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ABSTRACT

Loteprednol etabonate (LE) is a soft corticosteroid recently approved for the short-term treatment of signs and symptoms of dry eye disease. LE is available on the market as a suspension, which can release only limited amount of dissolved corticosteroid after application at the ocular surface. This study is focused on the development of an oil-in-water nanoemulsion (NE) to effectively deliver dissolved LE to the ocular surface, in order to promote its absorption. We newly developed an extended-throughput 3D model of the corneal epithelium for biocompatibility study, and an innovative approach to investigate the effect of biorelevant dilution on LE release from the NE oil phase. Castor oil, Capryol® 90, Kolliphor® EL and Soluplus® were selected as NE excipients based on their potential to dissolve LE. Design of experiments was successfully employed to develop biocompatible and physically stable NE with high LE content (0.15 %, *w/w*) and retention efficiency (87 % after 10 months of storage at room temperature). LE retention within oil droplets (above 90 % of the initial LE content) despite biorelevant dilution simulating tear turnover suggests the potential for direct LE absorption from the NE oil droplets into the lipophilic corneal epithelium. The results obtained encourage the extension of the studies in terms of *in vitro* permeability and *in vivo* eye-related bioavailability assessment to prove the potential of the proposed LE formulation.

1. Introduction

Dry eye disease (DED) is a multifactorial and self-perpetuating inflammatory disease of the ocular surface (Bron et al., 2017). Tear film instability and hyperosmolarity are considered to be the starting point of the disease, which leads to symptoms and compensatory responses. If the pathological process continues through a chain of events, it ultimately leads to ocular surface damage and a chronic inflammatory state of ocular surface epithelia (*i.e.*, cornea and conjunctiva). A large proportion of patients with DED usually have only mild disease signs and symptoms that can be adequately controlled with lubricant eye drops (Gupta and Venkateswaran, 2021). In severe cases of DED, patients require chronic therapy with immunomodulatory agents such as cyclosporine A or lifitegrast (Jones et al., 2017). However, many patients with mild DED have periodic flares or exacerbations of symptoms in response to specific triggers and may benefit from additional short-term therapy

Abbreviations: ALI, air-liquid interface; API, active pharmaceutical ingredient; BAC, benzalkonium chloride; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DDW, double-distilled water; DED, dry eye disease; DLS, dynamic light scattering; DoE, design of experiments; EE, entrapment efficiency; ELS, electrophoretic light scattering; FDA, Food and Drug Administration; HBSS, Hank's balanced salt solution; ICH, International Conference on Harmonization; LE, loteprednol etabonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and methylthiazolyldiphenyl-tetrazolium bromide, NE, nanoemulsion; PDI, polydispersity index; PES, polyethersulfone; RSD, relative standard deviation; RC, regenerated cellulose; RT, room temperature.

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when their DED symptoms flare up.

Topical corticosteroids are widely used to treat the inflammatory conditions of the ocular surface and multiple studies have shown the clinical benefit of their short-term use in the treatment of DED (Jones et al., 2017). However, their use carries the risk of complications (*e.g.*, ocular hypertension, cataracts, and opportunistic infections, even after short periods of use). Loteprednol etabonate (LE), a soft corticosteroid, was designed to be rapidly hydrolyzed by endogenous esterases to an inactive and non-toxic metabolite, *i.e.*, Δ1 cortienic acid, after exerting its pharmacological effects thus reducing the risk of elevated intraocular pressure (Bodor and Buchwald, 2005). A retrospective safety study, pointed out that LE has minimal effect on intraocular pressure when used topically to treat a wide range of ocular surface and intraocular inflammatory disorders, including DED (Jones et al., 2017; Sheppard et al., 2016).

Topical treatment of DED is hampered by the difficulty of delivering active pharmaceutical ingredients (APIs) to the affected cornea and conjunctiva, which is particularly challenging with poorly water-soluble APIs, such as corticosteroids. After application at the ocular surface, it is difficult to achieve and maintain an effective anti-inflammatory API concentration inside the target tissues, hence frequent administration is required (Shen et al., 2021). Drug solubility in the aqueous tear film, residence time of the API on the ocular surface and API permeability through the cornea and conjunctiva are considered to be the main factors affecting the bioavailability of an API in the ocular tissues after topical administration (Loftsson and Stefánsson, 2017).

In 2020, LE in the form of an ophthalmic suspension (0.25 %, EYSUVIS®, Kala Pharmaceuticals Inc.) was approved by the FDA for short-term (up to 2 weeks) treatment of signs and symptoms of DED (Nadelmann et al., 2022). The suspension is based on mucus-penetrating particle (MPP) technology to improve eye-related drug bioavailability, and it is applied four times a day (Mohamed et al., 2022; Nadelmann et al., 2022).

Ophthalmic oil-in-water nanoemulsions (NEs) have emerged as a useful formulation strategy to increase the eye-related bioavailability of poorly water-soluble APIs. NEs consist of oil nanodroplets dispersed in aqueous solution of surfactants, viscosity enhancing polymers, and other water-soluble components, such as preservatives, tonicity adjusting agents and buffering agents (Dong et al., 2020; Singh et al., 2020). Surfactants stabilize NEs by forming a monolayer on the surface of the oil nanodroplets. The interaction between the oil nanodroplets and the lipid layer of tear film supports prolonged residence time of APIs and excipients on the ocular surface and influences the rate and extent of API absorption (Gawin-Mikołajewicz et al., 2021). Depending on API and NE physicochemical properties, API can be released to the tear film from the NE oil droplets upon physiological dilution or it can partition directly to the lipophilic corneal epithelium (Tamilvanan and Benita, 2004).

Ophthalmic NE presents the formulation of choice for the treatment of DED. Namely, oil nanodroplets and surfactants themselves have a stabilizing effect on the compromised tear film of DED patients (Daull et al., 2020). A clinical trial conducted by Santen Pharmaceutical pointed out that NE without an API (vehicle) gave significantly better results than the reference marketed artificial tears in patients suffering from mild-to-moderate DED (Amrane et al., 2014).

Ophthalmic NE provides the potential to decrease required API dose promoting its penetration into the cornea (Shen et al., 2021). So far, among APIs used in DED therapy, only cyclosporine A has been successfully formulated and approved as ophthalmic NE (Ikervis®, Santen Pharmaceutical, and Restasis®, Allergan), leaving plenty of room for further research. In this study, we propose the development of ophthalmic NE loaded with LE for the short-term treatment of periodic flares of mild-to-moderate DED. The delivery of LE dissolved in NE is expected to promote its absorption to the affected ocular tissues in DED patients. The possibility of achieving complete solubilization of LE inside the formulation and maintaining LE in a solubilized state throughout its expected residence time on the ocular surface was investigated.

Formulating LE in a NE form represents a great challenge. Specifically, LE is less soluble in oils (Patel et al., 2016) when compared to cyclosporine A already available in the form of ophthalmic NEs (Lallemand et al., 2017). Solving such a complex task may bring general benefits in NE ophthalmic delivery of practically insoluble APIs.

In this study rational selection of excipients was performed to formulate LE-loaded NEs. Process and formulation parameters, as well as their levels, were determined to set a statistical design of experiment (DoE) for LE-loaded NE development. NEs were characterized in terms of droplet size and size distribution, zeta-potential, LE content, osmolality, surface tension, stability, and sterilization procedure suitability. For the screening of *in vitro* corneal biocompatibility, we newly developed and successfully employed an extended-throughput 3D model of the corneal epithelium. Furthermore, in this study we present an innovative approach to investigate the effect of biorelevant dilution on the release of API from the NE oil phase. Experimental setup applied in this study revealed the successful incorporation of practically insoluble LE into a NE formulation, as confirmed by high entrapment efficiency, formulation stability, corneal biocompatibility and robustness to biorelevant dilution.

