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ABSTRACT

Dry eye disease is a multifactorial condition characterized by a loss of homeostasis of the tear film. Among the various treatment approaches, the application of ophthalmic oil-in-water nanoemulsions with incorporated anti-inflammatory drugs represents one of the most advanced approaches. However, the liquid nature of nanoemulsions limits their retention time at the ocular surface. Transforming the nanoemulsions into a dry form that would disperse rapidly in the tear fluid would improve the retention of the drug at the ocular surface. The aim of this study was to investigate electrospinning as a method for the preparation of a solid eye preparation based on nanoemulsion loaded with the anti-inflammatory drug loteprednol etabonate. Four nanoemulsions differing in oil-to-surfactant ratios were incorporated in hydrophilic nanofibers based on polyethylene oxide, poloxamer 188, and Soluplus®. The dried nanoemulsions in the form of nanofibers dispersed readily on contact with aqueous medium, resulting in a dispersion of nanometre-sized droplets with average size comparable to the average droplet size of the initial nanoemulsions. A rheological study revealed the predominant elastic behavior of the dispersed nanofibers, which indicates the formation of a weak gel after the dispersion of the dried nanoemulsion in tear fluid at the ocular surface. The biocompatibility of the dried nanoemulsions in the form of nanofibers after a single and multiple-dose application was confirmed using the 3D HCE-T model of the stratified epithelium of the human cornea, suggesting that this innovative solid eye preparation could represent a new approach to the treatment of dry eye disease.

1. Introduction

Dry eye disease (DED), a multifactorial disease of the ocular surface, is characterized by a loss of homeostasis of the tear film. DED affects millions of people worldwide, with a significant impact on their quality of life (Bron et al., 2017). The economic burden and impact of DED on vision, work productivity, psychological and physical impact of pain, are substantial, particularly if considering the costs due to reduced work productivity. The prevalence of DED increases linearly with age, but a relatively high prevalence has recently been reported in younger adults and schoolchildren due to the huge increase in the use of digital devices (Stapleton et al., 2017). In this context, there is a growing need to develop new efficacious therapies for DED treatment.

The main therapeutic approach for the treatment of DED is currently the topical administration of ophthalmic products in the form of eye drops with or without an active pharmaceutical ingredient (Jones et al., 2017). The major disadvantage of eye drops is that they introduce an unphysiologically large amount of fluid into the eye, leading to overflow and extensive nasolacrimal drainage (Rohde et al., 2022). In addition, due to blinking, reflex tearing, and basal tear turnover, the residence time of the formulation at the ocular surface is limited to 14–17 min, with the majority of the applied dose being eliminated within the first 5 min (Agarwal and Rupenthal, 2023). Therefore, eye drops should be applied very frequently.

Oil-in-water (o/w) nanoemulsions (NEs) for ocular application represent one of the most important advancements in the treatment of

Abbreviations: DED, dry eye disease; HBSS, Hank's balanced salt solution; HCE-T, human coreal epithelial cells; LE, loteprednol etabonate; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NE, nanoemulsion; NF, nanofiber; P188, poloxamer 188; PEO, polyethylene oxide; PDI, polydispersity index.

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Table 1
Composition of investigated loteprednol etabonate-loaded NEs (LE - loteprednol etabonate, CO - castor oil, C90 - Capryol® 90, KOL -Kolliphor® EL, SP - Soluplus®).

NE formulation	LE [%, w/w]	CO [%, w/w]	C90 [%, w/w]	KOL [%, w/w]	SP [%, w/w]	Water [%, w/w]
LE-NE1	0.10	20	1	1	0.5	77.40
LE-NE2	0.15	20	5	5	1.0	68.85
LE-NE3	0.10	10	1	5	1.0	82.90
LE-NE4	0.10	15	5	5	1.0	73.90

various eye diseases, including DED (Lallemand et al., 2012; Singh et al., 2017). These nanodelivery systems, consisting of oil nanodroplets dispersed in the water phase and stabilized by surfactants, represent a reservoir for poorly water-soluble drugs and enable prolonged retention of the drug at the ocular surface (Gan et al., 2013; Jurišić Dukovski et al., 2020; Lalu et al., 2017) as well as enhanced absorption (Tamilvanan and Benita, 2004). Depending on the formulation composition, NE oil droplets may merge at the ocular surface with the lipid part of the tear film and thus play a key role in replenishing the deficient lipid layer of the tear film, which is one of the main characteristics of DED (Craig et al., 2017; Lalu et al., 2017; Willcox et al., 2017).

For the treatment of the DED, anti-inflammatory drugs, including non-steroidal anti-inflammatory drugs, glucocorticoids, and non-glucocorticoid immunomodulators, are particularly suitable for incorporation into NEs for ophthalmic application, as they reduce the ocular surface inflammation caused by the DED (Pilong et al., 2023).

Despite their promising attributes, the liquid nature of conventional NEs presents a challenge especially in terms of retention time at the ocular surface. It is assumed that mixing NEs with the tear film not only removes the formulation from the ocular surface but also compromises its stability (Gan et al., 2013). According to the literature, the retention time of eye drops at the ocular surface can be extended by the addition of viscosity-enhancing polymers, *in situ* gel-forming polymers, and mucoadhesive polymers to the formulation, or by reformulation into a semisolid or solid form (Grassiri et al., 2021). Recently, it has been shown that NEs can be converted to a dry form (El-Messery et al., 2020; Pilong et al., 2023; Sodalee et al., 2022), which may be a promising approach to improve the limited residence time of NEs at the ocular surface. To date, NEs have been converted into dry form by spray-drying (El-Messery et al., 2020; Li et al., 2024; Rehman et al., 2021; Rivera-Pérez et al., 2023; Shah et al., 2023; Sodalee et al., 2022), freeze-drying (El-Messery et al., 2020; Zhu et al., 2015), and foam-mat freeze-drying (Pilong et al., 2023; Ruengdech and Siripatrawan, 2022). These methods typically produce powder or foam products, intended either for oral (El-Messery et al., 2020; Pilong et al., 2023; Rehman et al., 2021; Rivera-Pérez et al., 2023; Ruengdech and Siripatrawan, 2022) or pulmonary (Shah et al., 2023; Zhu et al., 2015) administration. The dry form of NEs, suitable for ophthalmic application, is expected to disperse rapidly in the tear fluid at the ocular surface, forming a film that does not disturb the patient's vision.

Electrospinning could be an alternative to the currently available methods for the preparation of a dry product containing NEs. It uses a high-voltage electric field to produce fibers with nanosized diameter (*i.e.* nanofibers, NFs) from polymer solutions. Recently, it has shown promise as a drying method for nanoparticle dispersions, which can produce an easily redispersible non-powdered product with relatively small amounts of additional excipients (Dragar et al., 2023, 2022). During the electrospinning process, the electric field between the grounded collector and the metal nozzle connected to the high-voltage supply causes the elongation and bending of a viscoelastic jet of polymer solution, reducing the jet diameter to nanometer size (Reneker et al., 2007; Reneker and Yarin, 2008; Shepa et al., 2021). As the solvent evaporates during jet elongation and bending, the final product is deposited on the grounded collector in the form of NFs (Kajdič et al., 2019; Reneker and Yarin, 2008). These NFs exhibit unique properties such as a high surface area-to-volume ratio and the ability to form a highly porous three-dimensional mat with nanosized interfibrillar pores (Kocbek, 2012; Rošic et al., 2013). NFs have already been explored for various biomedical applications, including tissue regeneration, wound healing, and drug delivery (Rošic et al., 2013). By exploiting the advantages of their polymer matrix, NFs can serve as carriers not only for small molecular-weight drugs but also for biotherapeutics, such as peptides, proteins, probiotics, and cells (Rošic et al., 2013). Electrospinning could be used to produce NFs coated lenses for ophthalmic drug delivery as was already reported by the Sakpal's research group (Sakpal et al., 2022).

Since electrospinning enables the preparation of a dry non-powered product in the form of a NF mat that can be rapidly dispersed, it has the potential for use as an alternative drying method for NEs, overcoming the challenges associated with current drying methods. However, the question is whether the properties of NEs can be preserved during the electrospinning process and whether the electrospun product can be efficiently dispersed on the ocular surface after application. Therefore, this study aimed to investigate the possibility of using electrospinning as a method for the preparation of a solid ophthalmic formulation of NEs containing an anti-inflammatory drug loteprednol etabonate for the treatment of DED.

