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Delivery of riboflavin-5'-monophosphate into the cornea: can liposomes provide any enhancement effects?

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Abstract

Keratoconus is a progressive condition caused by the thinning of the cornea, which eventually deforms the front surface of the eye into a cone shape leading to ghosting, multiple images, glare and several other vision problems. Currently keratoconus is treated with UV-induced riboflavin-mediated collagen cross-linking, which requires a physical removal of the corneal epithelium under topical anesthesia. This study reports the penetration of riboflavin (Rb) and its more water-soluble form, riboflavin-5'- monophosphate (RbP), into the bovine cornea ex vivo. Using ex vivo bovine corneal tissues and 0.8 mg/mL drug solutions in phosphate buffer, it was established that RbP penetration into the cornea within 3 hours of diffusion experiment was greater (17.3 ± 0.8 μg) compared to Rb (10.4 ± 4.2 μg). In the cornea RbP was found to convert to Rb, which is mediated with enzymes present in this tissue. Several formulations including the conventional and propylene glycol-containing liposomes with encapsulated RbP have been developed and their effect on the drug penetration into the bovine cornea was evaluated. Encapsulation of RbP into the liposomes did not provide any statistically significant improvement in the penetration of RbP into the cornea.

Keywords: Keratoconus, Riboflavin, Cornea, Liposomes, Ocular drug delivery

1. Introduction

Keratoconus is a progressive condition caused by the thinning of the cornea, which eventually deforms the front surface of the eye into a cone shape, and can lead to ghosting, multiple images, glare and several other vision problems. It is the most common dystrophy of the cornea and according to recent estimates, 1 in 2000 people worldwide suffer from keratoconus [1]. Wollensak *et al.* [2] suggested a novel approach for treating keratoconus using ultraviolet-induced riboflavin-mediated collagen cross-linking (CXL) of the corneal stroma (Fig.1). In this procedure a riboflavin-5'- monophosphate (RbP) solution is applied to the eye and activated via UVA irradiation (370 nm), where it acts as a photosensitiser. During collagen cross-linking, RbP generates singlet oxygen species, which induce the formation of covalent bonds between collagen molecules, while riboflavin additionally protects the cornea from the damage from UVA radiation [3]. CXL treatment has been proven successful in strengthening and stabilizing the cornea since its biomechanical properties are primarily determined by the collagen fibers.

However, in order to achieve efficient penetration of RbP into the corneal tissue, it is currently necessary to remove the corneal epithelium under topical anesthesia. Common side effects of epithelial abrasion include pain, corneal edema, and mild stromal haze [4, 5]. Therefore, finding a way to improve delivery of the drug without epithelial abrasion would significantly simplify the treatment, speed up the recovery, and improve patient compliance.

There are two forms of this photosensitive drug, a hydrophilic ionic RbP that is currently used clinically in corneal cross-linking, and a less polar non-ionic riboflavin (Rb) with a lower solubility in water. In previous studies various approaches have been considered to deliver riboflavin to the cornea including the use of permeation enhancers such as Ca^{2+} sequestering compounds, which showed improvements in the permeability of Rb when applied on the intact epithelial barrier due to loosening of tight junctions [1, 6]. Morrison *et al.* [7] reported that it is also possible to both increase the solubility and enhance the corneal permeability of Rb with the use of β-cyclodextrin. Recently, Bottos *et al.* [8] demonstrated that with Rb- and RbP-based nanoemulsions it is possible to saturate the stroma with the concentration of the drug comparable to the levels required during the standard epithelium-off procedure. However, these nanoemulsions were able to show the comparable results only after 4 hours. Other attempts to enhance the corneal permeability of the drug involved Rb-loaded mucoadhesive films [9], preparation of water-dilutable microemulsions with RbP [10], use of ultrasound with Rb [11] and iontophoresis with

RbP [12]. Despite all the efforts, the efficacy of CXL treatment has still not been sufficiently improved and, therefore, the enhancement of transepithelial absorption of riboflavin remains an important issue.

One of the potential strategies to improve corneal permeability of the drug applied to the eye is the use of liposomal formulations. Liposomes are phospholipid vesicles with the size range of 20 nm to 15 μm. Their specific structure enables encapsulation of different active substances without chemical bonding or any prior chemical modifications. Hydrophilic drugs are entrapped in the inner aqueous phase or are electrostatically bound to the outer bilayer. Lipophilic drugs interact with the lipid region, and amphipathic molecules are deployed between these two regions. Due to their biodegradability, biocompatibility, non-toxicity and non-immunogenicity, liposomes have shown some advantages among several other drug delivery systems [11, 12]. Research has shown improvement in precorneal drug retention, transcorneal permeation and therapeutic efficacy when liposomes are administered topically on the surface of the eye [11, 13].

The use of liposomes could potentially be considered as a viable strategy to overcome the epithelial barrier and improve riboflavin permeability into the corneal stroma for the treatment of keratoconus. Therefore, in this work the conventional and propylene glycol-containing liposomes (PG) with riboflavin-5'-monophospahte have been developed and the transcorneal drug penetration was evaluated *in vitro*. Propylene glycol is currently used in ophthalmic solutions present on the market (Systane®) as a lubricant to treat patients with mild to moderate dry eye syndrome [14, 15]. The potential of conventional liposomes to increase *in vitro* corneal flux was already reported in the literature [18]. Additionally, PG liposomes were studied in transdermal and vaginal drug delivery and reported to provide some advantages such as smaller size diameter and higher encapsulation efficacy resulting in enhanced permeability of drugs through the skin [19].

2. Materials and methods

2.1. Materials

Lipoid S100 (soybean lecithin, >94 % phosphatidylcholine) and Lipoid S75 (soybean lecithin with 70 % phosphatidylcholine) were generously provided by Lipoid GmbH (Ludwigshafen, Germany). Riboflavin-5'-monophosphate (RbP), riboflavin (Rb), egg phosphatidylcholine (EPC), propylene glycol, sodium hexane-1-sulfonate monohydrate and glacial acetic acid were purchased from Sigma-Aldrich (Gillingham, UK). Sephadex G50 superfine was obtained from GE Healthcare (UK). Sodium chloride, potassium chloride, sodium phosphate, potassium dihydrogen phosphate, Minisart syringe filters (0.2 µm) and HPLC grade ethanol were purchased from Fischer Scientific (Hemel Hempstead, UK). Water used for all aqueous solutions was purified using a Milli-Q system. All other chemicals and reagents were of analytical grade or superior.