2. Materials and methods

2.1. Materials

Loteprednol etabonate (LE) was generously donated by JGL d.d. (Rijeka, Croatia). For preparation of NEs, the following excipients were used: castor oil (Fagron, Rotterdam, Netherlands), Miglyol 812 (Fagron), sesame oil (Sigma-Aldrich, St. Louis, USA), soybean oil (Sigma-Aldrich), squalane (Sigma-Aldrich), Capryol® 90 (Gattefossé, Saint-Priest, France), Kolliphor® EL (BASF, Ludwigshafen, Germany), Tyloxapol (Sigma-Aldrich), polysorbate 80 (Sigma-Aldrich), Soluplus® (BASF) and glycerol (Gram-mol, Zagreb, Croatia). Acetonitrile and isopropanol were purchased from Merck (Darmstadt, Germany). 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl-2H-tetrazolium bromide and methylthiazolyldiphenyl-tetrazolium bromide (MTT) was purchased from Biosynth (Compton, UK). All other reagents were of analytical grade and purchased from Fagron or Gram-mol. For the *in vitro* cell biocompatibility study Hank's balanced salt solution (HBSS) pH 7.4 was prepared as already described (Jurišić Dukovski et al., 2020).

2.2. Methods

2.2.1. Solubility testing

The thermodynamic solubility of LE was determined in different oils, oily vehicles and solubilizers/surfactants (Miglyol 812, soybean oil, castor oil, sesame oil, squalane, Capryol® 90, Kolliphor® EL, Tyloxapol, polysorbate 80) using the shake-flask method. An excess amount of LE was added to oil/oily vehicle/solubilizer/surfactant and obtained dispersions were incubated at 25 °C and mixed at 300 rpm with a magnetic stirrer during 48 h. The samples were then centrifuged for 30 min at 10,000·g (Heraeus Biofuge Stratos, Thermo Scientific, Waltham, USA) and the supernatants were filtered through polyethersulfone (PES) filter (0.2 μm pore size, Whatman, Little Chalfont, UK). The supernatants were diluted with isopropanol and analyzed for LE content using High Performance Liquid Chromatography (HPLC) as described in the Section 2.2.6.

2.2.2. Preparation of ophthalmic nanoemulsions

A microfluidizer (Model LM20, Microfluidics®, Westwood, USA) was used for preparation of NEs, as previously described (Jurišić Dukovski et al., 2023). The oil phase and the water phase were prepared separately: ingredients were weighed in a beaker and mixed with a magnetic stirrer and heated up to 25–80 °C, depending on the temperature chosen by DoE. The oil phase comprises LE, castor oil, Kolliphor® EL, and

Capryol® 90. The water phase consisted of double-distilled water (DDW) and Soluplus®. After mixing on a magnetic stirrer, the water and the oil phases were homogenized on an Ultra-Turrax® (IKA-Werke GmbH & Company, Staufen, Germany) for 5 min at 15,000 rpm to obtain a coarse emulsion. The coarse emulsion was then processed using the microfluidizer under the pressure of 1000–2000 bar and 5–10 homogenization cycles. The recirculation loop of the microfluidizer was cooled in a bath filled with ice cubes in order to prevent overheating of the sample. The osmolality of NEs was adjusted by glycerol to obtain values in the ophthalmically acceptable range between 270 and 305 mOsm kg^{-1}(Baudouin et al., 2013). Glycerol was chosen as the tonicity agent with insignificant effect on NE physicochemical properties (Jurišić Dukovski et al., 2020; Teixeira et al., 2017).

2.2.3. Statistical design of experiments (DoE)

The quality-by-design (QbD) approach was used to optimize the formulation and process parameters that affect the NE physicochemical properties, which are critical for the performance of a NE. Five formulation parameters (content of LE, castor oil, Capryol® 90, Kolliphor® EL, and Soluplus®) and three process parameters (mixing temperature of the oil and water phases, number of homogenization cycles, and pump pressure of the microfluidizer) were selected for the custom experimental design developed using JMP 14.0 statistical software (JMP®, version 14.0, SAS Institute Inc., Cary, NC, USA, 1989–2023). Droplet size, polydispersity index, zeta potential, drug content, viscosity and surface tension were investigated as responses. Droplet size, polydispersity index, zeta potential and drug content were determined at 4 timepoints (immediately after preparation and on the 15th, 30th and 60th day of storage at room temperature (RT)). Viscosity and surface tension were measured only once, *i.e.*, after NE preparation.

2.2.4. Viscosity, osmolality, and pH

The viscosity of the NEs was determined at a shear rate range from 20 to 100 s^{-1} at 25 °C using an Anton Paar MCR102 rheometer (Graz, Austria) equipped with a cone-plate measuring system (CP 50–1, cone angle 1°, trim position 102 μm) and calculated by the RheoCompass software. The surface tension was determined with Kruss K-100C tensiometer (Hamburg, Germany) using Du Noüy ring method. The osmolality of the NEs was determined using an OsmoTECH® Osmometer (Advanced Instruments, Norwood, USA). The pH of the NEs was determined using a Seven Multi pH/conductometer (Mettler Toledo, Columbus, USA) at 25 °C. All measurements were performed in triplicate.

2.2.5. Droplet size, size distribution and zeta potential analysis

The droplet size and polydispersity index (PDI) of the NEs were determined at 25 °C by dynamic light scattering (DLS) while zeta potential was determined at 25 °C by electrophoretic light scattering (ELS), using a Zetasizer Ultra (Malvern Panalytical, Malvern, UK). Each formulation was diluted 100-fold (*V/V*) with DDW (filtered through 0.45 μm PES filter) and the droplet size and PDI were measured using a disposable cuvette (DTS0012, Sarstedt AG & Co. KG, Nümbrecht, Germany) and the zeta potential was measured in a disposable folded capillary cell (DTS1070, Sarstedt AG & Co. KG). The value of dispersant viscosity used in the measurement was set to be equal to that of water at 25 °C, *i.e.*, 0.89 mPas. All measurements were performed in triplicate.

2.2.6. HPLC analysis of LE

The quantitative determination of LE was performed by a HPLC method using a 1260 Infinity II LC System with UV–VIS detector (Agilent Technologies, Santa Clara, USA). Separation was performed using an XBridge C18 (2.7 mm × 100 mm, 5 μm particle size) reverse-phase column (Waters Corporation, USA). The mobile phase was a mixture of DDW and acetonitrile in 42:58 (*V/V*) ratio and the elution was isocratic. The following HPLC conditions were used: flow rate 1 mL min^{-1}, column temperature 50 °C, injection volume 5 μL and detection wavelength 255 nm. System suitability was tested for every sequence, and it was evaluated based on the following criteria: relative standard deviation (RSD) of the detector response factor for two standard solutions was not >2.0 % and RSD of hexaplicate standard solution injection was not >1.0 %. The validation of the HPLC method was performed according to the International Conference on Harmonization (ICH) guideline Q2 (R1) and the method was confirmed to be linear ($R^2 \geq 0.99$), accurate (recovery 98–102 %) and repeatable (RSD of peak area ≤ 2.0 %).

2.2.7. Determination of LE content in NE formulations

The NE samples were filtered through a polyethersulfone (PES) filter (0.45 μm pore size, Whatman) to remove any undissolved LE. The filtrates were diluted with isopropanol 50 times (V/V) to obtain a transparent solution of all NE constituents, and analyzed for LE content actually entrapped within NE (LE_A) using HPLC, as described in the previous section. LE_A is expressed as a percentage weight ratio of LE in a NE formulation.