2. Materials and methods

2.1. Materials

All reagents used in this study were of analytical grade and purchased from commercial sources. Loteprednol etabonate was generously donated by JGL d.d. (Croatia). For preparation of NEs the following components were used: castor oil (Fagron, Netherlands), Kolliphor® EL (BASF, Germany), glycerol (Gram-mol ltd., Croatia), Capryol® 90 (Gattefossé, France), and polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer Soluplus® (BASF, Germany). The latter was used also for the preparation of NFs, which involved also polyethylene oxide (PEO; Mw, 400,000 g/mol, Sigma-Aldrich, USA) and poloxamer 188 (P188; Lutrol® F68, BASF, Germany. Acetonitrile was from J.T. Baker (Poland). The water used in the experiments was purified water obtained via reverse osmosis, except for the preparation of NEs where double-distilled water was used.

For cell culture experiments, Hank's balanced salt solution (HBSS; pH 7.4) was prepared as previously reported by Jurišić Dukovski et al., 2020. Human corneal epithelial cell line (HCE-T) was obtained from RIKEN Cell Bank (Japan). The culture medium (Dulbecco's Modified Eagle Medium with nutrient mixture F-12), along with insulin, epidermal growth factor, antibiotic-antimycotic solution, rat tail type I collagen, and human fibronectin was from Sigma-Aldrich (USA). Fetal bovine serum was from Capricorn (Germany) and dimethyl sulfoxide was from Applichem (Germany). 96-well insert plates with polycarbonate membranes (0.4 μm pore size) were purchased from Merck-Milipore (USA), benzalkonium chloride from Honeywell Fluka (Denmark), and (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT) from Biosynth (UK).

2.2. Preparation of ophthalmic nanoemulsions

The NEs were produced by microfluidization. The oil phase consisted of a mixture of castor oil, Capryol® 90, and Kolliphor® EL, while the aqueous phase consisted of Soluplus® dissolved in double-distilled

Table 2
Composition of polymer solutions for the preparation of pre-drying samples (PEO - polyethylene oxide, P188 - poloxamer 188, and SP - Soluplus®).

Polymer solution	PEO [mg/mL]	P188 [mg/mL]	SP [mg/mL]
A	64	64	0
B	64	32	32
C	64	16	16

water (Table 1). Loteprednol etabonate was dissolved in the oil phase. The oil and aqueous phase, heated up to 80 °C, were first mixed by magnetic stirring and then pre-homogenized with an Ultra-Turrax® (IKA-Werke GmbH & Company, Germany) at 15.000 rpm for 5 min. The resulting coarse emulsion was further homogenized with a microfluidizer (Model LM20, Microfluidics®, USA). The placebo NEs without loteprednol etabonate were prepared using the same procedure, without the addition of loteprednol etabonate (NEs labeled as NE1, NE2, NE3, and NE4). The batch size of each NE was 30 mL.

The prepared NEs were diluted in a 1:100 vol ratio with water and analyzed for average hydrodynamic droplet size and droplet size distribution using photon correlation spectroscopy (ZetaSizer Ultra, Malvern Panalytical Ltd., UK; DTS0012 cuvette), and for zeta potential using laser Doppler anemometry (ZetaSizer Ultra, Malvern Panalytical Ltd., UK;). The measurements were performed at 25 °C using cuvet DTS0012 in size measurements and cuvette DTS1070 in zeta potential measurements. All measurements were performed in triplicates and the results are expressed as average hydrodynamic droplet size, the average polydispersity index (PDI), and average zeta potential with the corresponding standard deviations.

2.3. Drying of nanoemulsions using the electrospinning method

2.3.1. Preparation of pre-drying samples

Polymer solutions were prepared by dissolving PEO, P188, and Soluplus® (Table 2) in water at 80 °C under moderate magnetic stirring. The obtained polymer solutions were cooled to room temperature and stirred overnight. The polymer solutions were then mixed with NEs in a 1:1 wt ratio to form pre-drying samples (Table 3). Pre-drying samples were diluted in a 1:100 vol ratio with water and analyzed for average droplet size and droplet size distribution using photon correlation spectroscopy as described in Section 2.2.

2.3.2. Electrospinning

The pre-drying samples were electrospun immediately after preparation using an electrospinning device in a horizontal configuration (Spinbox® system, Bioinicia, Spain). Each sample was loaded into a 5 mL plastic syringe (Chirana, Slovakia) that was placed in the syringe pump. The syringe was connected to a metal needle (Bioinicia, Spain; outer diameter, 0.7 mm) via a plastic tube (outer diameter, 1.3 mm). The needle was connected to a high-voltage generator (15 kV) and positioned 15 cm from a grounded collector wrapped in aluminum foil. Electrospinning was conducted at a sample flow rate of 1.77 mL/h for ~1 h at room temperature and relative humidity ≤ 45 %. The electrospun products were stored in a desiccator until further use.

2.4. Evaluation of electrospun products

2.4.1. Morphology of electrospun products

The morphology of dried NEs was examined using scanning electron microscopy (SEM; Supra35 VP, Carl Zeiss, Germany). Electrospun products were mounted on metal studs with double-sided conductive tape (diameter, 12 mm; Oxford Instruments, UK) and imaged at an accelerating voltage of 1 kV using a secondary electron detector. The diameters of at least 100 NFs were measured from representative SEM images using ImageJ (v1.53e) software (National Institutes of Health, USA), and the average NF diameter along with the standard deviation was calculated.

2.4.2. Drug content in electrospun products

To determine the drug content, approximately 50 mg of electrospun product was dissolved in 8 mL of ethanol/water mixture (50:50, v/v). Next, the solution was diluted 5-times with the ethanol/water mixture (50:50, v/v) and filtered using a 0.2 μm PES filter. The loteprednol etabonate concentration was determined by HPLC analysis (Agilent Infinity II 1260, Agilent Technologies, USA) using the XBridge C8 column (3.5 μm, 4.6 × 100 mm; Waters, USA) at 45 °C and acetonitrile/water (58:42, v/v) mobile phase at flow of 1 mL/min. The injection volume was 50 μL and the detector wavelength was set to 255 nm. The validation of the HPLC method was carried out according to International Conference on Harmonization (ICH) guideline Q2 (R1). The method was confirmed to be linear, accurate and repeatable. Based on the obtained results the drug content in electrospun product and the entrapment efficiency were calculated.

2.4.3. Residual moisture in electrospun products

The residual moisture in electrospun products was determined by thermogravimetric analysis using a TGA/DSC 1 STARe System (Mettler-Toledo, Switzerland). An electrospun product (5–10 mg) was weighed into a 70 μL aluminum oxide crucible and placed in the thermogravimeter cell under a nitrogen flow of 50 mL/min. The sample was heated from 30 °C to 95 °C at a rate of 30 °C/min. Then, the sample was held at 95 °C for 30 min before further heating to 250 °C at a rate of 20 °C/min. The residual moisture expressed as a percentage was calculated as the loss mass of the sample at a temperature of 30 °C to 110 °C devided by initial mass of the sample before termogravimetric analysis. All measurements were conducted in triplicate, and the results are presented as the average residual moisture with the corresponding standard deviation.

2.5. Dispersibility of dried nanoemulsions

The electrospun products were dispersed in water by vortex mixing

Table 3
Labeling of pre-drying samples and corresponding electrospun NFs. Placebo NEs are labeled with NE and number, while loteprednol etabonate-loaded NEs are labeled with LE-NE and number. NFs are labeled in the same manner as NEs, with NF instead of NE. Letters A, B, and C represent the polymer solution used for the preparation of the pre-drying sample and NFs.