Phosphate buffer, pH 7.4, and ion pair buffer were prepared according to procedures reported by Morrison *et al.* [20], and Anyakora *et al.* [21]. Ion pair buffer, used as a mobile phase, was prepared by dissolving 1.8822 g sodium hexane-1-sulfonate monohydrate in 2 liters of ultrapure water and adjusted to pH 3.0 ± 0.2 using glacial acetic acid. Phosphate buffer, used for Franz diffusion measurements, was prepared by mixing 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24g KH_2PO_4 with demineralised water (total volume 1000 mL).

2.2. Preparation of liposomes

In order to optimise entrapment efficacy of RbP, different types of lipids were used according to two methods of preparation (Table 1). The RbP content was kept constant in all preparations as well as liposomal suspension volume (5mL).

The first method that was used for the preparation of liposomes was "polyol dilution method", reported by Vanic *et al.* [19]. Lipid phase was prepared by dissolving phosphatidylcholine from egg (EPC) or soybeans (S75 or S100) in 1 g of propylene glycol at 60 °C under continuous stirring. 1 mL of RbP solution (50 mg/mL) was added to form an initial pro-liposome mixture. Liposomal suspension was diluted by the dropwise addition of water until the final volume of 5 mL.

The second method used for liposomal preparation was the conventional "film method". Phospholipids were dissolved in ethanol in a round-bottom flask. Organic solvent was then completely removed with the rotary vacuum evaporator (Rotavapor® R-124, Buchi, Switzerland). After the thin film was formed at the bottom of the flask, 5 mL of RbP solution (10 mg/mL) was added and was hand shaken for 20 minutes.

All the preparations were stored in a refrigerator (4 – 8 °C) overnight prior to vesicle size reduction. The liposomal formulations were extruded three times through 800-nm polycarbonate membrane filter (Whatman® Nuclepore™, Whatman International LTD, England) followed by three times extrusion through 200-nm membrane using Avanti mini extruder.

2.3. Particle size analysis and zeta potential measurements

Measurements of particle diameter, polydispersity index and zeta potential were conducted using Zetasizer Nano-ZS (Malvern Instrument, UK). The mean diameter and polydispersity index were measured by dynamic light scattering, while for the zeta potential measurements laser doppler micro-electrophoresis was used. Prior to data collection, samples were diluted with an appropriate amount of water, which was previously filtered through 200 nm Minisart filters. Each sample was measured three times at 25 °C and the mean value ± standard deviation was calculated. Before the measurements were performed, all liposomal formulations were sonicated for approximately 15 s in a sonication bath (F5200b, Decon Laboratories Limited, UK).

2.4. Entrapment efficacy

In order to determine the entrapment efficacy of liposomes containing RbP, non-encapsulated drug was separated from lipid vesicles by size exclusion chromatography according to the minicolumn centrifugation procedure [22]. Spin columns were placed in standard 1.5 mL Eppendorf vial and filled with 700 µL of Sephadex G-50 previously hydrated with demineralised water. Minicolumns were centrifuged at 200 g for 1 minute in order to remove water from Sephadex bed. 100 µL of PBS was added on the top of the bed and centrifuged for 2 minutes at 200g. This step was repeated twice. Once Sephadex gel was dry, spin columns were transferred to new Eppendorf vial and 0.1 mL of each liposomal suspension was added to the top. Subsequently, the columns were spun again at 200g to separate liposomal formulation from the free drug [22]. The eluates with liposomes were kept for HPLC analysis. Prior to HPLC assay lipid bilayer was disrupted with methanol. Free drug was recovered by washing the column with water and eluting by centrifugation at 600g [23]. The percentage of entrapped RbP was calculated using the following equation:

$$EE\% = \frac{W_{total} - W_{free}}{W_{total}} \times 100,$$

where EE is the entrapment efficacy; W_{total} represents the amount of the total drug, and W_{free} is the amount of non-encapsulated drug. This method of EE determination is commonly used in the literature for determining drug entrapment efficiency into liposomes; however, it has some limitations and can overestimate the results.

2.5. Permeability measurements

2.5.1. Comparative study of Rb and RbP penetration into and permeability through the bovine cornea

In this study the penetration of Rb and RbP into and permeability through the cornea was compared using experiments with Franz diffusion cells. Rb and RbP solutions were prepared in PBS at 0.8 mg/mL. Dissected bovine corneas (n=6) were mounted between donor and receiver chamber. The receptor compartment was filled with PBS (16.5 mL), ensuring no air bubbles were trapped under the cornea, and 1 mL of Rb or RbP was added to the donor chamber, which was then covered with a cling film to prevent evaporation. All apparatus were placed in water bath at 34 °C to mimic the physiological temperature at the cornea. Aliquots from the donor compartment were taken for HPLC analysis at the beginning of each experiment and after 3 hours to determine the amount of drug that went into

and through the cornea. Results are expressed in % as the mass difference at the beginning and at the end of the experiment. Additionally, after 3 hours of the diffusion experiment the aliquots were taken from the receptor compartment and analyzed. In order to determine the drug penetration into the tissue, the exposed part of the cornea was cut after each experiment, weighed and placed in a vial with 3 mL mixture of ethanol : water = 1 : 1 (by volume). After three days, 1 mL of the corneal extract was filtered through 0.2 μm syringe filter and the HPLC analysis was performed. The calculation of the drug amount was performed using a calibration curve prepared with a series of riboflavin standard solutions. The results are expressed as micrograms of Rb/RbP per mass of the cornea in grams. The experiments were conducted in triplicates, and samples were covered with aluminum foil to exclude light-induced degradation.

2.5.2. *In vitro* penetration study of RbP encapsulated into the liposomes

Although Franz diffusion cells are considered to be industry standard for permeability tests *in vitro*, problems caused by corneal swelling were reported previously [1]. Therefore, for the permeation studies of liposomal samples, the "whole eye" method was used (Fig. 1s, Supplementary Information). Briefly, the bovine eye was placed in bottle lids with the corneas facing up. Then Franz diffusion donor chamber was added at the top of the bovine eye and everything was secured with a cling film and placed in a water bath at 34 °C. To study the permeation of riboflavin into the cornea, 0.5 mL of liposomal preparation or control solution (10 mg/mL of RbP) was added in the donor chamber. After 1.5 h, the exposed part of the cornea was cut, weighed and placed in a vial with 3 mL mixture of ethanol : water = 1:1 (by volume). After three days, 1 mL of the corneal extract was filtered through 0.2 μm syringe filter and HPLC analysis was performed. All the experiments were conducted in triplicates, and samples were protected from light with aluminum foil.