The entrapment efficiency of LE inside the oil droplets ($EE_{oil\ droplet}$%) was determined by ultrafiltration. An aliquot of NE (0.5 mL) was placed in the ultrafilter-fitted centrifuge tube (Microcon®, MWCO 30 kDa, regenerated cellulose (RC), Merck-Milipore, Billerica, USA) and centrifuged at 2000 g for 1 h (Heraeus Biofuge Stratos). The amount of LE in the NE filtrate (aqueous phase of NE) was determined by HPLC, as described above. The following equation was used to calculate the LE entrapment efficiency inside the oil droplets ($EE_{oil\ droplet}$ %):

$$EE_{oil\ droplet}\ \% = \frac{m_t - m_f}{m_t} \times\ 100,$$

where m_t is the total amount of LE in the NE and m_f is the amount of LE in the NE filtrate.

2.2.8. Evaluation of NE biocompatibility In vitro

2.2.8.1. Cultivation of a 3D HCE-T model in 96-well plates. HCE-T cells (RIKEN Cell Bank, Tsukuba, Japan) were grown in DMEM/F12 medium (Sigma-Aldrich) supplemented with FBS (5 %, Capricorn, Düsseldorf, Germany), insulin (5 μg/mL, Sigma-Aldrich), dimethyl sulfoxide (0.5 %, Applichem, Darmstadt, Germany), epidermal growth factor (10 ng/mL, Sigma-Aldrich) and antibiotic-antimycotic solution (1 %, Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5 % CO_2.

Cells were seeded (1×10^4 cells per well) on polycarbonate membranes (0.4 μm pore size) of a 96-well insert plate (PSHT004, Merck-Milipore) precoated with rat tail type I collagen (Sigma-Aldrich) and human fibronectin (Sigma-Aldrich). The cells were cultivated submerged in the medium (75 μL in apical and 250 μL in basolateral chamber) for 5 days and were subsequently exposed for 3 days to the air-liquid interface (ALI) by removing the medium from the apical surface. The culture medium was changed every 2 days during the submerged conditions and every day during exposure to the ALI. Transepithelial electrical resistance (TEER) was monitored during submerged conditions and after exposure to the ALI using Millicell ERS-2 voltohmmeter (Merck-Milipore) equipped with an STX00 electrode.

2.2.8.2. Histomorphological characterization. The cells were fixed with 4 % paraformaldehyde (Sigma-Aldrich) at different time points after seeding. The cells were then washed with phosphate-buffered saline (PBS). Prior to preparing a cross section, the membrane was gently dried and placed in a Tissue-Tek embedding medium (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) and left for 2–3 min until the medium was frozen. Subsequently, 5-μm-thick cross sections were precisely sliced using a cryostat (CM1950, Leica, Eisfeld, Germany). The cross-sections were mounted onto glass slides with Fluoroshield™ mounting medium containing a cell permeable fluorescent dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) and the cover slips were sealed with a nail polish. The cell nuclei were imaged using ImageXpress Micro Confocal High-Content Imaging

System (Molecular Devices, San Jose, USA) with Nikon 10x CFI Plan Apochromat Lambda objective (Nikon, Tokyo, Japan) under 60 μm pinhole IXConfocal module disk geometry.

2.2.8.3. Cell viability assay. Cell viability was determined either with MTT or CellTiter- Glo® 3D Cell Viability Assay.

NEs were diluted 10 times (*V/V*) with HBSS buffer (pH 7.4) and the apical side of the cell model was exposed to the diluted NEs, for 30 min at 37 °C. Basolateral side of the cell model was immersed in HBSS for all tested samples. HBSS was used as a negative control and benzalkonium chloride (BAC, 0.005 % and 0.1 % (*w/V*)) was used as a positive control. After incubation, NE samples were removed, the inserts were washed with HBSS and the cell culture medium was returned to the basolateral side for 24 h, while apical side was exposed again to ALI. Thereafter, the cell viability was tested using two different cell viability assays.

Cell viability was determined by MTT assay as follows. MTT stock solution was prepared in PBS (5 mg mL^{-1}). The medium was removed from the apical and basolateral side, MTT solution diluted in the medium (0.5 mg mL^{-1}) added to each side (70 μL to the apical and 200 μL to the basolateral side) and the cell model was incubated for 45 min at 37 °C. The MTT solution was then removed, and the polycarbonate membranes with cells were carefully detached from the insert plate with tweezers and transferred to a 96-well plate with flat bottom (one membrane per well). Formazan crystals were dissolved by the addition of isopropanol (100 μL per well) and subsequent shaking on an orbital shaker. The absorbance of the formazan solution was then measured at 570 nm with a microplate reader (SpectraMax® i3, Molecular Devices).

CellTiter-Glo® 3D Cell Viability Assay (CTG 3D) (Promega Corporation, USA) was performed according to manufacturer's protocol. Namely, the medium was removed from the basolateral side and CellTiter-Glo® 3D reagent diluted 1:1 (*V/V*) with the medium was added to the apical side of the cell model. The plate was vigorously shaken on an orbital shaker to induce cell lysis. After shaking, the plate was left at RT for additional 30 min to stabilize the luminescent signal. Subsequently, the apical side samples were transferred to a 96-well opaque-walled plate (Nunc™ MicroWell™, Thermo Fisher Scientific) and the luminescence was recorded with a microplate reader (SpectraMax® i3).

2.2.9. Evaluation of NE physical stability

To perform the stability studies, NE samples were stored in sealed glass containers protected from light for 10 months at RT. At predetermined time points (15, 30, 60 and 300 days after NE preparation), droplet size, PDI, zeta potential and LE content were determined as described in the previous sections. In addition, NEs were visually inspected for any signs of phase separation.

Additional studies were performed to evaluate the physical stability of NEs in HBSS at 37 °C, reproducing the conditions of the cell viability studies performed. Representative NEs were diluted 10-fold (*V/V*) with HBSS buffer (pH 7.4) and incubated for 30 min at 37 °C (Biosan, Environmental Shaker ES 20/60). NE droplet size and PDI potential were measured at 37 °C using a Zetasizer Ultra.

2.2.10. NE sterilization

NEs were aseptically filtered through PES filter (0.2 μm pore size, Whatman) or steam sterilized at 2.2 bar and 120 °C for 15 min in a sealed glass container using Systec VX-100 autoclave (Linden, Germany). After each sterilization method NEs were examined visually and characterized in terms of droplet size, PDI, zeta potential and LE content.

2.2.11. Analysis of LE distribution between NE oil and aqueous phase under simulated tear turnover conditions

The analysis of LE distribution between NE oil droplets and aqueous phase was performed employing biorelevant dilution of NEs, simulating tear turnover *in vivo*. In the preliminary study, phosphate buffer saline (PBS) of pH 7.4 supplemented with Kolliphor® EL 5 % (*w/V*) (PBS + 5 % Kolliphor® EL) was selected as a dilution medium in order to ensure sink conditions using the same surfactant as in NEs. The sink conditions were used, determined on the basis of thermodynamic LE solubility data in PBS + 5 % Kolliphor® EL determined by shake flask method at 34 °C (Section 2.2.1).

The lead formulation (NE 21) was diluted with PBS + 5 % Kolliphor® EL in 1:1, 1:4, 1:9, 1:25, 1:38 and 1:64 vol ratios and incubated for 60 min at 34 °C.

The diluted samples were transferred to the upper chamber of a centrifuge tube fitted with ultrafilter (Microcon®, MWCO 30 kDa, RC, Merck-Millipore), and centrifuged at RT and 1000·g for 30 min (Heraeus Biofuge Stratos). The content of LE in the oil phase prior and upon NE dilution was determined indirectly based on the content of LE quantified in the filtrate (aqueous NE phase) by HPLC (described in the Section 2.2.6 and 2.2.7). The same procedure was performed with control formulation, namely NE21 formulation loaded with more water-soluble ibuprofen (IBU). The IBU quantification is described in the Supplementary materials.