NE formulation	Polymer solution A		Polymer solution B		Polymer solution C	
	Pre-drying sample	NFs	Pre-drying sample	NFs	Pre-drying sample	NFs
NE1	NE1/A	NF1/A	NE1/B	NF1/B	NE1/C	NF1/C
NE2	NE2/A	NF2/A	NE2/B	NF2/B	NE2/C	NF2/C
NE3	NE3/A	NF3/A	NE3/B	NF3/B	NE3/C	NF3/C
NE4	NE4/A	NF4/A	NE4/B	NF4/B	NE4/C	NF4/C
LE-NE1	LE-NE1	LE-NF1/A	/	/	/	/
LE-NE2	LE-NE2	LE-NF2/A	/	/	/	/
LE-NE3	LE-NE3	LE-NF3/A	/	/	/	/
LE-NE4	LE-NE4	LE-NF4/A	/	/	/	/

for up to 3 min to obtain NEs with a composition comparable to that of 100-times diluted initial NEs. The average droplet size and droplet size distribution were measured immediately after the dispersion of electrospun products in water using photon correlation spectroscopy as described in Section 2.2. The investigation of dry product dispersibility was performed in triplicate, and the results are presented as average with the corresponding standard deviation.

2.6. Rheological measurements of dispersed dried nanoemulsions

The rheological characterization of dispersed dried NEs in the form of NFs was performed with a Modular Compact Rheometer MCR 102 (Anton Paar GmbH, Austria) equipped with either cone (1°) - plate (diameter 25 mm, CP25–1) or parallel-plate (diameter 25 mm, PP25) measuring system and air-cooled Peltier temperature control system. To prepare a sample of dispersed dried NEs, 50 mg of each dried NF sample was added to 1 mL of simulated tear fluid, which was prepared according to Rohde et al., 2022. A fixed gap between the lower and the upper plate was set to 0.05 mm. All measurements were carried out at 34 °C.

A rotational test was carried out using a cone plate at a shear rate ranging from 0.01 s^{-1} to 10,000 s^{-1}. The linear viscoelastic range of dispersed dried NEs was determined after 5 min of equilibration by amplitude sweep test using the PP25 measuring system. The test was performed at a frequency of 1 Hz and in the amplitude range of 1 – 10,000 %. The limit of the linear viscoelastic range was calculated by RheoCompass software. The measurements were performed in duplicate.

A frequency sweep test of the dispersed dried NEs obtained by mixing NFs with simulated tear fluid was performed using the PP25 measuring system. The samples were equilibrated for 5 min. Storage and loss moduli were recorded in the range of frequencies from 20 to 1 Hz (angular frequency 125.6 to 6.3 rad/s), with the applied shear strain of 1 % (within the linear viscoelastic range). The measurements were performed in duplicate.

2.7. Evaluation of nanoemulsion biocompatibility in vitro

The biocompatibility evaluation of NEs was performed using a 3D corneal epithelial model based on the HCE-T cell line. The cells were cultured in Dulbecco's Modified Eagle Medium with nutrient mixture F-12 supplemented with fetal bovine serum (5 %, *v/v*), insulin (5 μg/mL), dimethyl sulfoxide (0.5 %, *v/v*), epidermal growth factor (10 ng/mL), and antibiotic-antimycotic solution (1 %) at 37 °C in a humidified atmosphere with 5 % CO_2.

Cells were seeded (1×10^4 cells per well) on membranes of a 96-well insert plate precoated with rat tail type I collagen (51 μg/well, 24 h incubation at 37 °C) and human fibronectin (4 μg/well, 20 min incubation at 37 °C). Cells were cultured in the medium (75 μL in the apical compartment and 250 μL in the basolateral compartment) for 5 days to form a confluent monolayer. The medium was then removed from the apical compartment and the cells were exposed to the air-liquid interface for 3 days to induce cell differentiation and the formation of multilayered epithelia. Transepithelial electrical resistance was measured under submerged conditions and after exposure to an air-liquid interface using a Millicell ERS-2 Vohmmeter with an STX00 electrode (Merck Millipore).

After 3-day cultivation under air-liquid interface conditions, the HCE-T corneal epithelial model was treated with single or multiple doses of dispersed dried NE and benzalkonium chloride, a preservative in ophthalmic formulations with well-known adverse effects on the ocular surface. First, the medium was removed from the basolateral compartment and replaced with HBSS. HBSS was also added to the apical compartment (75 μL apical side) to pre-wet the 3D corneal epithelial model at 37 °C before NE application. After 30 min, the HBSS was removed from the apical side and 75 μL of the diluted formulations were added to each well. The model was incubated for another 30 min at 37 °C.

For the treatments, the NEs were diluted 10-times with HBSS, while the NFs were dispersed in an amount of HBSS that the theoretical loteprednol etabonate concentration was comparable to that in the 10-times diluted initial NEs. HBSS was used as a negative control and benzalkonium chloride at three different concentrations (0.1, 0.01, and 0.005 % (w/*v*)) as a positive control. Each treatment was carried out in quadruplicate. After the 30-min treatment, the inserts were carefully washed with HBSS and the cell culture medium was returned to the basolateral side for 24 h In a multi-dose treatment, the model was treated on three consecutive days, repeating the same procedure in each step of treatment.

24 h after NE treatment, the medium was removed from the cell model and solution of MTT in cell culture medium (0.5 mg/mL) was added to the apical and basolateral compartments, and the model was incubated at 37 °C for 30 min. The MTT solution was then removed and formazan was dissolved by adding isopropanol and shaking the plate on an orbital shaker. The absorbance of each well was measured at 570 nm using a microplate reader (SpectraMax® i3, Molecular Devices, USA).

2.8. Statistical analysis

Statistical analysis was performed on the data obtained from the study on *in vitro* biocompatibility 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

In our previous studies, the formulation of loteprednol etabonate-loaded NEs has been thoroughly investigated to develop a suitable NE formulation for the treatment of DED (manuscript in preparation). Since NE formulations developed are low viscous liquid preparations, the aim of this study was to convert them into dry, non-powdered final dosage form, namely NFs, by electrospinning. These NFs are intended to be used in dry form and are expected to disperse rapidly in tear fluid after administration, increasing the viscosity of the tear film and prolonging the retention of the drug at the ocular surface. Moreover, electrospinning enables preparation of sterile NFs to comply with the pH. Eur. requirements for eye preparations (Rediguieri et al., 2016).

Based on the preliminary experiments, we have selected the four representative NE formulations with different compositions (Table 1), and their placebo counterparts, for the preparation of NFs. Selected NEs differ in loteprednol etabonate content, and oil-to-surfactant ratio, which may affect their performance *in vivo*. LE content in NEs was 0.10 % (w/w), except for LE-NE2, which had LE content of 0.15 % (w/w). LE-NE1 and LE-NE2 contained the highest amount of castor oil, namely 20 % (w/w), followed by LE-NE4 with 15 % (w/w) and LE-NE3 with 10 % (w/w) of castor oil (Table 1).

To form an easily dispersible dry non-powdered product by electrospinning, we adopted the formulation of hydrophilic NFs, which has been previously shown to enable rapid reconstitution in water (Dragar et al., 2023, 2022). In this formulation, PEO was used as NF-forming polymer, while P188 was added to enhance the dispersion of NFs in tear fluid. The weight ratio of both polymers in NF formulation was 1:1 as it was previously shown that this ratio results in the formation of easily dispersible hydrophilic NFs stable for up to 30 days at room temperature and relative humidity < 70 % (Dragar et al., 2023, 2022; Kajdič et al., 2020). Both polymers used for the preparation of NFs are hydrophilic, water-soluble, biocompatible, and approved for ophthalmic use by the U.S. Food and Drug Administration (FDA, 2024). PEO is used as a demulcent to protect and lubricate the ocular surface (Mohamed et al., 2022). It can improve one or more components of the tear film by increasing tear volume and stability and protects the eye

