ORIGINAL RESEARCH

Effective Ocular Delivery of Eplerenone Using Nanoengineered Lipid Carriers in Rabbit Model

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Background: Eplerenone (Epl) is a selective mineralocorticoid-receptor antagonist used for chronic central serous chorioretinopathy treatment. Our goal was to enhance the corneal performance of Epl-loaded nanostructured lipid carriers (NLCs) through surface modification using different coating polymers.

Methods: Epl-loaded modified NLCs (Epl-loaded MNLCs) were prepared by coating the surface of Epl-loaded NLCs using different polymers, namely hyaluronic acid, chitosan oligosaccharide lactate, and hydrogenated collagen. A $3^1 \times 4^1$ full factorial design was used to evaluate the effect of the surface modification on the properties of the prepared systems. Selected optimal Epl-loaded MNLCs were further evaluated for in vitro drug release, morphology, pH, rheological properties, corneal mucoadhesion, irritation, and penetration.

Results: Epl-loaded MNLCs were successfully prepared with high drug-entrapment efficiency and nanosized particles with low size distribution. Transmission electron microscopy revealed nanosized spherical particles surrounded by a coating layer of the surface modifier. The pH, refractive index, and viscosity results of the Epl-loaded MNLCs confirmed the ocular compatibility of the systems with no blurring of vision. The safety and ocular tolerance of the optimal MNLCs were confirmed using the hen's egg test on chorioallantoic membrane and by histopathological evaluation of rabbit eyes treated with the optimal systems. Confocal laser-scanning microscopy of corneal surfaces confirmed successful transcorneal permeation of the Epl-loaded MNLCs compared to the unmodified Epl-loaded NLCs, revealed by higher corneal fluorescence intensity at all time intervals.

Conclusion: Overall, the results confirmed the potential of Epl-loaded MNLCs as a direct approach for Epl ocular delivery.

Keywords: eplerenone, modified nanostructured lipid carriers, chronic central serous chorioretinopathy, surface modifiers

Introduction

Central serous chorioretinopathy (CSCR) is an intriguing ocular disease commonly causing visual impairment in the working-age population. CSCR is the fourth–most frequently encountered nonsurgical retinopathy after age-related macular degeneration, diabetic retinopathy, and retinal vein occlusion.[1] It is characterized by acute or chronic neurosensory detachments of the retina in the posterior segment of the eye associated with or without detachments of the retinal pigment epithelium causing central visual disturbance and blurring.[2,3] Examination of CSCR reveals subretinal fluid accumulation; however, the complex pathogenesis of CSCR is multifactorial and not fully understood.

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The use of mineralocorticoid antagonists as a potential treatment option has increased in several prospective and retrospective studies for CSCR treatment.[4] Their action is due to the existence of an independent renin–angiotensin–aldosterone system at the ocular level, with greater expression in retinal and uveal tissue and acting on the ocular vasculature, aqueous humor, and intraocular pressure control.[5,6] Eplerenone (Epl) is a selective mineralocorticoid-receptor antagonist currently used for the treatment of CSCR.[7] It is a class II drug according to the biopharmaceutical classification system, with low solubility and high permeability through biological membranes.[8] Epl has the ability to act on neuroretinal cell types,[9] modifying the aforementioned physiopathological processes.[10–12]

Topical formulations to treat intraocular diseases face obstacles, limiting their delivery, including precorneal loss factors (eg, tear dynamics, transient residence time in the cul-de-sac, and relative impermeability of the corneal epithelial membrane), resulting in poor bioavailability.[13] In addition, the residence time of the drug on the corneal surface and drug penetration across the cornea still needs to be increased.[14] Modifying viscosity has been adopted to prolong corneal residence time by applying hydrogels or in situ gelling systems.[15] However, this has resulted in hindering permeation, leading to imperfect results.[16] Therefore, the need for new strategies to improve drug bioavailability has increased. Lipid nanocarriers like nanostructured lipid carriers (NLCs) have shown excellent features for the ocular delivery of pharmaceuticals.[16–18] Furthermore, modification of NLCs using surface modifiers (coating polymers) improves ocular drug bioavailability through prolonging corneal residence time.[19–21] Surface modifiers are capable of enhancing the interaction between NLCs and ocular surface structures through hydrogen bonding and electrostatic and covalent bonding between the mucin layer covering the conjunctiva and/or the corneal surfaces of the eye and the applied surface modifier.[22] In this article, different surface modifiers are used: hyaluronic acid (HA), COSL, and hydrogenated collagen.

HA is an anionic biodegradable biopolymer with interesting capacity for ocular delivery, since it is a major constituent of the vitreous humor,[23] found throughout the retina,[24] present in the extracellular matrix of many soft connective tissues, and is responsible for maintaining tissue-structure integrity, together with other structural macromolecules.[25] HA is known to be similar to mucin in terms of viscoelasticity, biophysical properties, and

lubrication of the ocular surface. It has beneficial effects in providing long-lasting hydration and retention. The salt form of HA can easily cover the corneal epithelium.[26,27]

COSL is a cationic polymer of low molecular weight.[28] Unlike chitosan, COSL has high aqueous solubility, making it suitable for ocular application.[28] It has been reported that cationic polymers like the polycationic COSL have mucoadhesive properties due to their ability to develop electrostatic interactions with the negatively charged mucin, resulting in extended residence time on the ocular surface, along with the penetration-enhancement effect, leading to a significant improvement in ocular drug bioavailability.[28]

Collagen is one of the most useful biomaterials, with excellent biocompatibility, biodegradability, weak antigenicity, and high safety due to its biological characteristics. Collagen can be dispersed in aqueous media, forming a clear colloidal solution. Collagen-based nanoparticles have demonstrated excellent potential as a sustained-release formulation through both topical and oral usage.[29–31]

Previously, our team succeeded in preparing Epl-loaded NLCs, with small particles (134 nm) and polydispersity index (PDI; 0.31) and high entrapment efficiency (EE; 76%) and ζ-potential (ZP; −34 mV.[32] This NLCs were used as a base (core) for further surface modification to enhance the ocular delivery of Epl. In this study, three goals were achieved. The first was to develop Epl-loaded modified NLCs (MNLCs) according to a $3^1 \times 4^1$ full factorial design using Design-Expert software to study the outcome of design variables on formulation characteristics and select an optimal formula based on the desirability function. The second was extensive in vitro evaluation to confirm the safety of the optimal Epl-loaded MNLCs for ocular delivery. Third, the in vivo safety of the NLCs was evaluated using histopathology analysis and the Draize test, in addition to in vivo imaging using confocal laser microscopy to demonstrate the transcorneal permeation ability of the system through rabbit eyes. To our knowledge, ocular delivery of Epl for CSCR treatment has not been investigated in the literature.

Methods

Epl was obtained from Shenzhen Oriental Pharmaceutical. Imwitor 900K (glyceryl monostearate) was purchased from Changwei Pharmaceutical Excipients Technology. Pluronic F127 and Miglyol 812N was purchased from BASF. Hydrogenated collagen and HA were obtained from Acros Organics. COSL and porcine mucin powder

were obtained from Sigma-Aldrich. Ethanol and acetone were obtained from El-Nasr Pharmaceutical Chemicals. All other chemicals were of analytical grade.

Preparation of Epl-Loaded MNLCs

Epl-loaded MNLCs were prepared by emulsification-solvent evaporation using Miglyol 812N as liquid lipid and glyceryl monostearate as solid lipid (2:1 w:w liquid lipid: solid lipid), as per the method adopted by Shamma et al.[33]

In brief, 25 mg of Epl were dispersed in a weighted amount of Miglyol 812N, then added to a determined amount of molten glyceryl monostearate (kept at 80°C using a magnetic stirrer with a heating plate). Ethanol–acetone 10 mL (1:1 v:v) was added to the molten lipids maintained at 80°C until complete dissolution in the organic phase. The organic phase was then added to 20 mL aqueous solution containing Pluronic F127 as surfactant (0.43% w:v) to form a primary oil-in-water emulsion under stirring at 1,000 rpm for 1 minute using a magnetic stirrer. The emulsion obtained was subsequently subjected to 3 minutes of sonication using a probe sonicator (VCX 750 ultrasonic processor) adjusted to 40 W. The NLCs formed were stirred using a magnetic stirrer at 500 rpm for 2 hours at room temperature to allow the organic solvent to evaporate and tEpl-loaded NLCs to form.[33] Coating of the Epl-loaded NLCs was achieved using the surface modifiers (HA, COSL, and hydrogenated collagen). Weighted amounts of the modifiers were added in the dispersion and stirred at 300 rpm for 10 minutes to form surface-modified Epl-loaded NLCs.