2.2.12. Statistical analysis

Statistical analysis of *in vitro* biocompatibility data was performed using One-way ANOVA followed by a Tukey's test, with $P < 0.05$ set as the minimal level of significance. Analysis was performed with the GraphPad Prism software (GraphPad Software, Inc., San Diego, USA; www.graphpad.com).

3. Results and discussion

LE is a cortienic acid-based soft corticosteroid extensively used in the treatment of ophthalmic inflammatory conditions. Being a highly lipophilic compound (logP of 3.4, (Loteprednol etabonate, DrugBank online), LE penetrates the ocular tissues efficiently (Glogowski et al., 2014; Sheppard et al., 2016). However, the extent of LE permeation/penetration to the cornea is limited by its poor solubility. LE binds to the glucocorticoid receptor with 4.3-fold greater affinity than dexamethasone (Druzgala et al., 1991), rapidly hydrolyzes by esterases to an inactive and non-toxic metabolite, and therefore exerts potent anti-inflammatory effects with minimal propensity for side effects (Bodor and Buchwald, 2005; Sheppard et al., 2016). It is practically insoluble in water (reported solubility of 8 μgL^{-1} (Bao et al., 2017)). Due to poor water solubility, LE is available on the market as a micro/-nanoparticle suspension, which can release only limited amount of dissolved corticosteroid after application at the ocular surface.

This study aimed to develop of an ophthalmic NE to increase the availability of dissolved LE on the ocular surface, in order to promote its absorption. In a NE, poorly water-soluble drug is predominantly dissolved inside the oil droplets, and the amount of drug in the NE aqueous phase varies based on its equilibrium solubility (Dong et al., 2020).

3.1. Selection of NE excipients

The first step in the development of NE was the identification of NE excipients, that can fully solubilize LE in the formulation in the amount suitable for delivery of the adequate LE dose with a single eye drop. To perform initial excipient screening, the solubility of LE was tested in various oils, oily vehicles, solubilizers and surfactants. According to the results obtained (Table 1), castor oil, Capryol® 90 and Kolliphor® EL were selected for the development of LE-loaded NE formulation. Namely, solubility of LE was the highest in castor oil among the tested oils, in Kolliphor® EL among the tested surfactants, and in Capryol® 90 among all the tested excipients, so Capryol® 90 was chosen as an additional solubilizer.

As the solubility of LE in the selected NE excipients has been shown to be limited, challenges were expected with its incorporation into a NE formulation. Therefore, Soluplus® was additionally introduced as a novel polymeric solid excipient with excellent solubilization properties

Table 1
Thermodynamic solubility of LE in the tested NE excipients at 25 °C.

Tested NE excipient	Solubility at 25 °C (mg mL^{-1})
Castor oil	2.43 ± 0.12
Miglyol 812	1.30 ± 0.03
Sesame oil	0.36 ± 0.02
Soybean oil	0.42 ± 0.00
Squalane	0.03 ± 0.00
Capryol® 90	12.18 ± 0.34
Kolliphor® EL	11.49 ± 0.11
Polysorbate 80	10.51 ± 0.04
Tyloxapol	10.08 ± 0.13

Values are mean ± SD ($n = 3$).

for poorly soluble drugs. The proposed NE qualitative composition is innovative in relation to NEs described in the literature (Patel et al., 2016; Singh et al., 2020; Uner et al., 2023a) and suited for DED therapy considering beneficial effects of castor oil such as tear film lipid replenishment and tear film stabilization (Maïssa et al., 2010; Maulvi et al., 2024; Sandford et al., 2021).

The next step in the NE development process was fine-tuning of quantitative NE composition in conjunction with the optimization of production process. The applied approach for such purpose is described in the following sections.

3.2. Formulation development through DOE

In this study, NEs were prepared using a repeatable and reliable microfluidization technique, suitable for pharmaceutical scale up (Singh et al., 2017). DoE was employed in NE development by varying formulation (content of LE and selected excipients) and process (temperature, homogenization cycle number and pressure) parameters. The design space *i.e.*, lower and upper limits for parameter intervals, was defined based on literature reports and preliminary experiments performed. The level of theoretical LE content was set up to 0.20 %, *i.e.*, close to the LE content in the approved LE suspension (0.25 %, Eysuvis). The level of castor oil ranged from 20 % (generally recognized as the maximal oil content in ophthalmic NEs (Gawin-Mikołajewicz et al., 2021)) to 10 %, presenting relatively high oil content needed to solubilize sufficient dose of LE. The Kolliphor® EL level was set up to 5 % (*w/w*), representing its maximal concentration in the approved

Table 2
A sample sequence from the design of experiments (DoE) and the corresponding responses (measured immediately after NE preparation): droplet size (DS), polydispersity index (PDI), zeta potential (ZP), actual LE content (LE_A), viscosity (η) and surface tension (γ).

	Formulation parameters						Process parameters			Responses					
	LE_T (%, *w/w*)	CO (%, *w/w*)	C90 (%, *w/w*)	KOL (%, *w/w*)	SP (%, *w/w*)	DDW (%, *w/w*)	T (°C)	C	P (bar)	DS (nm)*	PDI*	ZP (mV)*	LE_A (%, *w/w*)	η (mPas)*	γ (mNm^{-1})*
NE1	0.1	20	3	5	0	71.9	25	10	2000	121.8 ± 1.7	0.06 ± 0.02	−42.6 ± 7.4	0.08	3.2 ± 0.1	35.4 ± 0.0
NE2	0.1	20	1	1	0.5	77.4	80	10	1000	216.9 ± 0.3	0.10 ± 0.03	−26.1 ± 0.7	0.10	2.3 ± 0.0	36.0 ± 0.2
NE3	0.15	10	1	1	0	87.85	25	5	1000	203.6 ± 1.5	0.14 ± 0.02	−35.9 ± 0.5	0.06	1.2 ± 0.0	37.4 ± 0.2
NE4	0.15	15	3	3	0.5	78.35	52.5	7.5	1500	143.5 ± 1.2	0.06 ± 0.02	−20.4 ± 0.4	0.12	2.4 ± 0.1	34.0 ± 0.4
NE5	0.2	20	1	5	1	72.8	25	5	1500	148.6 ± 0.3	0.05 ± 0.02	−24.2 ± 1.7	0.12	4.9 ± 0.0	36.5 ± 0.2
NE6	0.2	20	5	1	1	72.8	25	10	1000	250 ± 2.9	0.08 ± 0.01	−20.5 ± 0.5	0.16	3.2 ± 0.3	32.9 ± 0.1
NE7	0.1	10	1	5	0	83.9	80	5	2000	124 ± 2.1	0.15 ± 0.03	−23.1 ± 1.3	0.10	1.7 ± 0.0	34.9 ± 0.2
NE8	0.2	10	3	1	1	84.8	80	5	1000	200 ± 1.8	0.11 ± 0.01	−20.3 ± 0.4	0.16	2.1 ± 0.7	33.3 ± 0.1
NE9	0.2	10	5	5	0.5	79.3	25	5	2000	111.6 ± 2.5	0.12 ± 0.04	−20.5 ± 3.7	0.11	2.5 ± 0.3	31.9 ± 0.1
NE10	0.2	10	5	5	0	79.8	52.5	10	1000	101.1 ± 0.1	0.18 ± 0.03	−14.6 ± 0.8	0.14	2.1 ± 0.1	31.7 ± 0.2
NE11	0.2	20	5	1	0	73.8	80	5	2000	599.5 ± 36.5	0.22 ± 0.04	−30.8 ± 0.1	0.20	2.1 ± 0.0	33.7 ± 0.2
NE12	0.1	10	1	5	1	82.9	25	10	1000	134.1 ± 2.7	0.12 ± 0.02	−18.3 ± 1.5	0.11	2.1 ± 0.1	35.3 ± 0.2
NE13	0.2	10	1	3	1	84.8	80	10	2000	131.5 ± 3.4	0.09 ± 0.03	−17.7 ± 1.3	0.16	1.9 ± 0.0	35.6 ± 0.2
NE14	0.1	15	5	5	1	73.9	80	5	1000	137.5 ± 0.4	0.05 ± 0.00	−17.0 ± 1.0	0.10	4.2 ± 0.1	32.9 ± 0.2
NE15	0.2	15	1	1	0	82.8	25	10	2000	201.4 ± 1.3	0.09 ± 0.01	−24.4 ± 0.4	0.08	1.7 ± 0.1	37.0 ± 0.1
NE16	0.2	20	1	5	0	73.8	80	7.5	1000	144.4 ± 1	0.09 ± 0.00	−19.4 ± 1.2	0.13	2.9 ± 0.1	36.4 ± 0.4
NE17	0.1	10	5	1	1	82.9	25	7.5	2000	180.5 ± 3.4	0.09 ± 0.01	−18.3 ± 0.4	0.11	1.8 ± 0.0	32.2 ± 0.1
NE18	0.1	20	5	3	0	71.9	25	5	1000	177.5 ± 1.3	0.07 ± 0.02	−22.3 ± 0.7	0.11	3.4 ± 0.2	33.7 ± 0.2
NE19	0.1	20	1	1	1	76.9	52.5	5	2000	334.6 ± 3.1	0.12 ± 0.02	−25.0 ± 0.4	0.09	4.7 ± 0.5	34.6 ± 0.2
NE20	0.1	10	5	1	0	83.9	80	10	1500	219.4 ± 1.9	0.04 ± 0.03	−22.8 ± 0.8	0.10	1.6 ± 0.0	32.3 ± 0.1
NE21	0.15	20	5	5	1	68.85	80	10	2000	147.2 ± 0.4	0.05 ± 0.02	−16.8 ± 0.6	0.15	7.0 ± 0.3	33.9 ± 0.1