2.6. HPLC analysis

Concentrations of riboflavin-5'-monophosphate and riboflavin were determined by HPLC method according to the procedure reported by Morrison et al. [1]. Quantitation was achieved with run time of 10 minutes in the isocratic conditions using the mobile phase consisting of 20 % ethanol and 80 % ion pair buffer at 0.8 mL/min (30 °C). Stationary phase was Hamilton PRP-1 C_{18} column, 150 mm × 4.6mm, 5 μm (part number: 79425). The analyses were carried out using fluorescence detector with the excitation wavelength of 450 nm and emission wavelength of 523 nm. The injection volume was 0.1 μL, and data acquisition was done via Peaksimple (version 4.09, SRI Inc., USA). RbP peak was found to have the retention time of 1.8 minutes, whereas riboflavin peak appeared at 5.3 minutes. The method was validated presenting a linear calibration curve (for Rb: R^2=0.9998, y=1087.1x - 98.515; and for RbP: R^2 = 0.9953, y = 21347x – 35.852) over a drug concentration range of 0.00125 to 0.04 mg/mL for Rb and 0.00625 to 0.5 mg/mL for RbP, respectively.

2.7. Statistical analysis

Statistical analyses were performed using the statistical programming language *R* within the RStudio environment (version 0.98.1091, RStudio Inc., USA). Analyses of means of two groups were performed using the independent two-sample Student's t-test; group means were considered significantly different when $P < 0.05$, and annotated in figures with (*), while the tests which resulted in values of $P < 0.01$ were annotated as (**). When comparing three or more groups, one way between subjects ANOVA was first used to establish if statistically significant differences exist between group means. $P < 0.05$ was considered statistically significant, and further post-hoc analysis was performed using pairwise t-tests with the Bonferroni correction method to adjust for multiple comparisons problem: if a total of m pair-wise ad-hoc tests were performed, then $P < 0.05/m$ was required to consider the means of the two compared groups statistically significant [24].

3. Results and discussion

3.1. Comparative study of Rb and RbP penetration into the bovine cornea

The goal of this study was to develop a formulation which would achieve a desired concentration of riboflavin in the corneal stroma without the need for removal of the epithelium. The cornea represents the most significant barrier for absorption of drugs into the eye. The reasons for poor drug absorption by the cornea are two-fold: a small absorption surface area (~1 cm^2) and its complex multilayered structure [25]. The cornea is composed of five main layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane and the endothelium. Corneal drug absorption is influenced by drug characteristics such as lipophilicity, pKa value and molecular size [26]. Among these properties the octanol/buffer partition coefficient (logP), which represents the measure of lipophilicity, has been researched most extensively. It is reported that the preferred partition coefficient for corneal drug absorption is between 10 to 100, suggesting that lipophilic drugs are favored in corneal transport [27]. This is due to lipophilic nature of corneal epithelium, which represents a barrier to around 90 % of hydrophilic drugs [1]. Following ocular administration, the drugs with high partition coefficient will be quickly absorbed by the cornea. However, corneal permeability of molecules with low polarity is limited by the stroma, the hydrophilic inner layer of the cornea, and generally low amount of drug is expected to reach the aqueous humour of the eye [27]. Taking this into account, it could be expected that a less polar Rb (logP = -0.92 [28]) should have a higher penetration through the corneal epithelium compared to more hydrophilic RbP (logP = -1.20 [29]).

The penetration of both Rb and RbP into the bovine cornea was studied in this work using two methods of analysis. First, the total penetration was calculated based on the difference between the initial drug content in the donor compartment of Franz diffusion cell (FDC) at the beginning of each experiment and the amount left after three hours of its diffusion into the cornea. Second, the amount of riboflavin extracted from the cornea after completion of the FDC experiment was also evaluated. The amounts of RbP and Rb penetrated into the cornea within 3 hours are shown in Fig.2a. In contrast to our expectations, the total amount of RbP penetrated into the cornea was significantly higher compared to Rb ($p<0.05$, Student's t-test). After 180 minutes, 17.3 ± 0.8 μg of RbP was delivered into the cornea, and during the same time, the total permeation of Rb was 10.4 ± 4.2 μg. It should be

noted that HPLC analysis of the receiver compartment did not show any presence of Rb or RbP, indicating that there was practically no drug permeation through the cornea during this time and the entire drug penetrated into the cornea remains in this tissue.

Fig.2b shows the measurements of the amount of the drugs extracted from the cornea. The amount of Rb extracted from the treated corneas was 0.08 ± 0.05 μg compared to 0.05 ± 0.03 μg of RbP; there was no statistically significant difference between Rb and RbP (p>0.05). The levels of Rb and RbP absorbed by the corneas in this experiment were according to our expectations, since Rb is lipophilic and this resulted in easier penetration of the epithelium.

Possible explanation for the lower levels of Rb when measuring the total permeability could be the lack of correlation between partition coefficient and drug permeability rate as observed by Grass and Robinson [27]. It was observed that for the drugs with partition coefficient in the range of -1 to 0 the increase in logP value did not result in increase of corneal permeability. We assume that lower hydrophilicity of Rb would lead to its accumulation in the cornea. This assumption seems to be consistent with the work by Bottos *et al.* [8], which reported that Rb cannot be retained in the corneal stroma due to its lipophilic nature in contrast to its more hydrophilic counterpart - RbP. Furthermore, Prausnitz & Noonan [30] reported a comprehensive database of permeability measurements of the endothelium, stroma, epithelium, and other ocular tissues. Their study analysed more than 300 measurements from 40 different studies, and discussed the penetration of lipophilic and hydrophilic drugs through the eye. Even though the epithelium does represent a significantly stronger barrier for hydrophilic molecules, the study did not provide a definite conclusion on the impact of the endothelium on lipophilic molecules, while it did state that the endothelium does not present a significant barrier for hydrophilic compounds.

