive increase in PDI further indicates maintenance of a relatively uniform particle-size distribution. Similar physicochemical parameters are routinely used to assess the stability of NLC-based formulations, including ocular NLC systems. The zeta potential decreased slightly in magnitude from −37.5 ± 2.44 mV initially to −35.3 ± 2.11 mV after 3 months and −33.4 ± 2.17 mV after 6 months. Despite this modest reduction, the formulation retained a negative surface charge throughout the study. The limited change in zeta potential, together with the relatively small increase in particle size and stable PDI, suggests that substantial deterioration of the colloidal characteristics did not occur during storage. The entrapment efficiency remained high throughout the study, decreasing only from 96.07 ± 2.34% initially to 95.87 ± 2.11% after 3 months and ± 2.78% after 6 months. Thus, the formulation retained a high proportion of the encapsulated BH over the storage period, indicating good drug-retention characteristics of the NLC system. The viscosity profile also remained relatively stable. At 25 °C, the viscosity increased slightly from 66.7 ± 2.8 cP initially to 69.9 ± 3.7 cP at 3 months and 70.6 ± 2.5 cP at 6 months. At 37 °C, the viscosity decreased slightly from 1321.21 ± 38.6 cP initially to 1299.21 ± 37.8 cP and 1287.43 ± 35.6 cP after 3 and 6 months, respectively. Importantly, the marked difference between the viscosities at 25 and 37 °C was retained throughout storage, indicating preservation of the temperature-dependent rheological behaviour of the formulation. Overall, the optimized OP-BH-NLC-Tgel formulation exhibited satisfactory preliminary physicochemical stability under room-temperature

conditions (25–30 °C) over 6 months. Only minor variations were observed in pH, Tgel, particle size, PDI, zeta potential, entrapment efficiency, and viscosity, while the formulation remained clear and homogeneous throughout the study. These findings indicate that the optimized formulation retained its essential physicochemical and thermo-responsive characteristics during the investigated storage period [48].

Table 10. Stability evaluation of optimized OP-BH-NLC-Tgel during storage at room temperature for 6 months. Values are expressed as mean ± SD (n = 3).

Parameter	Initial	3 months	6 months
Appearance	Clear, homogeneous	Clear, homogeneous	Clear, homogeneous
pH	6.91 ± 0.07	6.94 ± 0.05	6.97 ± 0.03
Tgel (°C)	34.2 ± 0.2	34.3 ± 0.2	34.3 ± 0.1
Particle size (nm)	178.27 ± 3.23	180.90 ± 4.06	182.14 ± 3.97
PDI	0.265 ± 0.07	0.280 ± 0.03	0.244 ± 0.02
Zeta potential (mV)	−37.5 ± 2.44	−35.3 ± 2.11	−33.4 ± 2.17
Entrap. efficiency (%)	96.07 ± 2.34	95.87 ± 2.11	94.68 ± 2.78
Viscosity at 25 °C (cP)	66.7 ± 2.8	69.9 ± 3.7	70.6 ± 2.5
Viscosity at 37 °C (cP)	1321.21 ± 38.6	1299.21 ± 37.8	1287.43 ± 35.6