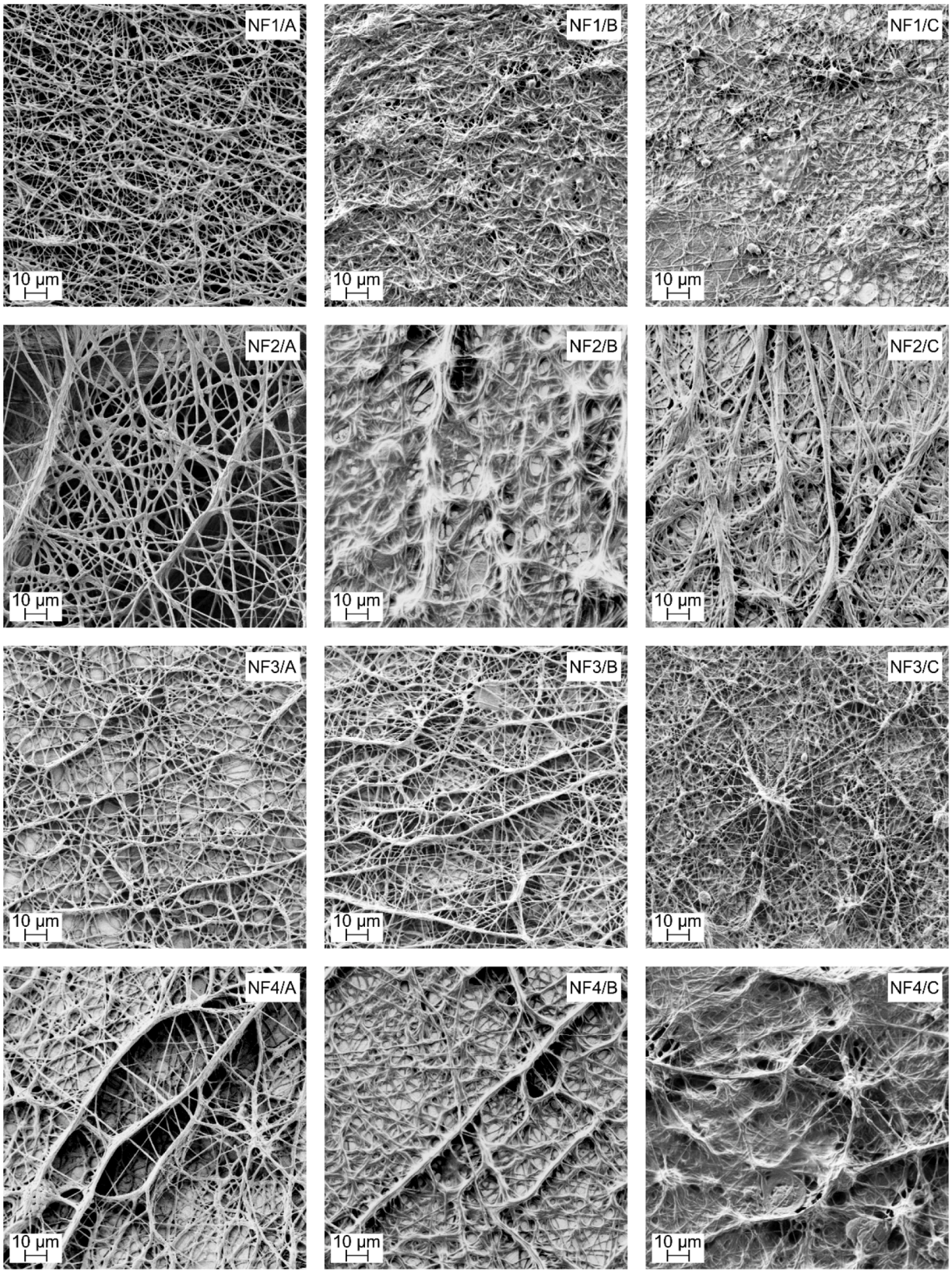

Fig. 1. Representative SEM images of electrospun products loaded with placebo NEs at lower magnification.

surface from desiccation, therefore, it is a functional ingredient in numerous artificial tear products on the market (*e.g.* Visine Dry Eye Relief (1 %), Systane® Ultra lubricant eye drops (0.4 %), Blink Tears (0.25 %)) (Semp et al., 2023). Thus, it may play an important role not only in the formation of NF but also in the treatment of DED. NFs are expected to disperse in tear fluid, thus polymers might affect the viscosity of tear fluid and might lead to the additional mucomimetic effect (Agarwal and Rupenthal, 2023). Since Soluplus® is an essential part of

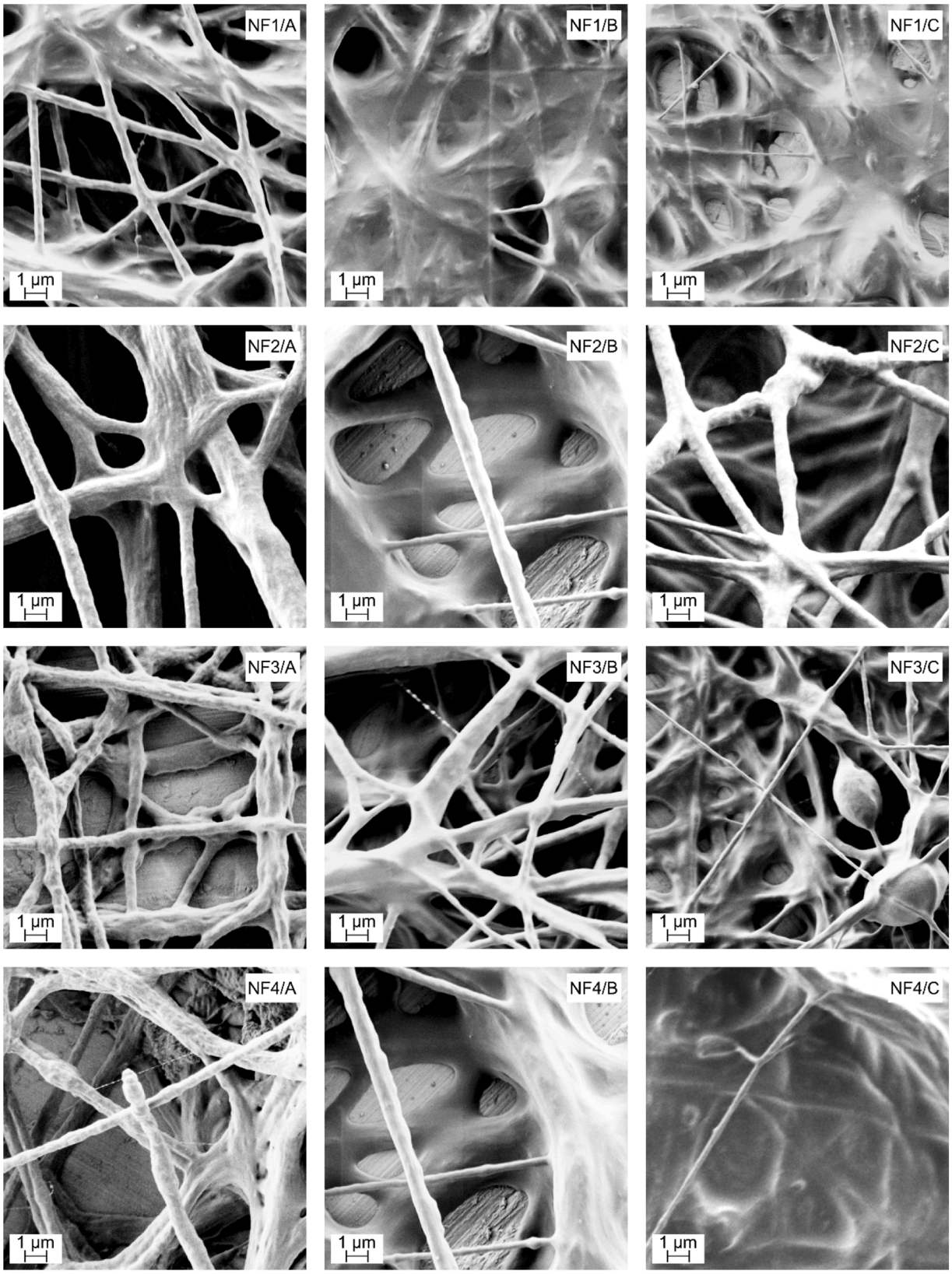

Fig. 2. Representative SEM images of electrospun products loaded with placebo NEs at higher magnification.