LE_T – theoretical LE content; CO – castor oil content; C90 – Capryol® 90 content; KOL – Kolliphor® EL content; SP – Soluplus® content, DDW – double distilled water content, T – mixing temperature of oil and water phase, C – number of homogenization cycles, P – pump pressure of the microfluidizer.

* Values are mean ± SD ($n = 3$).

ophthalmic drug products (FDA Database: Inactive ingredients). Levels of other NE constituents and process parameters were set to allow the formation of NEs with adequate properties for ophthalmic delivery and acceptable LE entrapment.

DoE generated 21 runs as presented in Table 2. The corresponding 21 LE-loaded NE formulations were prepared and characterized in terms of droplet size, PDI, zeta potential, LE content, EE, surface tension and viscosity.

Results on regression modeling are presented in Table 3. The parameters (covariates) were normalized to unitless intervals [−1, 1]. Within regression modeling equations, parameters with statistically significant impact on monitored response are marked with asterisk.

3.2.1. The overview of the main properties of LE-loaded NEs prepared according to DOE

LE-loaded NEs were successfully prepared within the DoE space, characterized by droplet size adequate for topical ophthalmic delivery ranging from 100 to 250 nm (Singh et al., 2020), with the exception of two NE formulations (NE19 and NE11; 334.6 nm and 599.5 nm droplet size, respectively; Table 2). PDI values were between 0.05 to 0.22 confirming their monodispersity (Table 2). This is of significant importance since monodispersity is one of prerequisites for NEs of adequate stability profile (Gupta et al., 2016). All the NEs were characterized with negative zeta potential, with the majority of NEs having zeta potential between −26 and −14 mV (Table 2). Negative zeta potential of NE prepared with nonionic surfactants has already been reported (Jurišić Dukovski et al., 2019; Uner et al., 2023a). NEs prepared within this study exhibited Newtonian behavior, thus their viscosity was constant regardless of the applied shear stress. All the NEs had low viscosity ranging from 1.2 to 7.0 mPas, indicating optimal properties for simple application. The surface tension of the NEs ranged from 31.7 to 37.4 mNm^{-1}, with the majority of NE formulations falling within the usual range of surface tension reported for approved ophthalmic dosage forms, *i.e.*, 34–71 mNm^{-1} (Han et al., 2016). All the prepared NEs were characterized with a pH suitable for ophthalmic delivery (pH from 4.45 to 5.50) (Ligório Fialho and da Silva-Cunha, 2004). NE osmolality was adjusted to 270–305 $mOsmkg^{-1}$ using glycerol as a tonicity agent.

Table 3
The results of the statistical analysis on droplet size, polydispersity index, zeta potential, actual LE content, viscosity and surface tension within the DoE.

Common DoE Response	Regression Model	Regression Analysis		
		R^2	RMSE	PRESS RMSE
Droplet size	$DS\ (nm) = 191.84^{*} + 40.81 \times CO^{*} - 68.64 \times KOL^{*} - 28.52 \times C + 21.5 \times P + 36.47 \times CO \times P^{*} - 39.01 \times C \times P^{*}$	0.76	63.70	84.38
PDI	$PDI = 0.098^{*} + 0.013 \times LE - 0.002 \times C90 - 0.014 \times SP^{*} - 0.013 \times C + 0.029 \times LE \times C90^{*} - 0.016 \times LE \times SP^{*}$	0.74	0.03	0.03
Zeta potential	$ZP\ (mV) = -22.90^{*} - 2.00 \times CO + 1.54 \times C90 + 3.21 \times SP^{*} - 1.38 \times P - 2.60 \times CO \times P - 2.72 \times KOL \times P$	0.60	5.03	6.73
Actual LE content	$LE_A\ (\%) = 0.116^{*} + 0.017\ LE^{*} + 0.01 \times C90 - 0.003 \times KOL + 0.017 \times T^{*} - 0,012 \times KOL \times C90^{*} + 0,012 \times LE \times T$	0.74	0.02	0.03
Viscosity	$Viscosity\ (mPas) = 2.80^{*} + 0.93 \times CO^{*} + 0.56 \times KOL^{*} + 0.66 \times SP^{*} + 0.29 \times CO \times KOL + 0.47 \times CO \times SP^{*}$	0.86	0.59	0.73
Surface tension	$Surface\ tension\ (mNm^{-1}) = 34.46^{*} + 0.47 \times CO^{*} - 1.57 \times C90^{*} + 0.36 \times C90 \times CO - 0.34 \times CO \times CO + 0.21 \times C90 \times C90$	0.86	0.75	0.89

R^2 = the coefficient of determination; RMSE = root mean square error; and PRESS = predicted residual error sum of squares. *Statistically significant parameters (individual and in interaction; $P < 0.05$).

In general, the formulation parameters varied within DoE were found to exert a stronger impact on the investigated NE responses in comparison with the employed process parameters. More detailed explanations are given below.

3.2.2. Theoretical loteprednol etabonate content (LE_T)

The content of LE used for the preparation of NE formulations (LE_T) had statistically significant impact on LE content actually entrapped within the prepared NEs (LE_A). More specifically, the increase in LE_T resulted in an increase in LE_A, indicating appropriately set design space. In addition, LE_T in interaction with each of the tested solubilizers (Capryol® 90 and Soluplus®) was found to influence PDI of NEs. Thus, at the highest LE_T (0.2 %, *w/w*) the increase in Soluplus® content (SP) resulted in PDI decrease, while at the lowest LE_T (0.1 %, *w/w*) PDI was constantly low regardless of SP. Furthermore, the increase in Capryol® 90 content (C90) in the NEs prepared at LE_T of 0.1 % and 0.2 % resulted in a decrease and an increase in PDI, respectively.