In Vitro Characterization of Epl-Loaded MNLCs

Particle Size, PDI, and ZP

Particle size (PS), PDI, and ZP were measured using dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments). Appropriate dilution before measurement was performed for each system to obtain suitable scattering intensity (1 mL system to 10 mL deionized water). Each system was measured three times.

Entrapment Efficiency

The EE (w:w) of Epl in the Epl-loaded MNLCs was determined indirectly through measuring the concentration of free drug in the aqueous phase of the MNLC dispersion. MNLC dispersion (1 mL)was centrifuged using a cooling centrifuge at 22,000 rpm for 1 hour at 4°C (Sigma 3-30 KS). The supernatant was separated and properly diluted

with ethanol, and the unentrapped drug concentration was estimated spectrophotometrically at 241 nm (Shimadzu UV-1601 PC). EE% was calculated:

$$The\ EE\% = \frac{W\ initial - Wfree}{W\ initial}x100$$

where: W initial is the total drug amount and Wfree the unentrapped drug amount.

Rheological Measurement

Rheological properties of the MNLCs were evaluated using cone-and-plate viscometry (Brookfield DVT-2). The temperature of the plate was fixed at 35°±0.1°C by connecting it to a thermostatic water bath. Samples (0.5 mL) of each system were added to the plate. The rate of shear was increased gradually, viscosity determined from instrument readings, and the systems were subjected to a continuous change in the shear rate (0.5–600 s^{-1}).[34]

Impact of Formulation Variables

A $3^1 \times 4^1$ full factorial design was employed for preparing Epl-loaded MLNCs using Design-Expert software version 7 (Stat-Ease). Two factors were evaluated in this design: the type of surface modifier, which was evaluated on three levels, and the second the concentration of surface modifier which was evaluated on four levels. As a result, 12 formulae were prepared and evaluated for the dependent variables: PS (Y_1), PDI (Y_2), ZP (Y_3), EE% (Y_4), and viscosity (Y_5; Table 1). One-way ANOVA was performed to test the significance of each factor ($p<0.05$) on the selected responses.

Optimized Epl-Loaded MNLCs

Design-Expert was employed to select the optimal Epl-loaded MNLCs by applying the desirability function. Constraints were applied to obtain Epl-loaded MNLCs with the smallest PS, PDI <0.5, and highest EE, ZP, and viscosity.

Further Investigations

In Vitro Release of Epl from Epl-Loaded MNLCs in Simulated Lacrimal Fluid

The release of two selected Epl-loaded MNLC types (HA-coated MNLCs and COSL-coated MNLCs) was compared to the release of the unmodified one (Epl-loaded NLCs) in the same conditions, in order to ascertain the effect of the surface modifier on Epl release. The in vitro release study was carried out using dialysis-bag diffusion. Exactly 2 mL of the optimized MNLCs (equivalent to 2.5 mg Epl) was put into the dialysis bag (molecular weight cutoff 12,000–

Table 1 Experimental Runs, Independent Variables, and Measured Responses of the $3^1 \times 4^1$ Full Factorial Experimental Design of Epl-loaded MNLCs

F (Sorted by Standard Order)	Type of Surface Modifier	Concentration of Surface Modifier (% w:v)	Y₁: PS (nm)	Y₂: PDI	Y₃: ZP (mV)	Y₄: EE% (% w:w)	Y₅: Viscosity (cP) at Shear Rate (20 [1/s])
MNLC-1	Hydrogenated collagen	0.3	152.75±15.20	0.333±0.01	−28.1±0.42	62.945±1.10	39.24±0.1
MNLC-2	COSL	0.3	282.25±32.31	0.382±0.01	+15.8±0.85	69.948±0.77	0±0
MNLC-3	Hyaluronic acid	0.3	167±42.57	0.416±0.16	−44.25±1.63	81.285±1.63	186.39±0.32
MNLC-4	Hydrogenated collagen	0.5	155.6±4.10	0.362±0.07	−24.1±0.57	63.865±1.89	39.24±0.56
MNLC-5	COSL	0.5	236.15±26.38	0.428±0.06	+20.65±0.49	70.583±9.08	78.48±0.98
MNLC-6	Hyaluronic acid	0.5	262.75±84.07	0.449±0.03	−48.1±1.13	79.025±1.05	333.54±0.15
MNLC-7	Hydrogenated collagen	0.7	127.773±9.15	0.381±0.13	−27.95±0.21	67.281±8.39	49.05±0.17
MNLC-8	COSL	0.7	222.95±11.10	0.433±0.03	+29.3±0.28	70.26±0.08	29.43±2.11
MNLC-9	Hyaluronic acid	0.7	261.75±66.40	0.434±0.04	−53.2±1.13	88.445±2.80	824.04±1.65
MNLC-10	Hydrogenated collagen	0.9	161.2±5.23	0.393±0.04	−30.944±0.93	76.905±7.96	39.24±1.24
MNLC-11	COSL	0.9	397.1±158.11	0.428±0.16	+46.35±1.77	74.357±6.73	9.81±0.98
MNLC-12	Hyaluronic acid	0.9	277.45±36.56	0.475±0.01	−54.4±0.49	90.775±3.57	1373.4±0.47

Notes: Data presented as means ± SD (n=3). All systems contained 1.25 mg Epl/1 mL.
Abbreviations: PS, particle size; PDI, polydispersity index; ZP, ζ-potential; EE, entrapment efficiency.

14,000 Da), then tightly closed at both ends. The filled dialysis bag was immersed in a bottle containing 100 mL simulated lacrimal fluid (pH7.4).[35] The release was carried out using a thermostatic horizontal shaker (GFL Gesellschaft für Labortechnik) at 50 rpm and 35°±0.5°C.[36] At determined time intervals (0.25, 0.5, 1, 1.5, 2, 4, 6, 8, and 24hours), 3 mL aliquots were withdrawn and replaced with an equal volume of fresh medium, in order to keep a constant volume.[37] Drug concentration was measured spectrophotometrically at 245nm with ultraviolet spectroscopy (Shimadzu UV-1601 PC) using simulated lacrimal fluid as a blank. The release profiles were drawn by plotting the percentage of cumulative drug released at each time point versus time. Experiments were repeated three times, and results are expressed as mean ± SD. Data obtained from the release of the drug from different NLCs were statistically analyzed to test significance ($p<0.05$).

Mucoadhesion of the Selected Nanosystems

The mucoadhesive properties of the two selected Epl-loaded MNLCs were evaluated to get an insight on adhesion behavior upon contacting the corneal surface. Exactly 1 mL of each selected system was mixed with 0.1% w:w aqueous mucin dispersion (1:40) and incubated for 24 hours at 35°±2°C. PS and ZP of the resulting dispersions were determined at three time points during the incubation (6, 12, and 24 hours), using the same procedure mentioned earlier, and compared to those obtained for plain mucin aqueous dispersion.[38]

In Vitro Irritation Testing

The possibility of irritation from the optimized MNLCs on the eye surface was studied in vitro using the hen's egg test on chorioallantoic membrane (HET-CAM).[39] The two selected Epl-loaded MNLCs were applied directly onto egg CAM and observed for any alteration or change to the membrane, simulating their effect on the eye mucosal membrane when applied in vivo.[39] The test involved three steps, as follows.

Preparing the Eggs

Fresh fertilized chicken eggs were incubated for 10 days at 37.5°±0.5°C and 66%±5% relative humidity. During this period, rotation of the eggs was done three times per day to avoid the embryo sticking to one side. After 10 days, the eggs were candled to ensure the viability of the embryos and to mark the air space using a pencil (Figure 1I). Infertile eggs were discarded. Before use, the eggshell around the air space was removed using a dental rotary saw and scissors. After exposure of the inner membrane, it was sprayed with saline for moisturization. Just prior to use, the inner membrane was removed using forceps to expose the CAM and considered ready for application of the test substances.