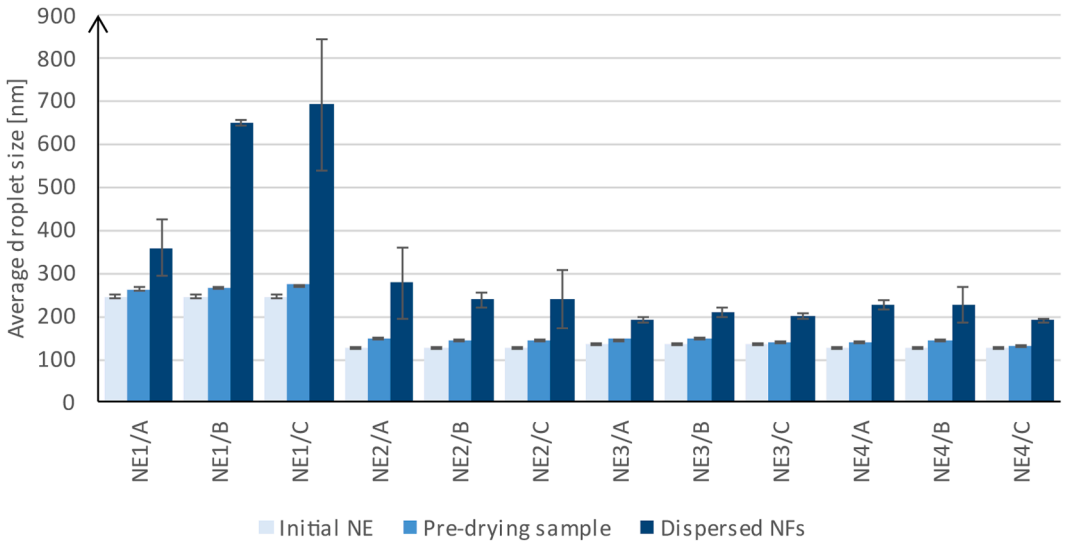

Fig. 3. The average hydrodynamic droplet size in the investigated initial placebo NEs, in pre-drying samples, and in dispersions of dried placebo NEs in the form of NFs. Data are expressed as the average ± SD ($n = 3$).

the NE composition, we decided to use it also in the formulation of NFs instead of a portion of P188 (polymer solution B; Table 2). We reduced the stabilizer amount in the final dry product by taking into account the amount of Soluplus® that was present in the initial NEs and thus used a lower amount of P188 and Soluplus® in the pre-drying samples (polymer solution C; Table 2).

3.1. Drying of placebo nanoemulsions

To evaluate the influence of NE and polymer composition on the electrospinning process and the properties of electrospun products, the placebo NEs (*i.e.*, without loteprednol etabonate) were mixed with different polymer solutions and electrospun as described in Section 2.3. The SEM images revealed that the electrospinning of all pre-drying samples resulted in the formation of NFs (Figs. 1 and 2). The morphology of the electrospun product might affect its dispersibility in tear fluid on the ocular surface. It was observed that the preparation of pre-drying samples from polymer solution A led to the formation of more uniform and smooth NFs without beads compared to other investigated polymer solutions. On the other hand, polymer solutions B and C were prepared with smaller amounts of P188, which was substituted with the equivalent amount of Soluplus® (Table 2) and resulted in the formation of beaded and partially fused NFs. Similar morphology was observed also for the formulation of NF with the NE1 in combination with polymer solution A (*i.e.*, NF1/A). The castor oil content, which varied in investigated NE formulations (Table 1), did not importantly affect the morphology of the electrospun products (Figs. 1 and 2). The average diameter of electrospun NFs with placebo NEs was between 500 ± 147 nm and 730 ± 278 nm.

The electrospun products loaded with placebo NEs were dispersed quickly (< 3 min) and efficiently in water, indicating that the difference in morphology of electrospun products did not importantly affect their dispersibility. The addition of polymers before drying and the drying itself may affect the characteristics of NEs, which are crucial for their stability and behavior of NEs on the ocular surface after application. The average droplet size was determined for pre-drying samples and dispersed NFs loaded with NEs and compared to values of initial NEs. The addition of polymer solution to NEs resulted only in a slight and statistically insignificant increase in droplet size (Fig. 3). The average droplet size in the dispersion of electrospun NFs was larger compared to the average droplet size of the initial placebo NEs, however, it was still in the nanometer size range (Fig. 3). The most significant increase in the droplet size was observed in dispersed electrospun products NF1/B or NF1/C. This could be associated with the morphology of the electrospun products, as these NFs exerted partially fused morphology (Figs. 1 and 2). Since the use of polymer solution A resulted in homogenous smooth non-fused electrospun NFs and the droplet size in the dispersion of dried NEs prepared from pre-drying samples with the polymer solution A was the most similar to the initial one, the formulation with the polymer solution A was selected as the most suitable. Thus, all further investigations with drug-loaded NEs were performed with NFs prepared from polymer solution A.

Average hydrodynamic droplet size of the studied initial placebo NEs, in pre-drying samples and dispersion of dried placebo NEs in the form of NF. Data are expressed as mean value

3.2. Drying of loteprednol etabonate-loaded nanoemulsions

SEM images of NFs with loteprednol etabonate-loaded NEs revealed that the presence of loteprednol etabonate did not importantly affect the NF morphology compared to the NFs with placebo NEs (Figs. 1, 2, and 4). Moreover, it was shown that the composition of loteprednol etabonate-loaded NEs did not affect the morphology of NFs (Fig. 4). Thus, the investigation of the morphology of NFs with loteprednol etabonate-loaded NEs confirmed that the composition of NEs does not affect the morphology of NFs. The average diameter of electrospun NFs with loteprednol etabonate-loaded NEs was between 490 ± 162 nm and 600 ± 185 nm. The dispersion time of NFs with loteprenol etabonate-loaded NEs was short, *i.e.* < 3 min, for all investigated formulations. Upon contact with an aqueous medium, dried NEs were easily and rapidly dispersed, resulting in dispersions with nanometer droplet size, *i.e.* NEs. The formulation LE-NF3 with the lowest oil phase content dispersed the most rapidly (< 90 s).

To evaluate the effect of the electrospinning process on the droplet size, we investigated the average hydrodynamic droplet size, PDI, and zeta potential of pre-drying samples and dispersed dried NEs. The addition of the polymers to initial loteprednol etabonate-loaded NEs resulted in a slight increase in average droplet size in pre-drying samples of LE-NE2, LE-NE3, and LE-NE4, while a significant increase in droplet size was observed in the pre-drying sample of LE-NE1, the NE formulation with the highest castor oil content (20 %, w/w) and a low surfactant content (Fig. 5A). The PDI also increased after the addition of polymers but did not exceed the value of 0.2 in the case of pre-drying samples of LE-NE2, LE-NE3, and LE-NE4 (Fig. 5B).