3.2.3. Castor oil content (CO)

Being the main oil phase constituent of the developed NEs, CO had a statistically significant impact on the majority of investigated NE responses. Thus, the increase in CO resulted in an increase in NE droplet size, viscosity and surface tension. It has been previously confirmed that the oils with high viscosity, such as castor oil, induce flow resistance within the interaction chamber of a microfluidizer, thereby diminishing the efficiency of droplet disruption which in turn results in the generation of larger oil droplets (Jurišić Dukovski et al., 2019). Moreover, the increase in CO enhanced the capacity of NE to retain incorporated LE. As described in the Section 2.2.3., LE_A was monitored during a 2-month storage period at RT with DoE regression analysis performed at 15, 30 and 60 days of storage (see Supplementary materials, Table S1). The amount of LE entrapped in a solubilized form inside the NE formulations, measured in the chosen timepoints increased with CO increase, as shown by regression models presented in Supplementary materials (Table S2).

3.2.4. Capryol® 90 content (C90)

Capryol® 90 used for the preparation of NE formulations had statistically significant impact on surface tension of prepared NE. More specifically, the increase in C90 resulted in a decrease in surface tension which may be explained by its surface activity. The increase in C90 enhanced the NE capacity to retain the incorporated LE (Table S2). The effect was more pronounced than that of CO. Such an observation is in agreement with the highest LE solubility in C90 (12.18 ± 0.34 mg mL^{-1}) when compared to other NE excipients.

3.2.5. Kolliphor® EL content (KOL)

The increase in KOL resulted in viscosity increase, probably due to its viscous nature (650 – 800 mPas at 25 °C, Safety data sheet for Kolliphor® EL, BASF). Being the main surfactant in the formulation, the increase in KOL decreased NE droplet size while at the same time no statistically significant effect on surface tension was observed. Such behavior may be explained by the fact that Kolliphor® EL concentration in developed NE formulations was well above its critical micellar concentration (0.02 %, *w/w* at 37 °C, Technical information for Kolliphor® EL, BASF).

3.2.6. Soluplus® content (SP)

As a water-soluble nonionic polymeric solubilizer, Soluplus® influenced several NE responses. Namely, the increase in SP resulted in a statistically significant increase in NE viscosity and a decrease in PDI. Regression modeling also revealed the decrease of zeta potential absolute value with increase of SP. Such an effect of a nonionic surfactant addition on NE ZP has already been described in the literature (Jurišić Dukovski et al., 2019). Moreover, SP was recognized as one of the parameters enhancing the retention of dissolved LE in the NE formulations

(Table S2).

3.2.7. Process parameters

Among the investigated process parameters (temperature, pressure and number of homogenization cycles), the critical role was that of oil and water phase heating temperature (T). Namely, variation of T within DoE (25–80 °C) revealed its statistically significant impact on LE NE content and retention efficiency, both recognized as critical quality attributes. Namely, increase in T resulted in an increase in LE_A, measured immediately after preparation as well as during 2-month storage of NEs.

The statistically significant impact of pressure and number of homogenization cycles was observed only in parameter interactions (Table 3). Thus, the increase in number of homogenization cycles at pressure of 2000 bar decreased the oil droplet size while at pressure of 1000 bar it induced no change in droplet size.

3.3. In vitro biocompatibility screening of developed NEs

Biocompatibility testing of DoE NE formulations was performed with three-dimensional (3D) model of the corneal epithelium that accurately represent its structure *in vivo*. Since the HCE-T cell line is the most extensively characterized human corneal epithelial cell line, 3D HCE-T model of the corneal epithelium was used in this study. Usually, 3D HCE-T model is cultured on 12-well insert plates (Juretić et al., 2017; Jurišić Dukovski et al., 2020). However, to test a large number of formulations as developed by DoE, an improved *in vitro* biocompatibility screening model with higher throughput is necessary. Therefore, one of the goals of this study was also to develop a 3D HCE-T cell model grown on 96-well insert plates.

The number of HCE-T cells seeded per well was adjusted to the membrane surface area of 96-well insert plates and the cells were initially cultured in cell culture media in both the apical and basolateral compartments. An increase in the TEER value indicated the formation of a confluent monolayer, and thus, the time at which the cell culture medium had to be withdrawn from the apical compartment. Exposure of the monolayer to ALI resulted in the formation of a stratified apical epithelial structure on the underlying polycarbonate matrix (Fig. 1), which is a characteristic of the corneal epithelium *in vivo* (Hahne and Reichl, 2011).

The developed model was initially evaluated in terms of its tolerance to BAC, a standard positive control in biocompatibility testing of topical ophthalmic formulations *in vitro* and *in vivo* (Kabashima et al., 2020; Vitoux et al., 2020; Yamashiro et al., 2021). BAC was used at two different concentrations, namely 0.005 and 0.1 % (*w/V*). Cell viability was evaluated using a standard colorimetric MTT assay. The results obtained showed a common toxic effect related to BAC. Precisely, the cells treated with BAC showed a concentration-dependent viability decrease, *i.e.*, the cell viability was 63.9 ± 2.4 % and 6.1 ± 2.6 % for BAC concentrations 0.005 and 0.1 % (*w/V*) respectively.

In the following step, results of MTT assay were compared to the results obtained with a luminescence-based assay characterized by a robust penetration into the microtissue and superior sensitivity (CellTiter-Glo® 3D Cell Viability Assay, Promega, CTG 3D assay) to evaluate the appropriateness of MTT as a reagent of choice for cell viability assessment in 96-well cell model. For this purpose, 4 preliminary NE formulations were prepared as described in the Section 2.2.2. The composition of each NE is shown in Table S3 of Supplementary materials. The tested formulations were diluted 10-fold with HBSS, presenting a common protocol for ophthalmic *in vitro* biocompatibility testing (Jurišić Dukovski et al., 2020; Kinnunen et al., 2014). The 10-fold dilution resembles the physiological dilution of a formulation that occurs 13 min after its instillation to the ocular surface (Jurišić Dukovski et al., 2023). No statistically significant difference was observed between the results obtained by MTT and CTG 3D assays when the cells were treated with the same NE formulations (Fig. 2). Therefore, MTT assay was selected for further DoE NE biocompatibility screening, as a more affordable, but still very convenient cell viability test.

The obtained results are presented in Fig. 2. Generally, DoE NEs were shown to be biocompatible, with cell viabilities above 80 % observed for the majority of investigated formulations. These results are in agreement

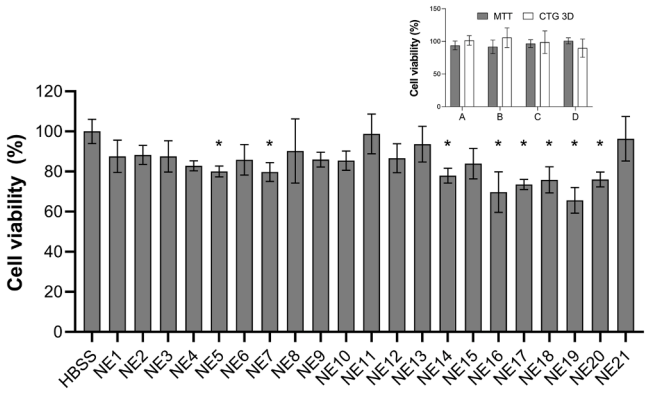

Fig. 2. *In vitro* 3D HCE-T model cell viability determined by MTT and CTG 3D assay after 30-min incubation with preliminary NE formulations (insert) and by MTT assay after 30-min incubation with 21 DoE NE formulations (main graph). The cells incubated in HBSS pH 7.4 were used as a negative control (100 % cell viability). Data are expressed as mean ± SD (n = 4–5). *Differs from the negative control ($P < 0.05$).