Application of Test Substances

Three systems were tested, (the two selected Epl-loaded MNLCs and Epl aqueous suspension). For comparison, 10% NaOH and saline were used as positive and negative controls, respectively. Exactly 0.3 mL of each tested
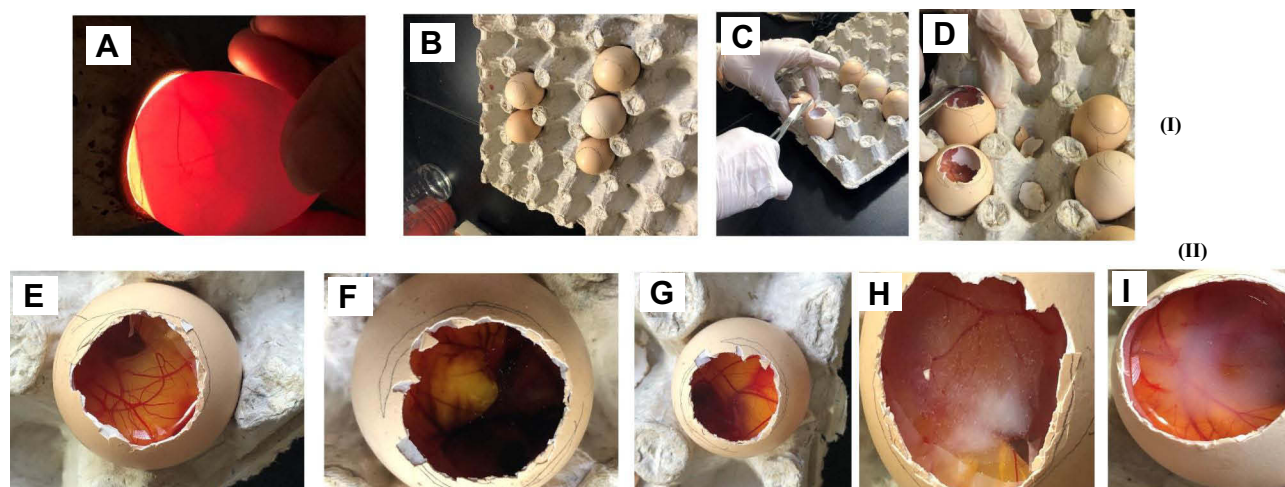
Figure 1 (**I**) Procedural steps for preparing the hen's egg on chorioallantoic membrane test (**A–D**). The egg was candled to check embryo viability (**A**) the air space marked (**B**), the external shell removed (**C**), and the inner membrane removed (**D**); (**II**) the observed responses to different systems application (**E–H**). Negative control (0.9% NaCl) (**E**); positive control (10% NaOH) (**F**); Epl aqueous suspension (**G**); Epl- loaded COSL-MNLCs (**H**); Epl loaded HA-MNLCs (**I**).

system was applied directly to the exposed CAM, covering at least 50% of its surface for 5 minutes.[40]

Visual Examination

After application of the test substances, the CAM was visually observed for any vascular change, such as hemorrhage, hyperemia, lysis, extravascular coagulation, or intravascular thrombosis.[41] Existence of any of these alterations reveals a possible tendency of the test compound to damage the eye mucosal membrane when applied in vivo. The CAM was photographed using a digital camera.

pH values

Measurement of pH values for the two selected systems was carried out with a Jenway 3505 pH meter (Bibby Scientific). Exactly 1 mL of each of the selected systems was dispersed in 9 mL distilled water, then the pH-meter electrode was immersed in the diluted dispersion and the value recorded.[37] Measurement was carried out three times.

Refractive Index

The refractive index of the two selected systems was determined using a Hilger and Watts 46.17/63707 refractometer.

Transmission Electron Microscopy

TEM was used for assessment of particle morphology of the two selected systems. One drop of each system was appropriately diluted and adsorbed on a carbon-coated copper grid for 10 minutes at room temperature to allow some of the particles to adhere to collodion. Filter paper was used to draw off the excess dispersion, then, a drop of phosphotungstic acid dye (negative stain) was applied for 1 minute, the excess removed, and the sample air-dried then examined with TEM (JEOL JEM 1400).[42]

Ocular Safety
Draize Test (Eye-Irritation Test)

The two selected Epl-loaded MNLCs were evaluated in vivo for ocular irritation or damage by observing any opacity, redness, inflammation, or increased tear production upon application on the eyes of albino rabbits.

Three male albino rabbits (2–2.5 kg) were assigned for evaluating each system. Rabbits were housed in accordance with National Institutes of Health guidelines, and all procedures in the study conformed to the guidelines of the Research Ethics Committee for Experimental and Clinical Studies at the Faculty of Pharmacy, Cairo University (PI 2682). Both eyes were examined before testing to confirm the absence of any defects, the lower lid was gently pulled away from the eyeball to form a cup into which the assigned dose (0.1 mL) of each of the selected Epl-loaded MNLCs was instilled into the right eye, and 0.1 mL normal saline solution was instilled in the contralateral eye to serve as control. The eyes (cornea, iris, and conjunctiva) were examined for any signs of irritation and scored as suggested by Draize.[43,44] An MNLC was considered nonirritant if it scored 0–3.9, slightly irritant if 4–8.9, moderately irritant if 9–12.9, and seriously irritant if 13–16.[45] Additionally, in order to evaluate the multi-instillation effect, the assigned dose was given every hour for 6 hours (six instillations) and

the eye structures examined for any irritation affecting the cornea (opacity), iris (inflammation), and conjunctiva (congestion, swelling, and discharge).[46]

Histopathological Examination of Rabbit Eyes

Thirty minutes after the last instillation, the rabbits were euthanized with an intravenous injection of sodium pentobarbital. Corneas receiving the selected MNLCs and control corneas receiving physiological saline were isolated, rinsed in physiological saline, and subsequently fixed in 10% formalin for 24 hours. Afterward, the isolated corneas were washed with tap water, then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in an oven for 24 hours. With a sledge microtome, paraffin–beeswax tissue blocks were prepared for sectioning at 4 μm thickness. The obtained tissue sections were collected on glass slides, deparaffinized, and stained with hematoxylin and eosin to be examined under light-electron microscopy.[47]

Corneal Visualization

Fabrication of MNLCs Labeled with Rhodamine B

The selected Epl-loaded MNLCs labeled with the fluorescent marker rhodamine B (RhB) were prepared by emulsification-solvent evaporation as previously mentioned, with a slight modification: 0.05% w:v RhB was added to the oil phase of the MNLCs. The rest of the procedure remained the same.

Transcorneal Visualization

Confocal laser-scanning microscopy (CLSM) was used to evaluate the ability of the tested formulations to boost drug corneal permeation,[48] in addition to demonstrating the ocular mucoadhesion of uncoated NLCs and the two selected Epl-loaded MNLCs.[49] They were evaluated by imaging the intensity of RhB and its depth at the surface of the eye using CLSM (Carl Zeiss LSM 710, differential interference contrast, fluorescence, and bright field). The three groups of male albino rabbits were used for evaluating each system (uncoated NLCs and the two selected Epl-loaded MNLCs). Healthy animals free of clinically observable abnormalities were housed singly in standard cages in a light-controlled room (12 hours light and 12 hours dark cycles) at 20°–24°C and 30%–75% relative humidity, with no restriction to food or water. On the day of the experiment, each group of rabbits received 0.1 mL of the tested formulations (uncoated NLCs, fluorescent Epl-loaded COSL-MNLCs, and Epl-loaded HA-

MNLCs) instilled in the eye using a micropipette. At predetermined time intervals after instillation (1, 2, 4, and 6 hours), the rabbits were euthanized with an intravenous injection of an overdose of sodium pentobarbital, given via a marginal ear vein, and the corneas removed, and fixed in physiological saline. The fluorescence intensity of RhB in the cornea was calculated after examination of the cornea using CLSM by taking mean intensity as the parameter,[49] and confocal images were managed and fixed.[50] Evaluation began from the outer corneal surface with the z-stack mode in the directions xy and xz. Surface by surface was imaged until complete RhB color disappearance.[51,52] Image acquisition and analysis were processed using ZEN 2.3 software.