3.2. Conversion of RbP to Rb in the cornea

The analysis of RbP and Rb penetration into the cornea allowed us to establish a conversion of RbP into Rb mediated by enzymes present in the cornea. As shown in Fig.3**,** the typical HPLC chromatogram of RbP shows two peaks. The first peak was found to appear after 1.8 minutes (t_r=1.8 min) and it corresponds to RbP, while the second peak belongs to Rb, with the retention time of 5.3 minutes (t_r=5.3 min). This is in agreement with prior work by Lam and Lowande [32], where the composition of 70 % riboflavin-5'-phosphate sodium salt was shown to be a mixture of 10 % riboflavin and 60 % phosphate salt. It is well known that RbP is converted to Rb by acidic or alkaline phosphatases due to its structural similarity to the nucleosides [32]. In order to confirm that this is happening in the cornea during the permeability test, the RbP solution was mixed with the homogenized parts of the cornea and its concentration was measured after 2 hours using HPLC. In comparison to their initial values (Fig.4), the concentration of RbP was reduced by 75 %, while the concentration of Rb increased by 12 % (p<0.05, n=3).

To confirm the role of enzymes in RbP conversion into Rb, the measurements were also carried out with the homogenized cornea, which was autoclaved for 30 minutes at 121 °C to ensure that enzymes lost their activity.

Again, the RbP solution was added, and samples were taken for HPLC analysis after one hour. Fig.2s (Supplementary information) shows no increase in the concentration of Rb after 2 hours compared to time zero. This suggests that the increase in the content of Rb is caused by conversion of RbP mediated by corneal enzymes.

Additional control experiment was conducted, where RbP solution was left for two hours at 37 °C and room temperature. No significant differences in the concentrations of Rb or RbP were found after two hours compared to the initial values (data not shown). Therefore, we assume that conversion of RbP to Rb is mainly caused by the corneal enzymes. This finding is important for better understanding of the processes taking place in the cornea, as well as for RbP detection during drug permeation tests through the cornea.

3.3. Preparation and characterisation of liposomes with RbP

Liposomal formulations of riboflavin were previously studied in tumor targeting and were reported to achieve improvements in riboflavin's photostabilization [33, 34]. However, to the best of our knowledge, there is no report in the literature on how incorporation of RbP into liposomal carriers will affect the transcorneal permeation of this drug. The main reason to investigate the potential improvement of RbP permeability through the eye after encapsulation in the liposomes, instead of investigating Rb, was the clinical relevance since RbP is currently predominantly used in the treatment of keratoconus. Furthermore, RbP has higher aqueous solubility, which allowed us to prepare more concentrated solutions and to achieve higher total corneal permeability as observed in earlier parts of the present work.

While developing liposomes containing RbP, our first objective was to optimise their composition. It is well known that composition of liposomes influences their size, polydispersity index, and charge - parameters that affect the performance of liposomes as an ocular drug delivery system. Therefore, in this study different classes of liposomes were developed. Conventional liposomes were prepared using "film method" and propylene glycol-containing liposomes (PG) were formed using "polyol dilution method". Liposomal formulations were prepared using three different types of phospholipids at different concentrations but keeping the amount of RbP equal in all preparations. Since egg and soybean lecithin are natural phospholipids widely used in ocular drug delivery, liposomes in the present study were formed using these phospholipids [13]. The composition and results obtained from physicochemical analysis are summarised in Table 1.

3.3.1. Size and polydispersity index of non-extruded liposomes

Firstly, we measured the size of the particles of the original liposomal preparation, before the extrusion was undertaken. Data obtained from this analysis are in agreement with other research in this field, further supporting the statement that liposomes prepared with "conventional film method" are larger in diameter (between 2156 ± 345 and 6837 ± 1602 nm)[19]. On the other hand, liposomes prepared by "polyol dilution method" were smaller in size (between 496 ± 13 and 1838 ± 184 nm). One-way ANOVA reports statistically significant differences between group means (p<0.05). Statistically significant differences were observed for both FM-EPC-100 and PD-S100-300 in comparison with PD-S75-300 liposomes. No statistically significant difference was found between FM-EPC-100 and PD-S100-300. These results are in agreement with recent studies published by Elmoslemany *et al.* [35], who suggested that interactions between propylene glycol and phospholipids increase liposomal flexibility and decrease the size of the particles [30, 19].

Another parameter influencing the size of the vesicles is the lipid charge. According to the literature charged lipids produce smaller and less lamellar particles [36]. Indeed, ANOVA tests confirm statistically significant difference in sizes ($P < 0.05$) when liposomes were formed from S75, which contains negatively charged phospholipids (496 ± 13 and 540 ± 9 nm), compared to almost neutrally charged S100 (1139 ± 121 and 1838 ± 184 nm).

Furthermore, non-extruded liposomes showed higher values of polydispersity index which indicates that the system has a broad distribution of particle sizes. The polydispersity index is a measure of the size distribution and according to the literature, liposomal formulation is considered to be heterogeneous if the value of polydispersity index is ≥ 0.3 [37].

3.3.2. Size and polydispersity index of liposomes after the size reduction

Since the particle size distribution can influence the permeability of topically applied drugs in ocular drug delivery, all formulations were extruded through 200-nm membrane in order to obtain homogenous size distribution and smaller size (Fig.3s, Supplementary Information). Although the eye can tolerate particles of up to 10 μm, in order to achieve greater permeability, it is recommended to have particles smaller than 200 nm [38]. Extrusion resulted in the desired range of vesicles size for the purpose of developed liposomal delivery system. The mean diameter of extruded liposomes was between 130 and 200 nm, with a decrease in polydispersity index value to less than 0.22. These formulations were used for all further measurements. Our previous study [20] has established that even 27 – 69 nm silica nanoparticles coated with polyethyleneglycol do not penetrate the intact bovine cornea. Therefore, the penetration of 130-200 nm liposomes into the cornea is also likely to be hampered. However, the deformable nature of the liposomes could potentially facilitate their permeability.