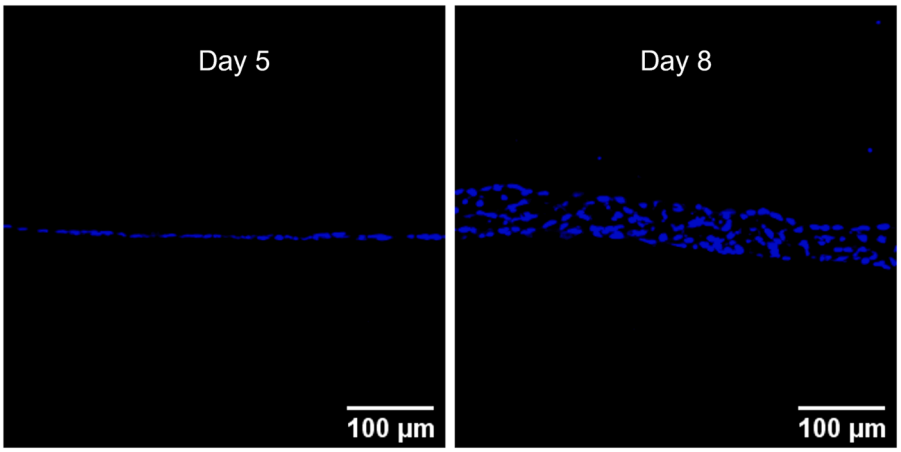

Fig. 1. Cross-sections of the HCE-T cell model during the initial cultivation in submerged conditions (day 5) and after 3 days at ALI cultivation (day 8). The cell nuclei were stained with DAPI.

with the available data on the safety of NE excipients in ophthalmic delivery (Maïssa et al., 2010; Varela-Garcia et al., 2018; Jurišić Dukovski et al., 2019; Yang et al., 2022). Prior to cell viability studies, representative NEs were shown to be stable in terms of droplet size and PDI when diluted and incubated in HBSS for 30 min at 37 °C (see Supplementary materials, Table S4).

3.4. Selection of the lead NE formulation

Results of DoE characterization and *in vitro* biocompatibility studies disclosed the suitability of the majority of prepared NEs for ophthalmic administration. In addition, stability studies revealed no significant changes in crucial physicochemical properties, such as droplet size and PDI, during a 2-month storage for any of NE formulations (Fig. 3). Therefore, the selection of the lead NE was finalized on the basis of LE content and LE retention efficiency, the critical quality attributes optimization of which is crucial for successful LE delivery to the affected ocular tissues in DED patients. Conclusively, as NE21 had high LE_A immediately after preparation (0.15 %) and after 10-month storage (0.13 %, referring to 87 % of the initial LE_A, Fig. 4), it was chosen as the lead formulation. It can be seen from the Table 2 that four NEs (NE6, NE8, NE11 and NE13) showed higher initial LE_A (0.16 %−0.20 %) than the selected NE21, however they showed more pronounced drop in LE content during storage (Table S1 and Fig. 4). This finding is in line with previous reports on even higher initial LE content in NE developed by Uner et al. (approximately 0.45 %; (Uner et al., 2023a)) that was followed by prominent decrease (up to 60 % of initial LE content) during storage (Uner et al., 2023b). That might be explained by supersaturation of the excipients with LE during NE preparation, resulting in metastable formulation, thus some LE has precipitated during storage (Sarheed et al., 2020).

In order to verify the selection of the lead formulation and prediction value of regression models, two formulations with higher LE content (LE_A) than in the lead formulation were derived. The formulation and process parameters of derived formulations as well as predicted responses are shown in Table 4. The derived samples were prepared and characterized with the aim to assess the accuracy of the models obtained (Table 4). The suitable fit between experimental and predicted data confirmed the output value of regression models. Moreover, it confirmed NE21 to be the lead formulation since initially high LE content (LE_A t_0) of derived NEs started to decrease in 15 days of storage at room temperature.

The lead formulation was further characterized in terms of its resilience to sterilization. Two commonly used sterilization methods were compared: steam sterilization and membrane filtration using a 0.2 μm sterile PES filter. The results obtained are presented in Table 5. High temperature (120 °C) during the steam sterilization significantly affected the physicochemical properties of NE21, especially in terms of LE content, which dropped from the initial 0.15 % to 0.09 %. Also, a change in the nanodroplet size was noticed, which increased from 147.2 nm to 182.9 nm, while PDI was shown to be practically unaffected by

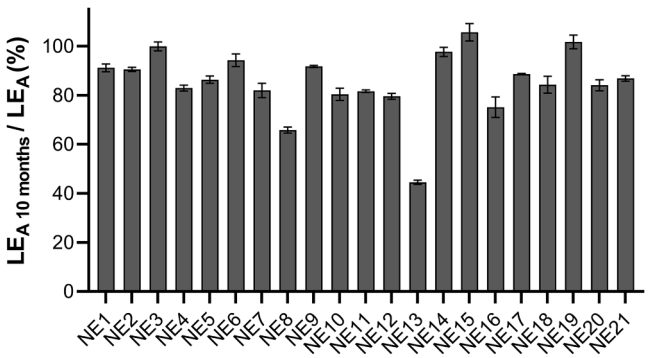

Fig. 4. LE retention capacity during 10 months of storage at RT, expressed as a percentage of the initial LE_A. Data are expressed as mean ± SD (n = 2).

steam sterilization. It was already reported that steam sterilization of NEs containing castor oil can result in hydrolysis of the certain castor oil lipids, leading to the release of free fatty acids which could then compromise the NE stability (Jumaa and Müller, 1999). LE itself is extremely stable at high temperatures, with melting point at 220–224 °C (Loteprednol etabonate, DrugBank online). However, when LE is incorporated inside a NE formulation, it appears that the high temperature maintained throughout the sterilization cycle increases LE distribution from the oil droplets to the aqueous NE phase (possibly due to the increase in LE aqueous phase solubility). After the end of sterilization cycle and subsequent cooling of the NE, the NE aqueous phase probably becomes supersaturated with LE, causing LE to precipitate. Apart from the LE content and nanodroplet size, steam sterilization changed the visual appearance of NE21 – creaming was observed and NE color turned from the milky-white to yellowish. On the other hand, membrane filtration using 0.2 μm PES filter neither affected the tested NE parameters, nor the visual appearance of the NE. Thus, membrane filtration was confirmed to be suitable sterilization method for NE21 for ophthalmic LE delivery.

3.5. LE distribution between NE oil and aqueous phase under simulated tear turnover

Recently performed NE *in vitro* release (IVR) studies evaluated drug distribution between NE and an aqueous buffer separated by a semi-permeable membrane, over 12 to 48 h (Attia et al., 2024; Lim et al., 2016; Srivastava et al., 2024). Nonetheless, it is generally understood that upon the instillation of a NE to the ocular surface, the aqueous phase merges with muco-aqueous layer of the tear film, being constantly diluted due to the tear turnover while the oil droplets may eventually fuse with the lipid layer (Gan et al., 2013; Jurišić Dukovski et al., 2023). Therefore, the frequently used IVR methods do not truly reflect such *in vivo* behavior of a NE. Thus, a biorelevant *in vitro* method for assessing the impact of the real timeframe NE dilution in the tear film, as a driving

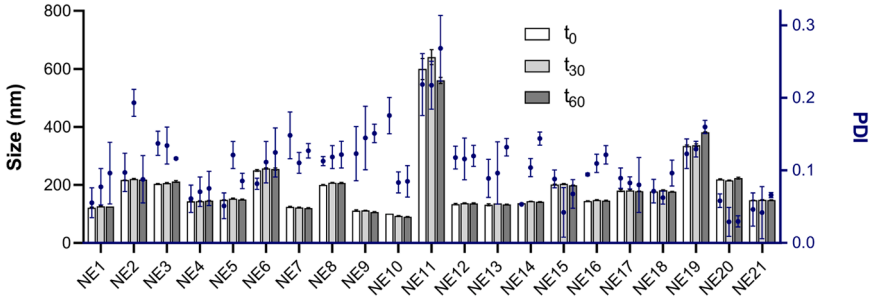

Fig. 3. Droplet size (bars) and PDI (points) measured immediately after NE preparation (t_0), after 30 days (t_{30}) and after 60 days (t_{60}) of storage at RT. Data are expressed as mean ± SD (n = 3).