Results and Discussion

Preparation of Epl-Loaded MNLCs

Epl-loaded NLCs were developed using emulsification-solvent evaporation using solid and liquid lipids. The liquid oil used (Miglyol 812N) has been reported to be well tolerated by the eye,[28,53,54] while the solid lipid, glyceryl monostearate, is a monoglyceride previously reported as safe for ocular drug delivery.[55] In general, these lipids are already permitted by European and US regulatory authorities for topical applications, because they are well established in various dosage forms and have a status of generally being recognized as safe.[56] Pluronic F127 was used as emulsifier, due to its ability to act as a thickening agent to increase formulation residence time on the ocular surface and to reduce physiological drug drainage. A nonionic emulsifier, Pluronic F127 is known to cause negligible or no ocular irritation.[57]

Factorial Design Analysis

A $3^1 \times 4^1$ full factorial design was used and statistically analyzed using Design-Expert software. Each factor's levels were set based on preliminary trials. The model selected was two-factor interaction. Table 2 shows the independent variables, their respective levels, and the model summary statistics of the full factorial design used for optimization of Epl-loaded MNLCs. Adjusted and predicted R^2 were found to be within 0.2 difference, and this was attained in all responses.

Effect of Formulation Variables on PS

For ocular administration, PS is an important parameter to assure patient convenience and efficient and safe administration. Particles intended for ocular applications should not

Table 2 Independent Variables, their Levels, and Summary Statistics of the $3^1 \times 4^1$ Full Factorial Design Used for Optimization of Epl-loaded MNLCs

Factors (independent variables)							
Type of surface modifier	Chitosan oligosaccharide lactate	Hyaluronic	Hydrogenated collagen				
Concentration of surface modifier (% w:v)	0.3	0.5	0.7	0.9			
Responses (dependent variables)	R^2	Adjusted R^2	Predicted R^2	Constraints	P	Adequate precision	
Y_1: PS (nm)	0.9985	0.9915	0.8459	Minimize	0.0069	41.924	
Y_2: PDI	0.9185	0.8879	0.8243	Less than 0.5	0.0001	16.330	
Y_3: ZP (mV)	0.9703	0.9456	0.8164	Maximize	0.0002	19.364	
Y_4: EE%	0.9161	0.8846	0.8061	Maximize	0.0001	15.254	
Y_5: Viscosity (cP)	0.9756	0.9553	0.8493	Maximize	0.0001	19.891	

Abbreviations: PS, particle size; PDI, polydispersity index; ZP, ζ-potential; EE, entrapment efficiency.

exceed 10 μm.[58] Nanometric drug-delivery systems ensure less ocular irritation and better patient convenience, with much improvement in corneal penetration and retention time.[46]

As presented in Table 1, the PS of the prepared Epl-loaded MNLCs ranged from 127.77±9.15 to 397.10 ±158.11 nm, which is acceptable for ocular administration.[59] According to the ANOVA results (Table 2), the type of surface modifier and concentration had significant effects on the PS of the Epl-loaded MNLCs (*p*=0.0026 and 0.0069 for effect of type and concentration of surface modifier, respectively).

The largest PS was observed in HA-coated Epl-loaded MNLCs, followed by COSL-coated ones, then hydrogenated collagen Epl-loaded MNLCs (Figure 2). This may be attributed to differences in viscositys which appear to increase in the same manner (as will be discussed later). Kamal et al[60] reported that higher increase in PS during surface modification can be due to the effect of a surface modifier on the viscosity of the external aqueous phase. Similar results were also reported by Foucher et al[61] for poly-ε-caprolactone nanospheres coated with HA.

Increasing the concentration of the surface modifier significantly increased the PS of the prepared Epl-loaded MNLCs. This can be attributed to the increased cross-linking and interaction occurring at the surface of the NLCs with the surface modifier leading to observable PS enlargement.[62,63] It is worth mentioning that the MNLCs had an extra transparent layer coating the outer surface (as would be confirmed later by TEM), its accounting for the

increase in PS by increasing the concentration of the surface modifiers.[64] These results are in agreement with Neslihan et al,[65] where an increase in PS was observed with increased COSL concentration in the coating of ofloxacin-loaded ophthalmic NLCs.

Effect of Formulation Variables on PDI

PDI values represent the width of PS distributions, where smaller values are indicative of a stable monodispersed system in medium.

Table 1 shows the PDI values of the prepared Epl-loaded MNLCs, which ranged from 0.333±0.01 to 0.475±0.01. According to ANOVA results (Table 2), the type and concentration of surface modifier had significant effects on the PDI values of the prepared Epl-loaded MNLCs (*p*=0.0001 and 0.0013, respectively). The largest PDI value was observed upon using HA as a surface modifier, followed by COSL and finally hydrogenated collagen (Figure 2). These results conform with the PS results, where the PS of MNLCs increased in the same manner.

ANOVA results showed that increasing the concentration of surface modifier resulted in significantly higher PDI values. This may be due to particle bridging as, manifested by increasing the PS with increasing the concentration of surface modifier. This is in agreement with Jain et al.[66]

Effect of Formulation Variables on ZP

The high magnitude of absolute ZP provides an indication of better stability of the dispersion in an aqueous environment and ensures long-term storage without any settling.[67]
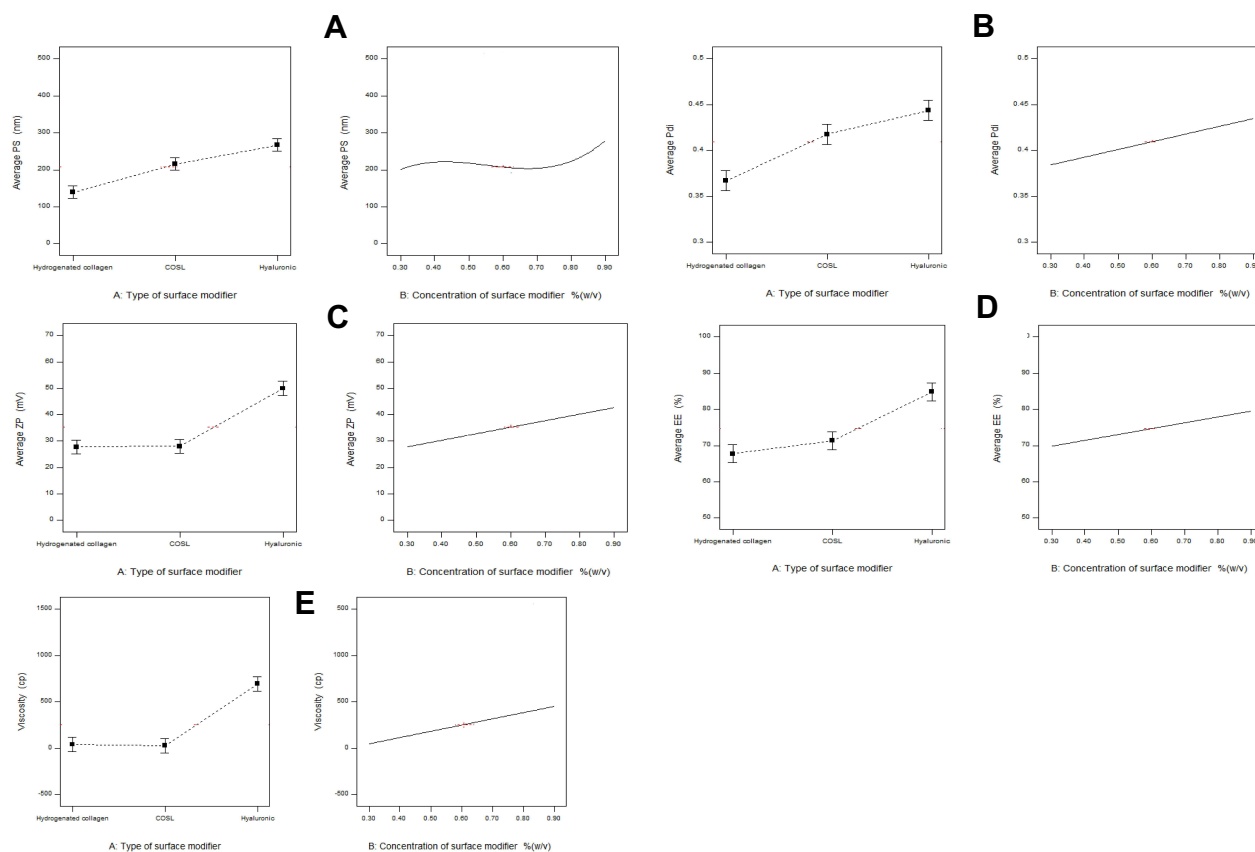
Figure 2 Line plots of significant effects of type of surface modifier and its concentration on (**A**) PS, (**B**) PDI, (**C**) ZP, (**D**) EE%, and (**E**) viscosity.