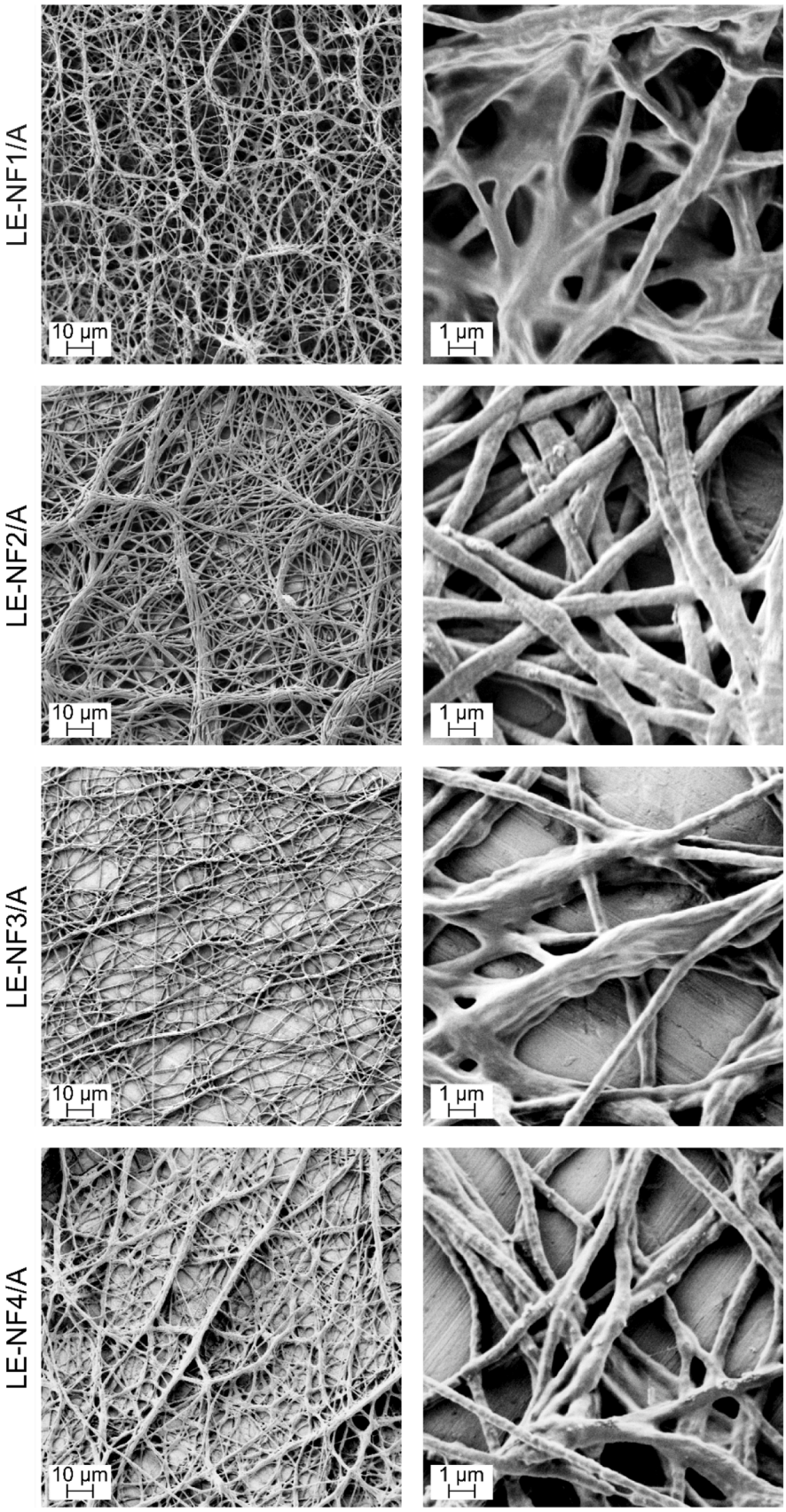

Fig. 4. Representative SEM images of electrospun products with loteprednol etabonate-loaded NEs prepared from polymer solution A at lower (left) and higher (right) magnification.

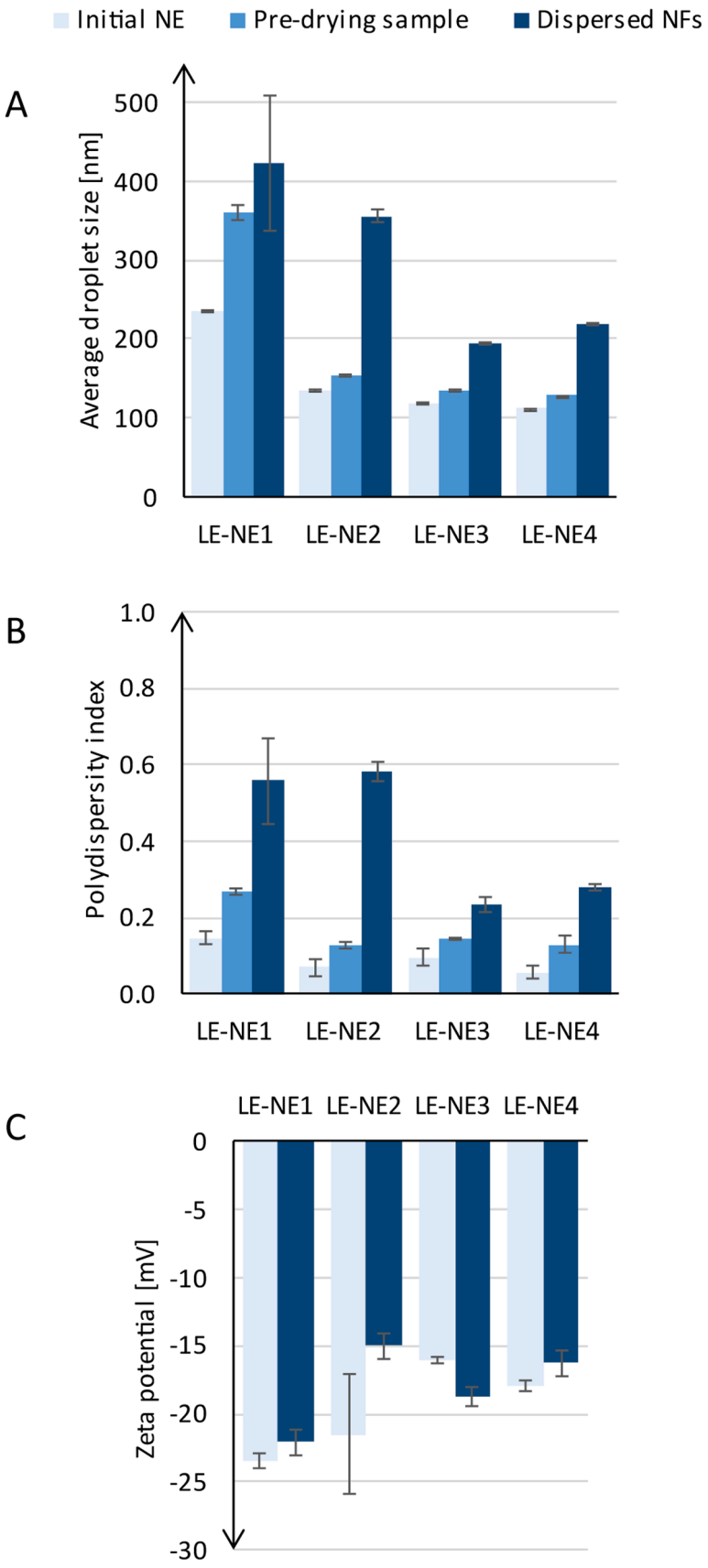

Fig. 5. (**A**) Average hydrodynamic droplet size, (**B**) polydispersity index, and (**C**) zeta potential of the initial loteprednol etabonate-loaded NEs, pre-drying samples, and dispersed dried NEs in the form of NFs. Data are expressed as the average ± SD ($n = 3$).

Table 4
Drug content and residual moisture in electrospun products with loteprednol etabonat-loaded NEs. Data are expressed as the average ± SD ($n = 3$).

NF formulation	Drug content [%]	Entrapment efficiency [%]	Residual moisture [%]
LE-NF1/A	0.258 ± 0.004	91.93	1.069 ± 0.459
LE-NF2/A	0.296 ± 0.004	88.01	1.502 ± 0.828
LE-NF3/A	0.274 ± 0.001	82.87	0.846 ± 0.404
LE-NF4/A	0.239 ± 0.002	94.21	1.538 ± 0.284

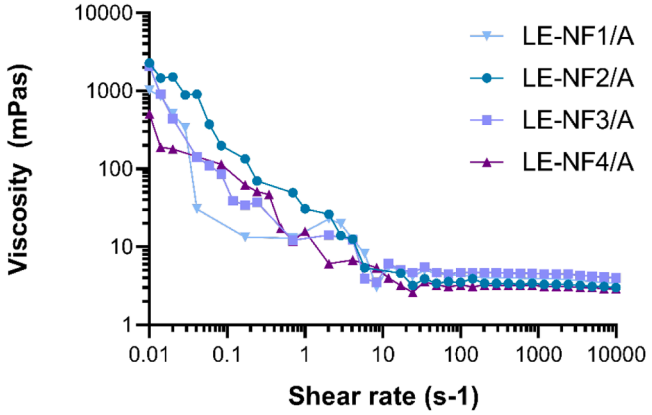

Fig. 6. Viscosity curves of dried loteprednol-etabonate-loaded NEs in the form of NFs dispersed in aqueous medium at the shear rate range of 0.1 – 10,000 s^{-1}. The graph shows a representative curve for each sample of dispersed NFs.