Table 4
Experimental and predicted values of droplet size (DS) and LE content (LE_A) of statistical model-derived formulations (NE_O) immediately after preparation (t_0) and after 15 days of storage at RT (t_{15}). Values in brackets refer to 95 % confidence interval.

	Formulation parameters					Process parameters			Responses					
	LE_T (%, *w/w*)	CO (%, *w/w*)	C90 (%, *w/w*)	KOL (%, *w/w*)	SP (%, *w/w*)	T (°C)	C	P (bar)	Experimetal DS t_0 (nm)*	Predicted DS t_0 (nm)*	Experimetal LE_A t_0 (%, *w/w*)	Predicted LE_A t_0 (%, *w/w*)	Experimetal LE_A t_{15} (%, *w/w*)	Predicted LE_A t_{15} (%, *w/w*)
NE_O1	0.2	20	5	5	0	80	9	1000	150.2 ± 1.04	112 (36.13–188.54)	0.20	0.20 (0.17–0.23)	0.19	0.18 (0.17–0.19)
NE_O2	0.2	20	5	5	1	80	5	1000	134.4 ± 0.52	95.5 (7.80–183.32)	0.20	0.20 (0.17–0.24)	0.19	0.19 (0.18–0.20)

Table 5
Physicochemical properties of NE21 after preparation, after membrane filtration and after steam sterilization.

	Freshly prepared	Filtered	Steam sterilized
Droplet size (nm)	147.2 ± 0.4	151.2 ± 1.1	182.9 ± 9.5
PDI	0.05 ± 0.02	0.05 ± 0.02	0.07 ± 0.07
Zeta potential (mV)	−16.8 ± 0.6	- 13.12 ± 1.02	- 17.17 ± 1.07
LE content (%)	0.15 ± 0.00	0.15 ± 0.00	0.09 ± 0.00

Data are expressed as mean ± SD ($n = 2$).

force for API release from the oil droplets, was developed within this research. NE21 was mixed with the release medium in 1:1, 1:4, 1:9, 1:25, 1:38 and 1:64 vol ratio. The employed ratios simulated the successive *in vivo* NE dilution occurring 4, 9, 13, 18, 20 and 24 min after instillation (Jurišić Dukovski et al., 2023). Namely, the tear turnover rate has been estimated to be around 16 % min^{-1} (Willcox et al., 2017), and the usual ratio between the volume of the tear fluid and an eye drop ranges from 7:25 to 7:50. The period of 24 min is the realistic timeframe for LE absorption into the corneal epithelium, because the dynamic environment of the eye surface rapidly eliminates applied formulation by means of tear flow and eye blinking.

To assess if the method can successfully discriminate between APIs differing in aqueous solubility, the same procedure was performed also with NE21 formulation in which LE was substituted with ibuprofen (IBU). Namely, LE is practically insoluble in aqueous medium while IBU has high solubility at biorelevant pH (7.4), since it is a weak acid. PBS pH 7.4 with Kolliphor® EL (5 %, *w/w*) was used as the release medium. Kolliphor® EL concentration in PBS pH 7.4 was equal to its concentration in NE21 formulation. LE and IBU thermodynamic solubilities in PBS pH 7.4 + 5 % Kolliphor® EL evaluated in this study were 0.12 mg mL^{-1} and 4.6 mg mL^{-1}, respectively. According to that, sink conditions for IBU and LE in the LE distribution studies under the stimulated tear turnover were obtained after the dilution of 1:1 and 1:25 vol ratio, respectively.

The results of the analysis refer to the percentage of LE or IBU retained within the NE oil droplets upon each point of dilution (Table 6). In case of LE, the biorelevant dilution did not trigger its release from the oil droplets to the release medium, as it can be seen from the LE content inside the NE oil droplets, which remained above 90 % of the initial LE content despite the excessive dilution. The employed biorelevant dilution allowed a distinction between LE and IBU release profiles, confirming the applicability and prediction value of this method. Namely, IBU content in the oil droplets continuously decreased with successive NE dilution (Table 6), which is in line with its higher aqueous solubility, when compared to LE. The obtained results strongly suggest that practically insoluble LE would favorably be absorbed directly from the NE oil droplets to the lipophilic corneal epithelium rather than being released to and absorbed from the tear film (Tamilvanan and Benita, 2004). This observation is of key importance for conducting LE NE absorption/-permeability studies and can be useful in similar investigations related to NE ophthalmic delivery of other practically insoluble APIs.

Table 6
The percentage of LE and IBU content retained within the oil droplets upon each point of NE dilution with PBS pH 7.4 + 5 % Kolliphor® EL.

Dilution volume ratio (NE21:dilution medium)	$LE_{oil\ droplet}$ (%)	$IBU_{oil\ droplet}$ (%)
Undiluted NE21	93.05 ± 3.93	92.26 ± 2.13
1:1 (4 min)*	98.12 ± 0.93	89.00 ± 1.18
1:4 (9 min)*	95.74 ± 3.87	77.89 ± 2.53
1:9 (13 min)*	99.98 ± 1.35	73.14 ± 1.70
1:25 (18 min)*	99.00 ± 1.02	70.61 ± 2.01
1:38 (20 min)*	97.68 ± 2.25	68.36 ± 0.46
1:64 (24 min)*	99.54 ± 0.07	67.23 ± 2.80

* values in brackets refer to the theoretical timepoints after installation *in vivo* that correspond to the employed dilution volume ratios. Values are expressed as mean ± SD ($n = 3$).

4. Conclusions

In this work, practically insoluble LE was successfully incorporated into a NE. The QbD approach enabled the selection of the lead formulation and revealed the formulation and process parameters with the greatest impact on the LE content and LE retention efficiency. The results highlighted the lead NE which showed favourable physicochemical properties for ophthalmic use, long-term physical stability (10 months) and the ability to be easily sterilized by membrane filtration. The extended-throughput 3D HCE-T model was successfully developed to demonstrate the lead NE biocompatibility. The dilution/ultrafiltration method was established to evaluate the robustness of the lead NE to biorelevant dilution. The results obtained suggest that LE may be absorbed directly from the NE oil droplets into the lipophilic corneal epithelium, encouraging further *in vitro* dynamic permeability and *in vivo* eye-related bioavailability studies to provide proof-of-concept.

CRediT authorship contribution statement

Josip Ljubica: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bisera Jurišić Dukovski:** Writing – review & editing, Methodology, Investigation. **Iva Krtalić:** Writing – review & editing, Methodology. **Marina Juretić:** Writing – review & editing, Methodology. **Maša Sufundžić Kučuk:** Writing – review & editing, Methodology. **Igor Petriček:** Writing – review & editing. **Drago Špoljarić:** Writing – review & editing, Formal analysis. **Luka Bočkor:** Writing – review & editing, Visualization, Methodology, Investigation. **Petra Kocbek:** Writing – review & editing, Methodology, Investigation. **Anita Hafner:** Writing – original draft, Methodology, Conceptualization. **Ivan Pepić:** Writing – review & editing, Methodology, Conceptualization. **Jasmina Lovrić:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2025.107077.

Data availability

Data will be made available on request.

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