Table 1 shows the ZP of the prepared Epl-loaded MNLCs, which ranged from 15.80±0.85 to 54.40±0.49 mV (absolute value). According to ANOVA results, type and concentration of the surface modifier had significant effects on ZP (p=0.0001 and 0.0008, respectively). Increasing the surface-modifier concentration led to a significant increase in ZP (absolute value). The largest absolute ZP values were observed with HA-coated systems (negatively charged), followed by COSL (positively charged) and hydrogenated collagen (negatively charged), which showed approximately the same absolute values (Figure 2). The change in ZP was concentration-dependent. Increasing surface-modifier concentration led to an increase in ZP, in agreement with Zhang et al,[54] who reported increased ZP upon increasing the concentration of Eudragit RS 100 (used for coating of NLCs).

Noteworthily, coating of Epl-loaded NLCs with only COSL resulted in the surface charge changing to positive (as manifested by positive ZP values) due to the adsorption of polycationic chitosan on the particle surface,[68] whereas HA increased the negative surface charge of Epl-loaded NLCs due to the polyanionic nature of the HA molecules. This high ZP enhanced the electrostatic stabilization of the system due to electrostatic repulsion among similarly charged MNLCs, providing a "stealth" characteristic that can mimic the prolonged precorneal retention of the NLCs, which might be due to the possible interaction of HA-coated NLCs with the hyaluronan receptors present on the corneal and conjunctival epithelial cells.[62]

Effect of Formulation Variables on EE

Epl EE in the prepared Epl-loaded MNLCs ranged from 62.94%±1.10% to 90.77%±3.57% w:w (Table 1).

According to ANOVA results, the type and concentration of surface modifier had significant effects on the EE of Epl-loaded MNLCs (p=0.0001 and 0.0035, respectively, Table 2). The highest EE was observed with HA-coated Epl-loaded MNLCs, followed by COSL-coated ones then hydrogenated collagen Epl-loaded MNLCs. These results correlated well with the PS results, as previously

discussed, where the highest PS was observed with HA-coated Epl-loaded MNLCs, followed by COSL-coated ones then hydrogenated collagen Epl-loaded MNLCs. EE of drugs from lipid nanocarriers is highly dependent upon interfacial area, surface charge, and nanoparticulate dimensions.[69] Previous reports in the literature have shown that increasing the PS of nanoparticles leads to an increment in the EE of nanocarriers.[70]

Increasing the concentration of the surface modifiers led to a significant increment in EE. For COSL-coated ones, high EE might be attributed to the ionic interaction between the COSL and the anionic segment in the formulation, which efficiently prevents drug escape from the nanoparticles and imparts the MNLCs better stability.[71] However, the HA in this product was in the form of sodium hyaluronate and collagen in the form of hydrogenated collagen. Positively charged ions from the monovalent alkali metal series, such as Na^+ and H^+, act as counterions to anionic structure of NLCs (negatively charged), thus being absorbed on the surface-dominating negative sites and preventing drug escape and further increment in EE.

Effect of Formulation Variables on Viscosity

Table 1 shows the viscosity values of the prepared Epl-loaded MNLCs. Each value represents the viscosity at a constant shear rate 20 (1/s) and ranges from 0±0 to 1,373.4±0.47 cP. According to ANOVA results, the type and concentration of surface modifier had significant effects on viscosity (p=0.0001 and 0.0011, respectively). As illustrated in Figure 2, higher viscosity was observed with HA-coated systems, followed by COSL coated ones and finally hydrogenated collagen Epl-loaded MNLCs. This is due to capability of HA to produce high viscosity at low concentrations, owing to its high molecular weight and random-chain structure.[72] Aqueous HA solutions have been reported to have non-Newtonian properties with high viscosity.[73] The highest molecular weight reported for HA is 4,000 kDa,[74] followed by COSL (3,000 kDa)[56] and finally hydrogenated collagen (300 kDa),[75] as described by the product label. Increasing the concentration of the surface modifier led to a significant increment in viscosity, owing to increased polymer-chain entanglement.[76]

Selection of the Optimal System

The optimization process is directed to tailor independent formulation variables to produce a high-quality system with the optimum physicochemical properties.[77] The desirability function was applied to select the optimum system. Desirability constraints for the optimum systems were minimizing PS, maximizing EE, maximizing ZP (absolute), maximizing viscosity, and achieving PDI <0.5. Two selections with desirability values near 1 were chosen as optimized systems (a negatively charged system with a desirability value of 0.937 and a positively charged one with desirability of 0.720). Furthermore, to confirm the efficacy of the model, the two systems were prepared, characterized, and compared with the predicted responses.

Negatively charged HA-coated Epl-loaded MNLCs (Epl-loaded HA-MNLCs) were prepared using 0.85% w:v HA, and showed EE of 90.77%±3.57%, PS 255±36.55 nm, PDI 0.40±0.01, ZP −54.2±0.494 mV, and viscosity 1,200±9.81 cP. Positively charged COSL-coated Epl-loaded MNLCs (Epl-loaded COSL-MNLCs) were prepared using 0.84% w:v COSL, showed EE of 70.11% ±0.084%, PS 285±5.03 nm, PDI 0.42±0.15, ZP 30 ±0.28mV, and viscosity of 40.24±7.12 cP.

As shown in Table 3, there was strong similarity between the observed and predicted values of the two selected systems, confirming the validity of the optimization process. Based on the aforementioned results, both Epl-loaded HA-MNLCs and Epl-loaded COSL-MNLCs were promising candidates for further investigation.

In Vitro Release in Simulated Lacrimal Fluid

In vitro drug release provides an indication of the delivery system in vivo.[36] Figure 3 shows the release profiles of the two selected systems (Epl-loadedEpl-loaded HA-MNLCs and Epl-loadedEpl-loaded COSL-MNLCs) over 24 hours compared to unmodified NLCs. The unmodified NLCs showed an initial burst effect in the first 2 hours, due to the diffusion of the surface-attached drug, followed by sustained drug release, due to the diffusion of the entrapped drug from inside the vesicles to the surrounding aqueous medium.[78,79] The two selected modified systems showed superiority in sustained drug release when compared to the unmodified one, where only 66.264%±2.081% and 75.937%±0.118% Epl had been released from Epl-loadedEpl-loaded HA-MNLCs and Epl-loadedEpl-loaded COSL-MNLCs, respectively, after 8 hours. Several studies have reported that chitosan coating leads to an improvement in drug loading,

Table 3 Predicted Values of Responses for Optimized Systems (Epl-loaded HA-MNLCs and Epl-loaded COSL-MNLCs) Compared to Actual Values, along with Prediction Intervals

|	Validity Parameters	Y_1: PS (nm)	Y_2: PDI	Y_3: ZP (mV)	Y_4: EE (%)	Y_5: Viscosity (cP)
Epl-loaded HA-MNLCs	Predicted value	265.54	0.464	−54.47	88.94	1190.78
	95% prediction interval	240.72–290.36	0.44–0.48	48.76–60.18	84.75–93.12	1,025.4–1,356.16
	Actual values	255±36.55	0.40±0.01	−54.2±0.494	90.77±3.57	1,200±9.81
Epl-loaded COSL-MNLCs	Predicted value	318.92	0.437828	40	75.2155	38.0875
	95% prediction interval	294.37–343.48	0.419–0.45	34.46–45.53	71.09–79.33	36.78–40.03
	Actual values	285±5.03	0.42±0.15	30±0.28	70.11±0.08	40.24±7.12

controlling drug release, mucoadhesion, enhancing cellular uptake, and targeting delivery.[63] Li et al[71] reported that coating of NLCs with chitosan oligosaccharides and carboxymethyl chitosan showed prolonged release characteristics compared to uncoated NLCs. The prolonged-release profile in the Epl-loadedEpl-loaded HA-MNLCs due to HA coating increases the thickness of nanocarriers, which increases the distance of drug diffusion, hence the slow release.[80] Moreover, the increased viscosity obtained by the addition of surface modifiers is thought to lessen NLC motion in the formulation, hence slower drug release.[65] One way-ANOVA revealed a significant difference in Epl released in 24 hours (Q_{24}) between unmodified NLCs and the two selected MNLCs (p=0.001and 0.003 for Epl-loadedEpl-loaded HA-MNLCs and Epl-loadedEpl-loaded COSL-MNLCs, respectively). Q_{24} was 99.481%±3.219% for the unmodified NLCs, and 75.555%±0.16% and 83.128%±0.859%) for Epl-

loadedEpl-loaded HA-MNLCs and Epl-loadedEpl-loaded COSL-MNLCs, respectively.