3.3.3. Zeta potential measurements

Zeta potential measurements are frequently used to describe the stability of the colloidal system [39]. Large negative values of zeta potential (< -30 mV) are expected to result in greater physical stability of the liposomal system due to the repulsion between the charges of the same polarity. This parameter is especially important to consider during the storage of the formulation. Furthermore, charge on the surface might influence its interaction with cells and biological environment [40].

provides the results obtained from zeta potential measurements, which indicate good colloidal stability of the liposomes prepared in this work. Each liposomal formulation with RbP has negative values of zeta potential. It can be seen from the data that more negative values are observed when liposomes were prepared from S75 compared to S100 (ANOVA, $p<0.05$). This can be explained with the fact that both phospholipids S75 and S100 do not consist of pure phosphatidylcholine, but they are mixtures of lipids containing negatively charged phospholipids as well. As expected, when liposomes were formed from S100, which has higher content (more than 94 %) of neutrally charged phosphatidylcholine, low-negatively charged values of zeta potential were observed (-3mV). However, despite these low values of zeta-potential, these liposomes show excellent colloidal stability, which is confirmed by their small sizes (126±1 and 132±1 nm) and low values of polydispersity indices (0.168±0.008 and 0.173±0.007, respectively). It is believed that colloidal stability of these liposomes is due to the factors other than the surface charge. On the other hand, S75 consists of negatively charged phospholipids resulting in a sharp decrease of zeta potential values (-47.5 mV) [41].

3.4. Entrapment efficacy

Entrapment efficacy of the drug into the liposomes is usually influenced by several factors, including drug's partition coefficient, drug's molecular mass, method of their preparation and lipid composition [42]. Studies have shown that hydrophilic drugs have lower encapsulation in the liposomes compared to the encapsulation

efficacy of the lipophilic drugs [43], which is mainly due to the small volume of the inner aqueous core. Since the RbP has a logP value of -1.20, we expected low encapsulation levels.

In order to determine the amount of drug encapsulated in the liposomes, the mini-column centrifugation method was used [22]. This method is reported to be advantageous over other methods used for separation of free drug from the liposomes since there is no dilution of liposomal formulation, numerous samples can be analyzed at the same time, and, finally, free drug can be easily recovered in a small volume of water [23]. Fig.4 shows that the encapsulation of RbP was found to be very low for the liposomes prepared by classical "film method". The entrapment efficacy of only 3.5 to 9.6 % is most likely due to aforementioned hydrophilic nature of the drug. On the other hand, the entrapment of 19.9 to 41.8 % of RbP occurred when liposomes were prepared by the "polyol dilution method". These results corroborate the study of Pavelic et al. (2001), which suggested that high entrapment efficacy of hydrophilic drugs using polyol dilution method is a consequence of the first steps of the preparation procedure, where a lipid-polyol solution is exposed to the highly concentrated drug solution [44]. Among the liposomes prepared with polyol dilution method, entrapment efficacy was found to be higher when higher amount of lipid was used.

3.5. *In vitro* penetration of RbP encapsulated into the liposomes

In the current study we compared the *in vitro* penetration of RbP encapsulated into the liposomes into the bovine cornea with 10 mg/mL aqueous solution of the drug. Since the treatment of keratoconus requires the delivery of riboflavin-5'-monophosphate into the corneal stroma, permeability measurements in this study were focused on the penetration into the cornea, as opposed to measuring permeability through the cornea. Hence, after the penetration test was performed using the "whole eye method" [1], the exposed part of the cornea was cut, measured and left in a mixture of ethanol and water in order to extract the absorbed drug from the cornea.

With the same amount of drug per dose, none of the formulations showed statistically significant improvement in ocular penetration of RbP compared to the levels observed in the corneas treated with control solution. Among the used liposomal preparations, the highest corneal drug absorption was observed for the liposomes that were prepared with the conventional film method. Although Fig.5 shows that there was a slight increase in the amount of RbP absorbed by corneas in some measurements for the formulation FM-S75-400, T-test reveals that there is no significant difference compared to the control values in the case of conventional liposomes (ANOVA, $p>0.05$). On the other hand, three liposomal formulations prepared with the "polyol dilution method" showed a significantly lower permeation than the control formulation (ANOVA, $p<0.05$). In reviewing the literature, similar data was observed in the case of hydrophilic drugs [34, 35]. Singh and Mezei [7, 34] reported that dihydrostreptomycin sulfate encapsulated in liposomes did not show any improvement in its corneal permeation. The authors assumed that this might be explained by the mechanism of interactions between liposomes and surface of the eye. There are four possible ways for liposomes to interact with the cells: adsorption, fusion, lipid exchange and endocytosis. The authors of the aforementioned study suggested that the main interaction between the liposomes

and the surface of the eye comes from adsorption and lipid exchange, which seems not to be suitable for hydrophilic drugs because of its rapid escape in the surrounding solution. Therefore, the authors emphasised the importance of the characteristics of the encapsulated drug to the overall performance of liposomes as drug carriers [45].

Data presented in this work further support the assumption that physicochemical characteristics of encapsulated drug play an important role in the transport of the drug through the eye. Corneal absorption of the drug entrapped in the liposomes depends on its location within the liposomes, and this seems to be particularly disadvantageous for hydrophilic molecules which are located in the aqueous core of the liposome [45]. The liposomes prepared using conventional “film method” have shown significantly higher potential to deliver RbP into the cornea compared to the liposomes prepared using “polyol dilution method”. However, these liposomes did not provide any permeability enhancement compared to the aqueous solution of the drug.

4. Conclusions

This study was focused on the development of novel formulations of riboflavin and riboflavin-5'-phosphate for their efficient topical delivery to the eye to facilitate corneal cross-linking in the treatment of keratoconus. The permeability of these drugs into and through freshly excised bovine cornea was studied in vitro and it was found that drug diffusion is very slow, confirming the need for advanced formulations with enhanced drug permeability. It was established that riboflavin-5'-monophosphate is converted to riboflavin in the cornea and this process is mediated with enzymes present in this biological tissue. This finding has significant implications for detection of riboflavin-monophosphate in the permeability measurements and understanding of the processes and behavior of riboflavin-5'-monophosphate in the cornea.

Six different liposomal formulations of riboflavin-5'-monophosphate were developed in this work and studied for their ability to facilitate drug permeability into the cornea. However, there was no statistically significant improvement in the penetration of riboflavin-5'-monophosphate into the cornea when the drug is encapsulated in the liposomes. These results might be explained by the hydrophilic nature of riboflavin-5'-monophosphate and are in agreement with several previous studies using other hydrophilic drugs.

Conflict of interest

The authors declare no conflict of interests. No animal or human studies were carried out by the authors for this article

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Legends to figures

Fig. 1. Schematic illustration of corneal cross-linking (CXL) treatment of keratoconus: (a) intact cornea; (b) cornea with physically removed epithelium; (c) topical application of RbP on de-epithelialized cornea; (d) irradiation of cornea saturated with RbP by UVA; (e) additionally cross-linked cornea with improved mechanical properties.