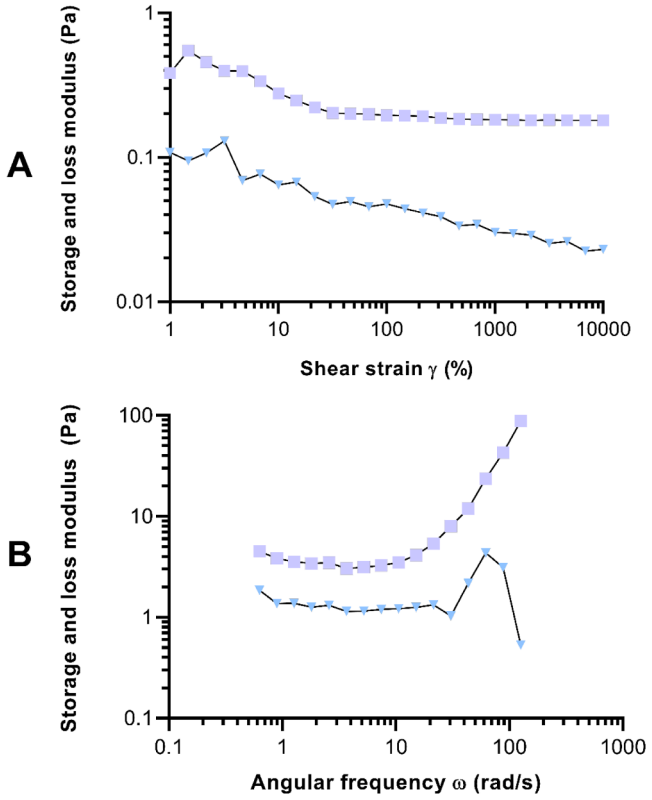

Fig. 7. Storage (G', squares) and loss (G"; triangles) moduli as the function of shear strain (A) and angular frequency (B) of representative dispersed dried NE in the form of NFs, namely LE-NF2/A. The graphs show the mean values of two replicates.

The average hydrodynamic droplet size in dispersed dried loteprednol etabonate-loaded NEs was bigger compared to the average hydrodynamic droplet size of initial loteprednol etabonate-loaded NEs (Fig. 5A). The increase was most pronounced in the case of formulation LE-NF2, characterized by the highest investigated castor oil (20 %, w/w) and surfactant content (6 %, w/w). The average hydrodynamic droplet size of NE formulations with lower castor oil content (LE-NE3 and LE-NE4, 10 % (w/w) and 15 % (w/w), respectively) increased to about 200 nm while it increased up to 420 nm in the case of the formulations with the highest castor oil content (LE-NE1 and LE-NE2, 20 % (w/w)). The increase in average hydrodynamic droplet size in the dispersion of NFs and its correlation with the amount of oil phase has already been reported in the literature (Arecchi et al., 2010; Gordon et al., 2015). The slight increase in average hydrodynamic droplet size in the case of dried loteprednol etabonate-loaded NE with a lower oil phase content indicates that the droplets do not coalesce during the electrospinning process. However, the significant increase in droplet size at higher oil phase contents can be explained by the effect of the electrospinning process, which can inherently cause aggregation of oil droplets due to shear forces during the elongation of liquid jet or due to electrostatic interactions induced by the applied voltage (Arecchi et al., 2010; Gordon et al., 2015). In our study, this effect was observed in the changes in PDI in dispersed NF samples (Fig. 5B). PDI increased up to 0.3 in the case of the formulations with lower castor oil content (LE-NE3 and LE-NE4) and almost to 0.6 in the case of the formulations with the highest castor oil content (LE-NE1 and LE-NE2). All NEs were characterized by a negative zeta potential and no pronounced change in zeta potential was observed after dispersion of NFs in water (Fig. 5C).

The drug content assesment revealed 83–94 % incorporation efficiency of loteprednol etabonate into NFs (Table 4), which is in

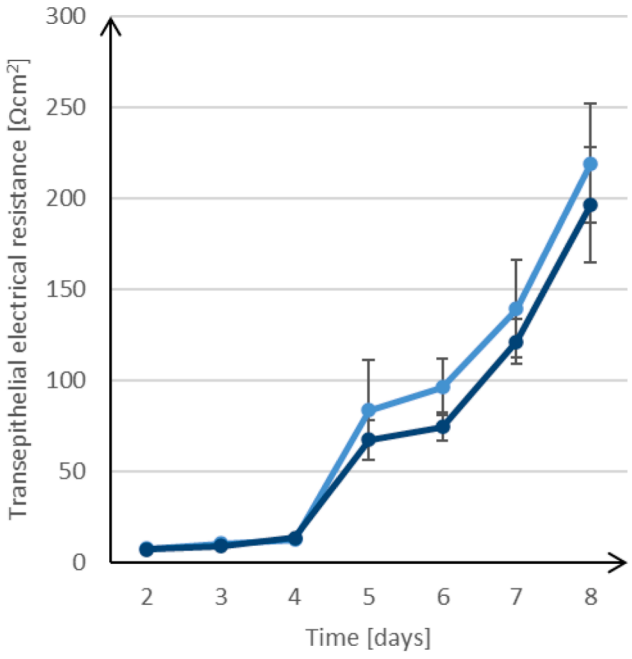

Fig. 8. The transepithelial electrical resistance of the 3D HCE-T model as a function of time after cell seeding on a 96-well plate insert (n = 8–96).

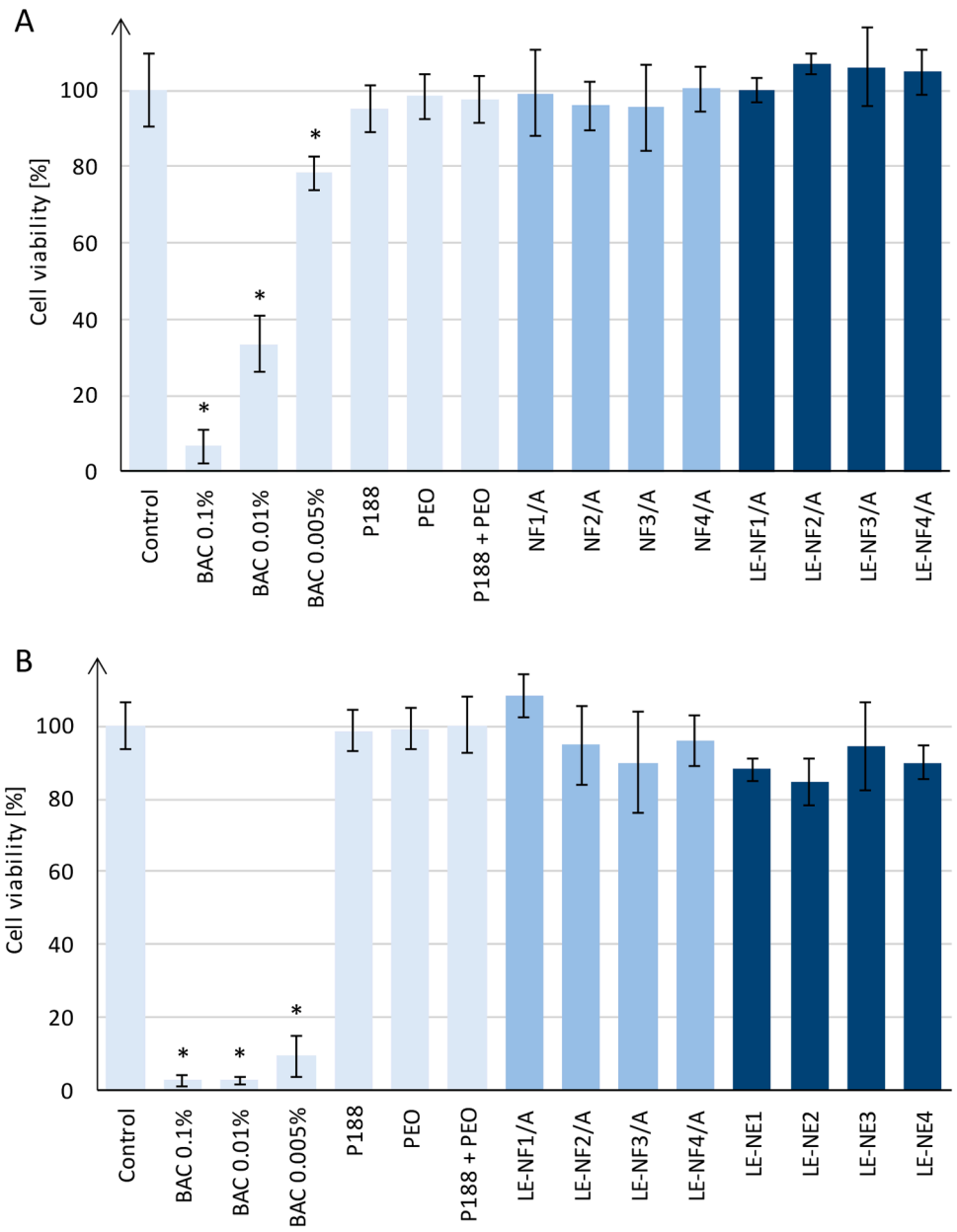

Fig. 9. Viability of HCE-T cells after (**A**) single-dose and (**B**) multiple-dose treatment with initial loteprednol etabonate-loaded NEs, NFs with loteprednol etabonate-loaded NEs, pure polymers (P188 and PEO), and benzalkonium chloride (BAC) in different concentrations. Cells treated with HBSS pH 7.4 were used as a control, representing 100 % cell viability. Data are expressed as mean ± SD ($n = 4$). *Indicates statistically significant difference compared to the control ($P < 0.05$).

agreement with our previous studies reporting almost 100 % drug incorporation efficiency into hydrophilic nanofibers (Dragar et al., 2024). Furthermore, we confirmed that the NFs with loteprednol etabonate-loaded NE were dry as they contained no >2.3 % (w/w) residual moisture (Table 4). This finding is also in line with our previous studies, showing that hydrophilic NFs may contain a small amount of residual moisture, typically below 3 % (w/w), which is affected by the type of drug and/or other material (*e.g.*, nanoparticles) loaded (Dragar et al., 2024, 2022).