Mucoadhesion

Evaluation of mucoadhesive properties was critical in indicating the ocular bioavailability of the selected systems (Epl-loadedEpl-loaded HA-MNLCs and Epl-loadedEpl-loaded COSL-MNLCs) along with their corneal retention behavior. The PS and ZP of mucin mixed with the two selected MNLCs (1:40 v:v) were measured using 0.1 w:v mucin solution as a reference. The PS for the two selected systems underwent a gradual increase till the end of incubation (Figure 4A). On the other hand, ZP of Epl-loadedEpl-loaded COSL-MNLCs reduced to a negative charge after only 6 hours and continued to drop till approximately reaching the same negative charge as pure mucin at the end of incubation (Figure 4B). The increase in PS and decrease in ZP of Epl-
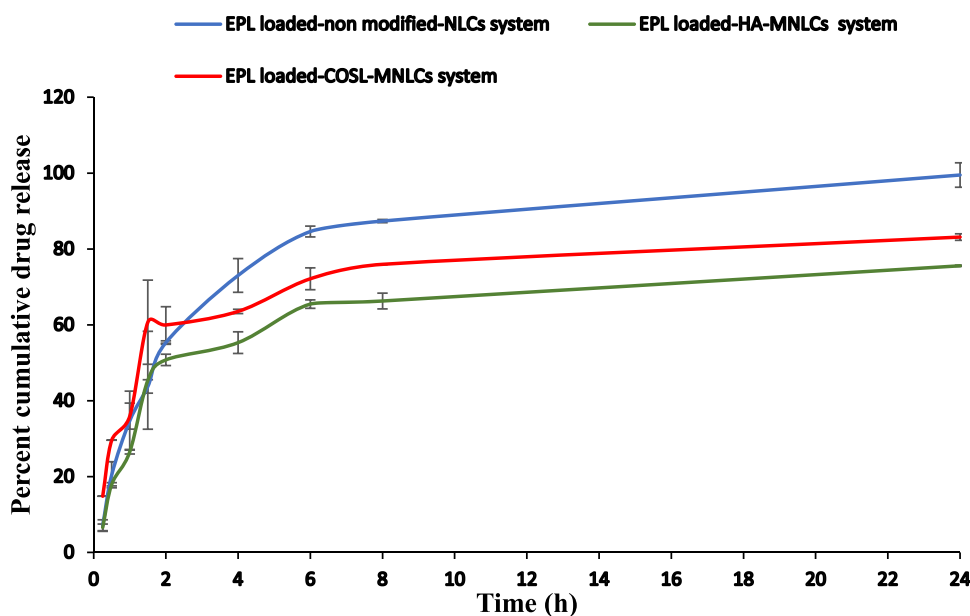
Figure 3 Cumulative release profile of Epl from loaded unmodified NLC system, Epl loaded HA-MNLCs and Epl loaded COSL-MNLCs.
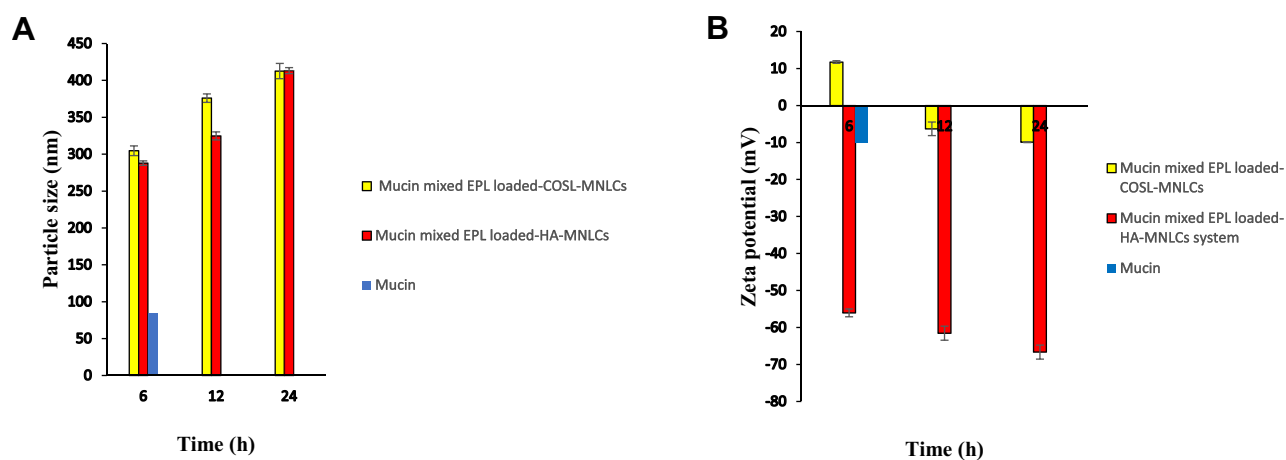
Figure 4 Mucoadhesion results: (**A**) PS measurements; (**B**) ZP measurements.

loadedEpl-loaded COSL-MNLCs could be attributed to electrostatic interaction between positively charged surface layer with negatively charged sialic groups of mucin as previously shown,[81] which can lead to an increase in retention time on the mucosal surface. For Epl-loadedEpl-loaded HA-MNLCs, interaction occurred between the mucin and the HA due to the physical entanglements and hydrogen bonding between hydrophilic functional groups of HA, such as carbonyl and hydroxyl groups and mucin,[76,82,83] which might be responsible for PS enlargement. On the other hand, ZP increased in the negative direction upon mixing Epl-loadedEpl-loaded HA-MNLCs with mucin. These negative values are attributed to HA because of its anionic nature, due to the presence of carboxylic groups. The mucin dispersion also presented a negative charge (−10 mV) due to the oligosaccharide chains, which confer a negative charge through carboxyl and sulfate groups, the charge similar to previous studies −10 mV.[84,85] As such, an increase in retention time on the corneal surface was expected for both systems, leading to an improvement in the efficacy of the ocular treatment.

In Vitro Irritation Testing

The HET-CAM test was used to detect possible irritation that may occur to ocular tissue upon application of the optimized systems (Epl-loadedEpl-loaded COSL-MNLCs and Epl-loaded HA-MNLCs), predicting their potential safety. Perfect vascularization of CAM tissue (arteries, veins, and capillaries) clearly shows an inflammatory reaction in response to injury, so was used to mimic the eye's conjunctival tissue.[86] As illustrated in Figure 1II, the positive control (10% NaOH) was severely irritant, resulting in severe

hyperemia and hemorrhage. On the other hand, 0.9% NaCl, used as the negative control, produced no irritant responses, as indicated by normal tissue vascularization (Figure 1II). The same results were obtained with the optimized systems and Epl suspension, where neither exhibited irritant effects on the CAM or any vascular damage. These findings confirm the safe and nonirritant characteristics of the optimized Epl-loaded MNLCs as a potential platform to deliver Epl efficiently to ocular tissue.

pH Values

The pH values of the two selected systems were found to be 6.42±0, and 7.39±0.12 for Epl-loadedEpl-loaded COSL-MNLCs and Epl-loadedEpl-loaded HA-MNLCs, respectively. The pH measurement confirmed the compatibility of these systems with the normal pH of tear fluid (7.4).[87]

Refractive Index

Refractive index values for ocular preparations are an important indicator of discomfort that may occur due to blurred vision after administration of ocular preparations. The light-refraction index for ophthalmic preparations should be <1.5 to prevent any discomfort in vision.[88,89] The refractive index was found to be 1.28 and 1.37 for Epl-loadedEpl-loaded COSL-MNLCs and Epl-loadedEpl-loaded HA-MNLCs, respectively, within the acceptable range and indicating no effect on vision.