Fig. 2 (A) Total drug penetrated into the cornea calculated from the difference in the concentration in FDC donor chamber before and after three hours exposure to bovine cornea. *p<0.05, Student's t-test, n=3. **(B)** Amount of Rb and RbP extracted from the cornea after three hours of in vitro penetration experiment. p>0.05, Student's t-test, n=3.

Fig. 3. Overlaid HPLC chromatograms for RbP from the experiment with the homogenized cornea. The typical chromatogram for RbP shows two peaks. The RbP peak appeared after 1.8 min and the Rb peak was found to have retention time of 5.3 min. The red curve represents the measurement at time zero. The blue curve shows the change in concentrations after 2 hours exposure to homogenized cornea at 37 °C. Insert: Scheme of RbP conversion into Rb

Fig. 4. Entrapment efficacy of RbP in various liposomal formulations. FM, film method; PD, polyol dilution method; EPC, egg phosphatidylcholine; S75, soybean lecithin with 70 % phosphatidylcholine; S100, soybean lecithin, >94 % phosphatidylcholine. *p<0.05, **p<0.01, One way ANOVA followed by a Bonferroni post hoc test, n=3.

Fig. 5. Amount of RbP extracted from the bovine corneas treated with different liposomal formulations compared to RbP aqueous solution at 10mg/mL. *p<0.05, Student's t-test, n=3. FM, film method; PD, polyol dilution method; EPC, egg phosphatidylcholine

Supplementary Information

Delivery of riboflavin-5'-monophosphate into the cornea:

can liposomes provide any enhancement effects?

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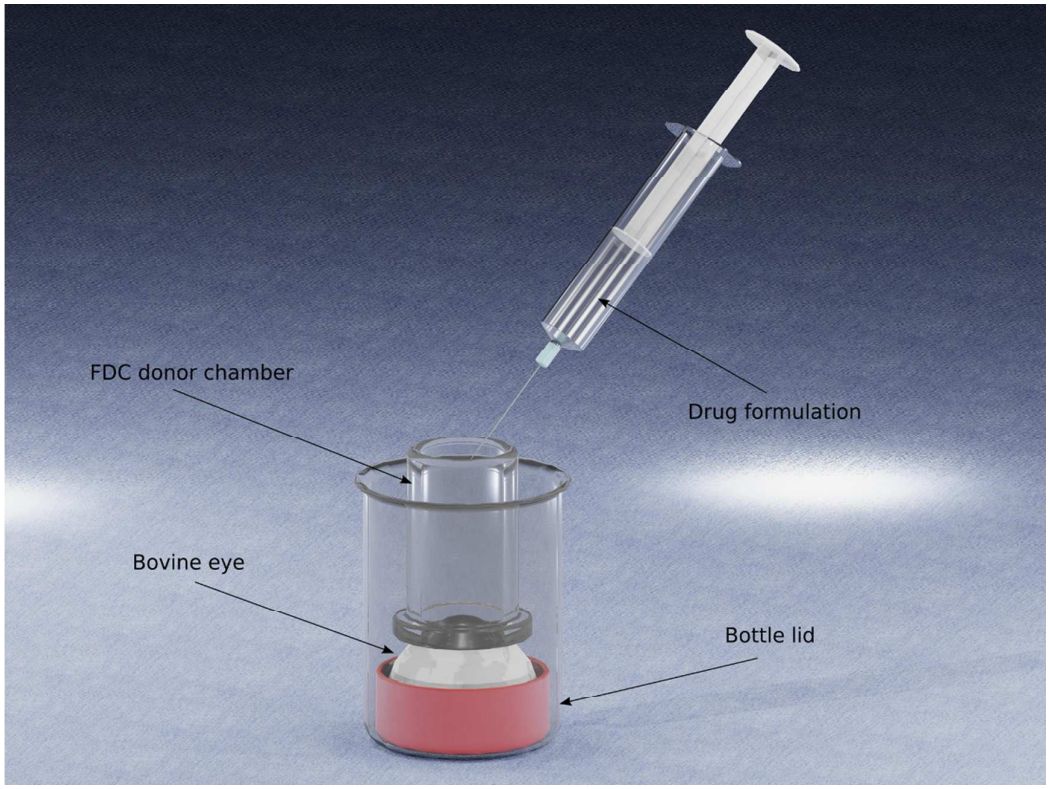

Fig 1s. Whole eye method for studying drug in vitro penetration.