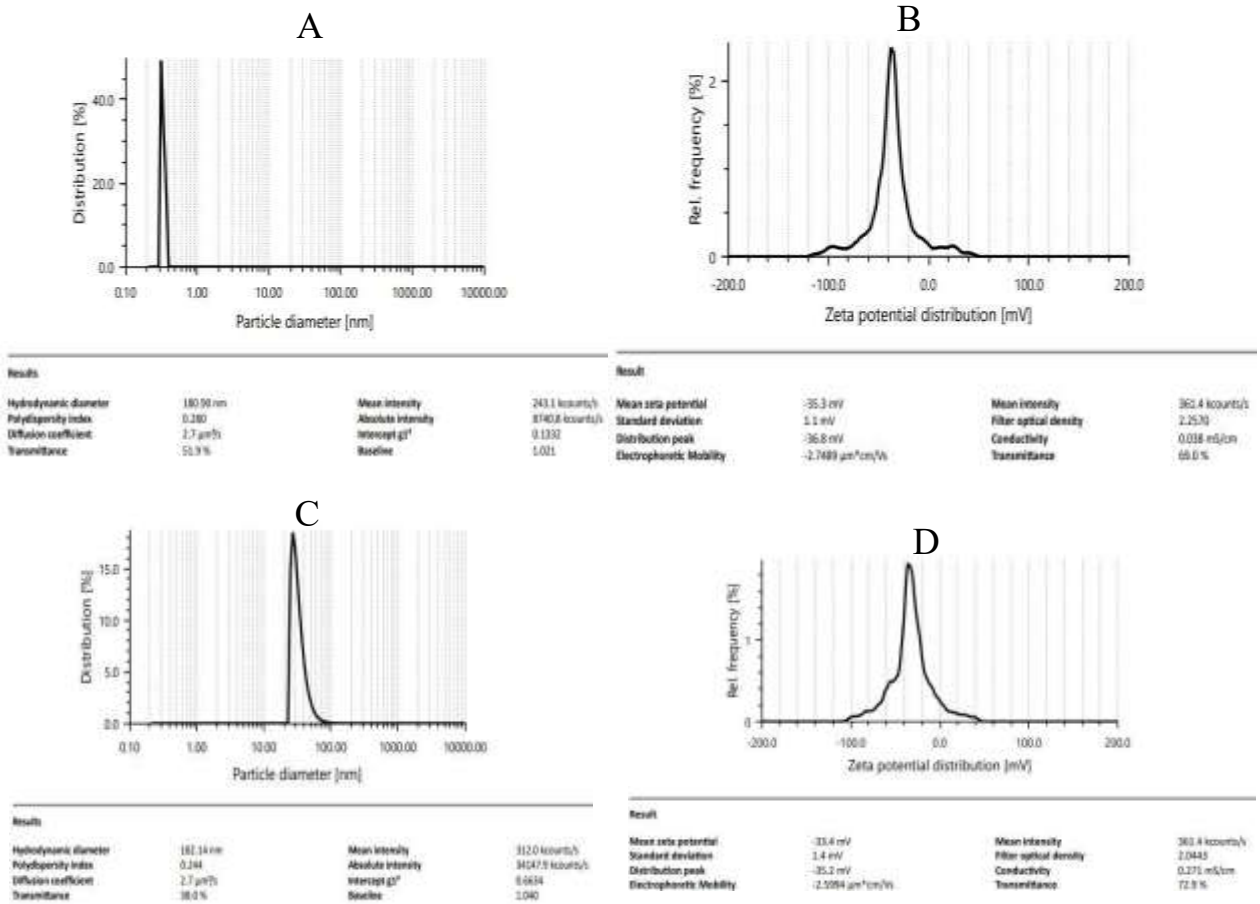


Figure 6: Stability evaluation of the optimized OP-BH-NLC-Tgel formulation after 3 & 6 months of storage. (A) Particle size & PDI after 3 months (B) Zeta potential after 3 months (D) Particle size & PDI after 6 months (D) Zeta potential after 6 months

CONCLUSION

The present study demonstrated the feasibility of a one-pot strategy for integrating NLC formation with thermo-responsive in-situ gel development, thereby eliminating the isolation and transfer of the intermediate NLC dispersion. This approach provided a simplified and integrated manufacturing sequence while retaining the desirable characteristics of both the lipid nanocarrier and thermo-responsive gel matrix. The optimized OP-BH-NLC-Tgel exhibited appropriate nanoscale characteristics, high drug entrapment, temperature-responsive gelation near the ocular surface temperature, and pseudoplastic rheological behavior, indicating its suitability for topical ocular administration. Importantly, integration of the NLC within the thermo-responsive matrix resulted in a delivery system capable of modulating BH release and improving its interaction with the corneal surface. The substantially lower initial drug release compared with Betoptic® 0.25%, together with enhanced transcorneal permeation and approximately 2.48-fold greater corneal drug retention, suggests that the combined nanocarrier–gel architecture can overcome some of the limitations associated with conventional topical BH administration. The preservation of corneal architecture following ex vivo exposure further supports the preliminary local compatibility of the developed system. The formulation also maintained its physicochemical and thermo-responsive characteristics during the 6-month preliminary stability study, supporting the robustness of the developed formulation under the investigated storage conditions. Overall, the findings establish one-pot fabrication as a promising approach for the development of NLC-based thermo-responsive ocular nanogels, with potential to simplify processing while providing controlled drug release and enhanced corneal delivery. Further studies, particularly long-term stability, in vivo ocular pharmacokinetics, and pharmacodynamic evaluation, are required to establish the clinical translational potential of OP-BH-NLC-Tgel for glaucoma therapy.

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