Transmission Electron Microscopy

Figure 5 shows the TEM images of Epl-loadedEpl-loaded HA-MNLCs (Figure 5A) and Epl-loadedEpl-loaded COSL-MNLCs (Figure 5B). The two selected MNLCs
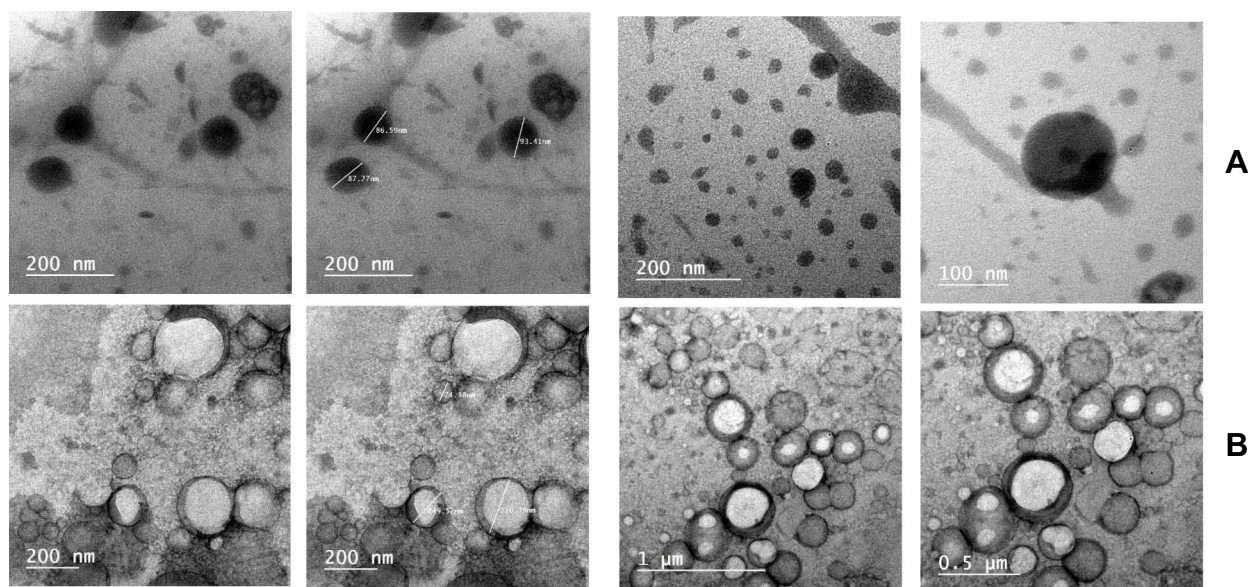
Figure 5 Transmission electron microscopy: (**A**) Epl loaded HA-MNLCs; (**B**) Epl loaded COSL-MNLCs.

were discrete spherical particles with a surface-adhering layer (surface modifier), as revealed by presence of a layer around each nanoparticle. In addition, the particles were separated from one another, assuring their stability.

In Vivo Safety Assessment
Draize Test (Eye Irritation)

Unlike the other ingredients used in our study and to the best of our knowledge, this was the first use of Epl in an ocular formulation.

Initial ocular irritation testing is of critical importance, due to the eye's sensitive nature. Special attention should be paid to evaluating irritation and toxicity to avoid any damage to ocular tissue.[58,90] Rabbits are preferred for irritation tests, due to their large eyes with well-described anatomy and physiology, ease of handling, low cost, and availability.[91] The Draize test revealed that the selected Epl-loaded MNLCs were nonirritant and safe for ophthalmic administration. Upon visual examination, corneas treated with Epl-loaded MNLCs did not show any sign of ocular irritation, inflammation, redness, increased tear production, or corneal edema over the saline solution that was used as a blank during the observation period, and thus were scored 0 according to the Draize scoring system.[43] (Figure 6). These observations suggested high ocular tolerance and were further confirmed by histopathological examination.

Histopathological Examination

This revealed no histopathological alteration in the cornea (outer and inner lining of the epithelium and stroma in between Figure 7a_2 and a_3) iris (Figure 7b_2 and b_3), retina, choroid, or sclera (Figure 7c_2 and c_3) in the treated group when compared to the control (Figure 7a_1–c_1). The absence of any ocular irritation and intolerability suggests that the two selected systems were well tolerated and can be used safely for ocular application.

Transcorneal Visualization

The interaction of the two selected modified systems (Epl-loadedEpl-loaded HA-MNLCs, and Epl-loadedEpl-loaded COSL-MNLCs) with corneal epithelia was investigated by observing the corneal samples using CLSM after administration of the systems into rabbit eyes in vivo at determined time intervals (1, 2, 4, and 6 hours), where they were prelabeled with fluorescent marker molecules ofRhB to provide visual evidence of cellular location. CLSM is considered s quantitative analysis test for absolute marker penetration.[48]

Figure 8I shows corneal epithelia exposed to RhB-labeled unmodified NLCs and RhB-labeled MNLCs at different time intervals. CLSM images using selective green filters confirmed cell uptake for the two selected Epl-loaded MNLCs and the unmodified NLCs, where transfer of the fluorescent marker was promoted by formation of a complex between the nanoparticles and the

Figure 6 Photographs showing normal rabbit eyes after instillation of 0.1 mL physiological saline in a control eye, Epl loaded HA-MNLCs, and Epl loadedCOSL-MNLCs (instillation repeated every hour for 6 hours).
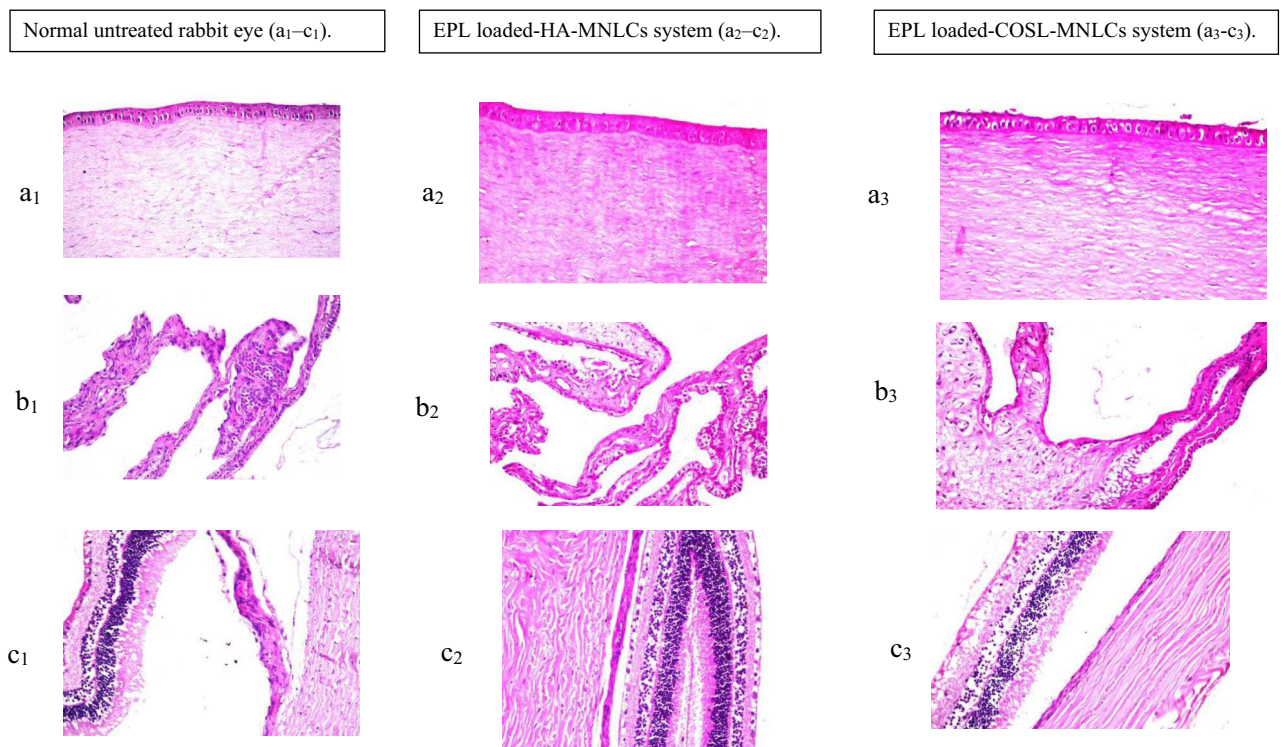