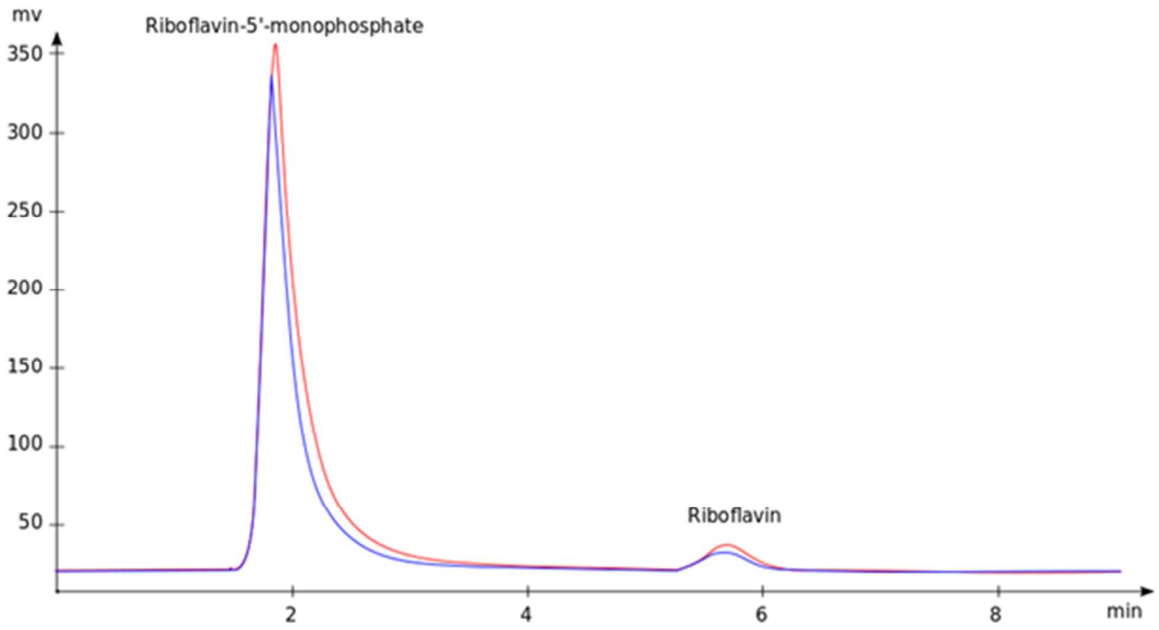

Fig. 2s Overlaid HPLC chromatograms for RbP from the experiment with autoclaved homogenized cornea and schematic representation of the conversion of RbP into Rb. The chromatogram for RbP shows two peaks. The RbP peak appeared after 1.8 min and the Rb peak was found to have retention time of 5.3 min. The red curve represents measurement at time zero. The blue curve shows the change in concentrations after 2 hours exposure to homogenized cornea previously autoclaved.

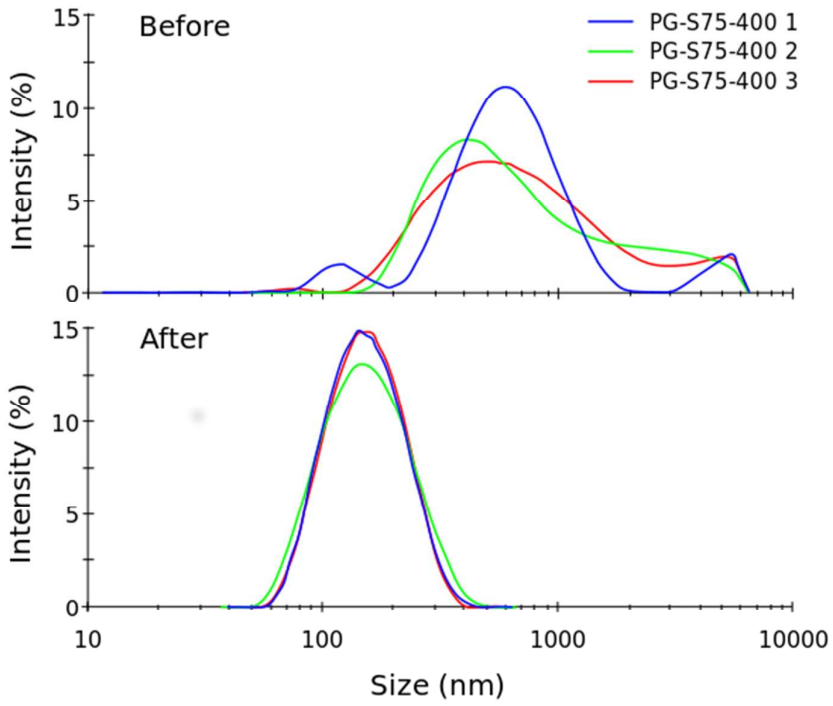

Fig. 3s Dynamic light scattering size distributions of liposomal preparation (PG-S75-400) before and after the size reduction performed by extrusion through 200-nm membrane

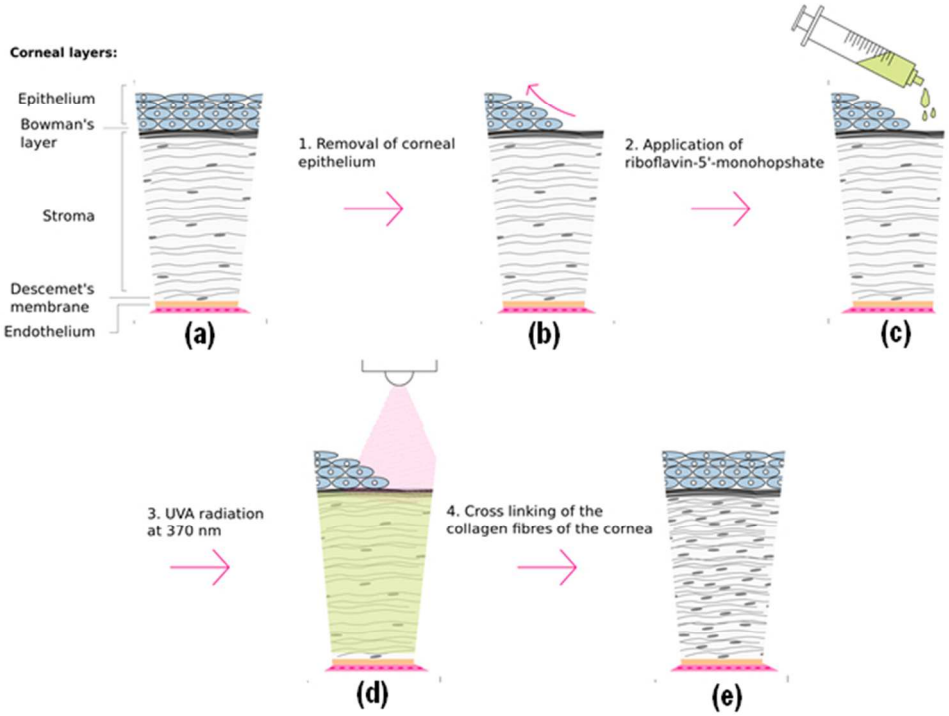

Fig. 1. Schematic illustration of corneal cross-linking (CXL) treatment of keratoconus: (a) intact cornea; (b) cornea with physically removed epithelium; (c) topical application of RbP on de-epithelialized cornea; (d) irradiation of cornea saturated with RbP by UVA; (e) additionally cross-linked cornea with improved mechanical properties.