Rheological characterization of dispersed NFs in aqueous medium was carried out to examine the effect of dried NEs on the tear fluid properties after administration. Fig. 6 shows viscosity curves of dispersed NFs obtained by a rotational test in the shear rate range of 0.01 – 10,000 s^{-1}. All samples exhibited shear-thinning behavior, with the high initial viscosity at low shear rates decreasing to around 3 mPas at shear rates above 100 s^{-1}. PEO and P188, NF-forming polymers in developed dry NEs, are known to form viscoelastic aqueous solutions with shear-thinning properties (Al Sabbagh et al., 2020; Zaitoon and Lim, 2020). Such characteristics are favorable in ophthalmic formulations since high shear rates (3000 – 40,000 s^{-1}) correspond to physiological eye blinking (Destruel et al., 2020). By blinking, the dispersed NE formulation can be evenly distributed over the ocular surface. The ophthalmic application of dried NE in the form of NFs would increase the viscosity of tear fluid, allowing longer retention of the formulation at the ocular surface (Rohde et al., 2022).

To assess the viscoelastic behavior of dispersed NFs, oscillatory tests were performed. Linear viscoelastic range was up to the amplitude (shear strain) of 2.5 % for all dispersed NF formulations, as was determined by the amplitude sweep test. Oscillatory measurements above this strain destroyed the sample structure. Hence, a frequency sweep test was carried out at the amplitude of 1 % to provide non-destructive conditions. Both oscillatory tests revealed the predominant elastic

behavior of the tested dispersed NFs (Fig. 7), with the storage modulus (*G'*) higher than the loss modulus (G") at the entire frequency range.

These results indicate that the dispersion of dried NEs in tear fluid at the ocular surface will result in the formation of a weak gel, with an average tanδ value of 0.280. There are many approaches described in literature, aiming to surpass the limitations of liquid eye preparations related to the fast clearance of the formulation from the eye due to blinking and tear turnover, several of which are based on *in situ* gelling systems (Andrews et al., 2009; Anumolu et al., 2009; Fernández-Ferreiro et al., 2015; Krtalić et al., 2018; Priya et al., 2023). P188 as a building block of NFs is a thermosensitive polymer that undergoes a sol-gel transformation at physiological temperature and is widely used in such systems due to its biocompatibility (Huang et al., 2016; Krtalić et al., 2018; Li et al., 2018; Qi et al., 2007; Zeng et al., 2018). The weak gel that would form *in situ* after the application of dried NE in the form of NFs to the ocular surface may be considered highly beneficial, as it would enable prolonged LE retention at the ocular surface without discomfort to the patient. The formulation of hydrophilic NFs with incorporated NE thus represents a multifunctional approach to address the problems associated with the use of liquid eye preparations, in particular eye drops.

3.3. Biocompatibility of electrospun nanofibers with loteprednol etabonate-loaded nanoemulsions

The biocompatibility of the dried NEs in the form of NFs after a single and multiple-dose application was performed using the 3D HCE-T model of the stratified epithelium of the human cornea cultured on 96-well insert plates. The biocompatibility of the NFs was compared to the biocompatibility of the corresponding initial loteprednol etabonate-loaded NEs and with the biocompatibility of the NF-forming polymers, namely PEO and P188. The HCE-T cells were exposed to the air-liquid interface when a confluent monolayer was formed and a sharp increase in transepithelial electrical resistance was measured (Fig. 8), *i.e.* on day 5 after seeding. Air-liquid interface induced cell differentiation and after 3 days of exposure to it (day 8 post-seeding), the formation of a multilayered epithelium was confirmed by microscopic imaging of cell layer cross-sections stained with 4′,6-diamidino-2-phenylindole (Jurišić Dukovski et al., 2023).

At physiological tear turnover (16 %/min), the conventional eye drop solution dilutes approximately 10-times within 13 min and 200-times within 30 min after administration at the ocular surface (Willcox et al., 2017). To better mimic the *in vivo* behavior of eye formulation designed to remain longer at the ocular surface, it is rational to increase the treatment time and decrease the dilution factor *in vitro* settings. Therefore, the 3D HCE-T model was treated for 30 min with initial loteprednol etabonate-loaded NEs 10-times diluted with HBSS, while NFs with loteprednol etabonate-loaded NEs were dispersed in HBSS to obtain a dispersion with the theoretically- equivalent loteprednol etabonate concentration to the one in diluted initial loteprednol etabonate-loaded NE. Benzalkonium chloride, which is known for its toxic effect on corneal epithelial cells *in vivo* and *in vitro* (Kabashima et al., 2020; Vitoux et al., 2020; Yamashiro et al., 2021), was used as a positive control of NE formulation toxicity at three different concentrations (0.1, 0.01 and 0.005 % (w/*v*)). The NF-forming polymers were dissolved in HBSS in the concentration corresponding to the one in the dispersion of loteprednol etabonate-loaded NFs, as pure polymers (PEO, P188) or as their mixture in weight ratio 1:1. The viability of the 3D-HCE-T model was assessed using the colorimetric MTT assay and expressed relative to the negative control represented by cells treated with HBSS. Treatment of cells with a single dose of benzalkonium chloride resulted in a concentration-dependent decrease in cell viability, whereas single-dose treatment with initial NEs, dispersed NFs, and NF-forming polymers in the same experimental setting did not affect cell viability (Fig. 9). Multiple-dose treatment of cells with initial loteprednol etabonate-loaded NEs, dispersed NFs, and NF-forming polymers for three consecutive days resulted in cell viability comparable to negative control. However, the treatment of cells with benzalkonium chloride in all tested concentrations had a detrimental effect on their viability (Fig. 9). Based on these results it can be concluded that dried NEs in the form of hydrophilic NFs represent in the tested concentration a biocompatible formulation suitable for ophthalmic application.

4. Conclusions

In the present study, we showed that electrospinning can be used as an innovative method for the transformation of NEs into dry, non-powdered product, that disperses easily and forms a nanodroplet dispersion upon contact with an aqueous medium. The biocompatibility of the dried loteprednol etabonate-loaded NEs in the form of NFs after a single and multiple-dose application was confirmed using the 3D HCE-T model of the stratified epithelium of the human cornea. The weak gel that would form *in situ* after the application of dried NE in the form of NFs to the ocular surface may be considered highly beneficial, as it would allow prolonged retention of loteprednol etabonate at the ocular surface without causing discomfort to the patient. Based on these findings, it can be concluded that dried NEs in the form of hydrophilic NFs represent a biocompatible formulation suitable for ophthalmic use. This innovative solid eye preparation, incorporating NE with the anti-inflammatory drug loteprednol etabonate, thus can represent a new approach for the treatment of DED.

CRediT authorship contribution statement

Josip Ljubica: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Črt Dragar:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Tanja Potrč:** Writing – original draft, Formal analysis. **Mirjam Gosenca Matjaž:** Writing – review & editing, Formal analysis. **Mirjana Gašperlin:** Writing – review & editing, Project administration, Funding acquisition. **Laura Nižić Nodilo:** Writing – original draft, Methodology. **Ivan Pepić:** Writing – review & editing. **Jasmina Lovrić:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Petra Kocbek:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

Data will be made available on request.

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