Figure 7 Histopathological stained sections (hematoxylin and eosin) of normal untreated rabbit eyes (a_1–c_1)and rabbit eyes treated with Epl loaded HA-MNLCs (a_2–c_2) and Epl loaded COSL-MNLCs (a_3–c_3), where "a" represents the outer layer of the cornea, "b" the irisand ciliary process, and "c" the retina, choroid, and sclera at magnification 40×.

biomembranes of the cornea, which led to staining of the corneal cells under investigation.[92]

Figure 8II shows the penetration of RhB (measure of depth in z-direction) from unmodified NLCs and the two selected MLNCs, as shown in an improvement in RhB penetration of 38 μm and 22 μm in HA- and COSL-MNCLs, respectively, compared to 16 μm in the unmodified system. Our finding was in agreement with a
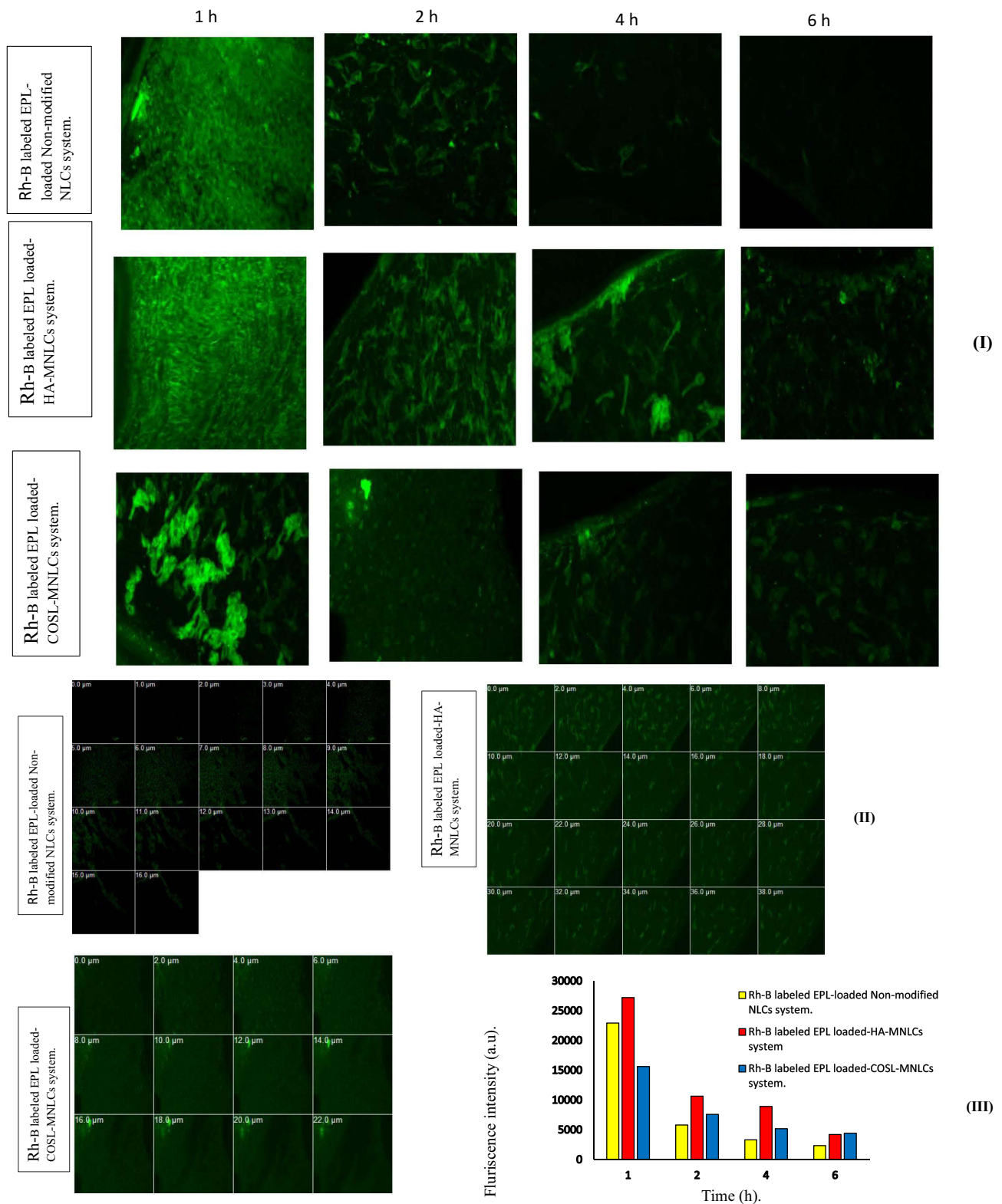
Figure 8 (**I**) Confocal laser-scanning microscopy of rabbit eyes at the corneal and scleral surfaces for RhB-labeled Epl-loaded unmodified NLCs, RhB-labeled Epl loaded HA-MNLCs, and RhB-labeled Epl loaded COSL-MNLCs; (**II**) confocal laser-scanning microscopy of rabbit corneas showing the depth of RhB; (**III**) mean fluorescence intensity of RhB-labeled Epl-loaded unmodified NLCs, RhB labeled Epl loaded HA-MNLCs, and RhB-labeled Epl loaded COSL-MNLCs at the time points indicated on the bar graph.

previously published work, where improved RhB penetration to a depth of 114 μm from ocular proniosomal gel was achieved.[93]

Upon measuring the fluorescence intensity at the predetermined time intervals after administration, images of the cornea treated with RhB-labeled unmodified NLCs and RhB-labeled MLNCs showed significant fluorescence intensity beginning at 1 hour, as depicted in the bar plot (Figure 8III). As time increased, fluorescence intensity was found to drastically decrease for the unmodified NLCs, whereas the decrease in fluorescence intensity was only gradual for the modified ones. After 2 hours, results revealed 1.8- and 1.3-fold higher fluorescence intensity in corneas treated with HA- and COSL-MNCLs, respectively, compared to cornea treated with unmodified NLCs. After 4 hours, there was 2.68- and 1.56- fold higher fluorescence intensity in corneas treated with HA- and COSL-MNCLs, respectively, compared to corneas treated with unmodified NLCs. This higher retention of the two selected modified systems can be attributed to the presence of surface modifiers (HA and COSL), which helped in enhancing the retention in conjunctival regions and the surface of the eye. This result is in agreement with previous work utilizing cell surface–associated ocular mucin and chitosan oligosaccharide.[94]

This improved retention could be due to the presence of secreted mucin and cell surface–associated mucin, which is abundantly present across the conjunctival and corneal regions of the eye, perhaps resulting in retention of a more significant amount of the modified systems available across the surface of the eye, due to ionic interactions (as discussed previously).[95,96] On the other hand, the unmodified NLCs had comparatively lower retention, which could be due to the impact of the clearance mechanisms of the eye, such as high tear-turnover rate and blinking, which otherwise would not significantly affect a mucoretentive system like the RhB-labeled MLNCs. Noteworthily, results revealed the superiority of HA-MNLCs over COSL-MNLCs, as revealed by higher corneal fluorescence intensity at all time intervals.

Based on the aforementioned results, we can reach the conclusion that NLC surface modification can enhance transcorneal Epl delivery and prolong precorneal retention time, thereby leading to higher ocular concentration.

Conclusion

In the present study, surface-modified Epl-loaded NLCs were designed and investigated for their in vitro characteristics, in vivo safety and tolerability, and in vivo transcorneal visualization using CLSM. Successful transcorneal delivery of the optimal Epl-loaded MNLCs was confirmed by higher fluorescence intensity for a longer time and greater depth in rabbit cornea than the unmodified NLCs. The final conclusions represent a direct approach for Epl ocular delivery. Furthermore, this could replace the necessity of oral use. Higher Epl concentration at the application site enhances efficiency and has fewer side effects.

Disclosure

The authors report no conflicts of interest in this work.

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