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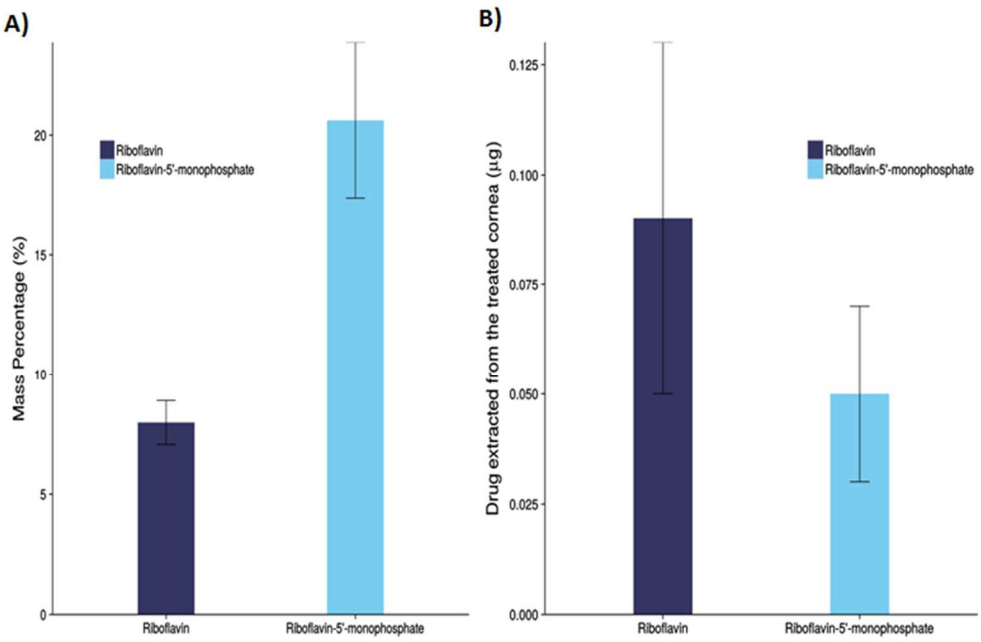

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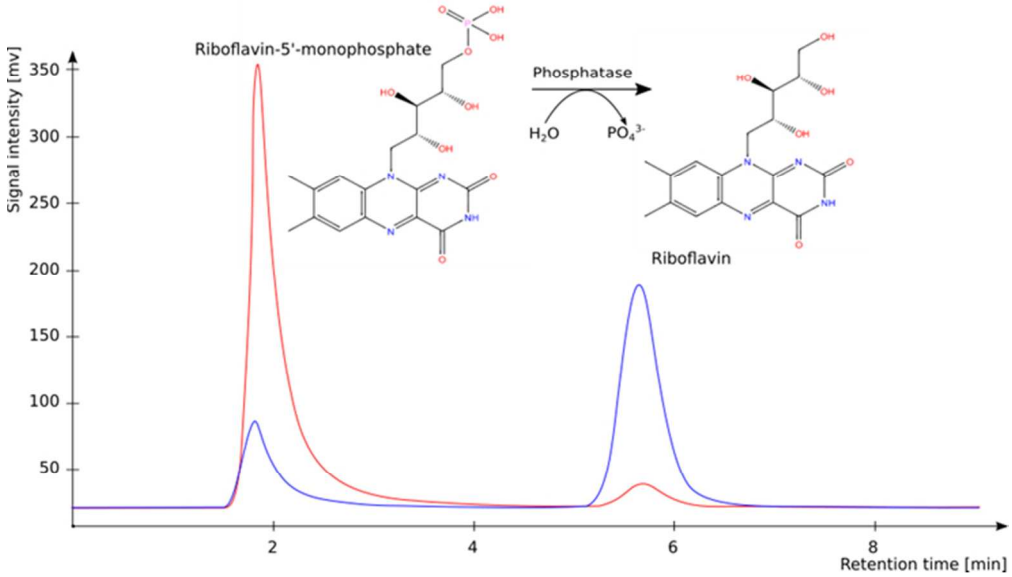

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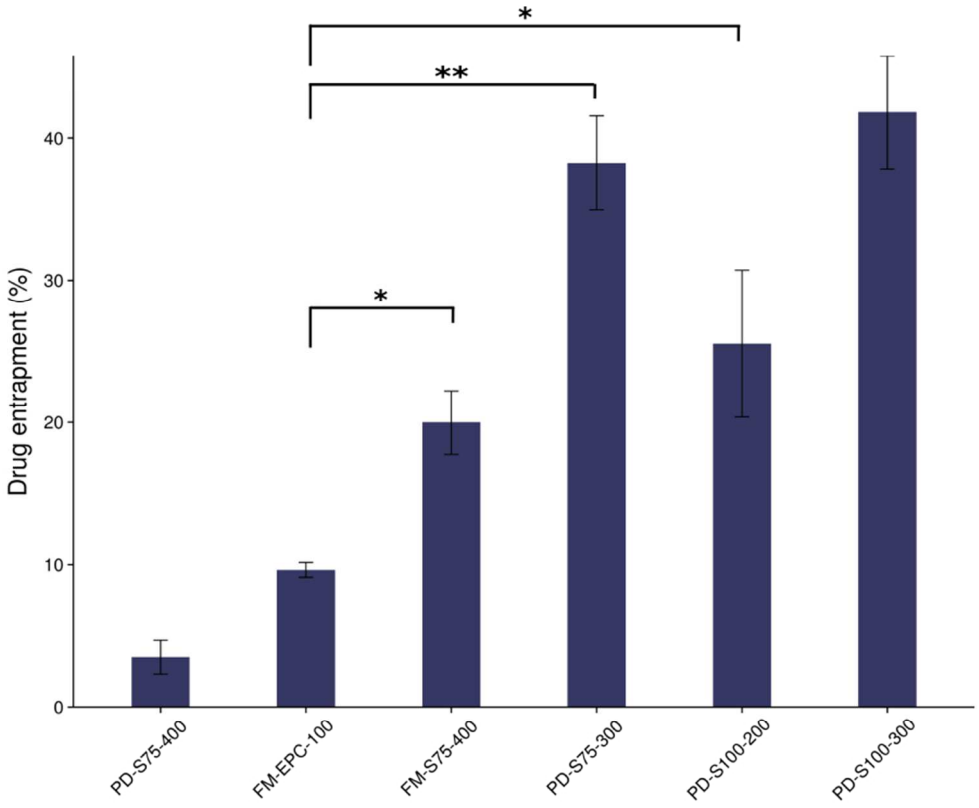

Fig. 4. Entrapment efficacy of RbP in various liposomal formulations. FM, film method; PD, polyol dilution method; EPC, egg phosphatidylcholine; S75, soybean lecithin with 70 % phosphatidylcholine; S100, soybean lecithin, >94 % phosphatidylcholine. *p<0.05, **p<0.01, One way ANOVA followed by a Bonferroni post hoc test, n=3.

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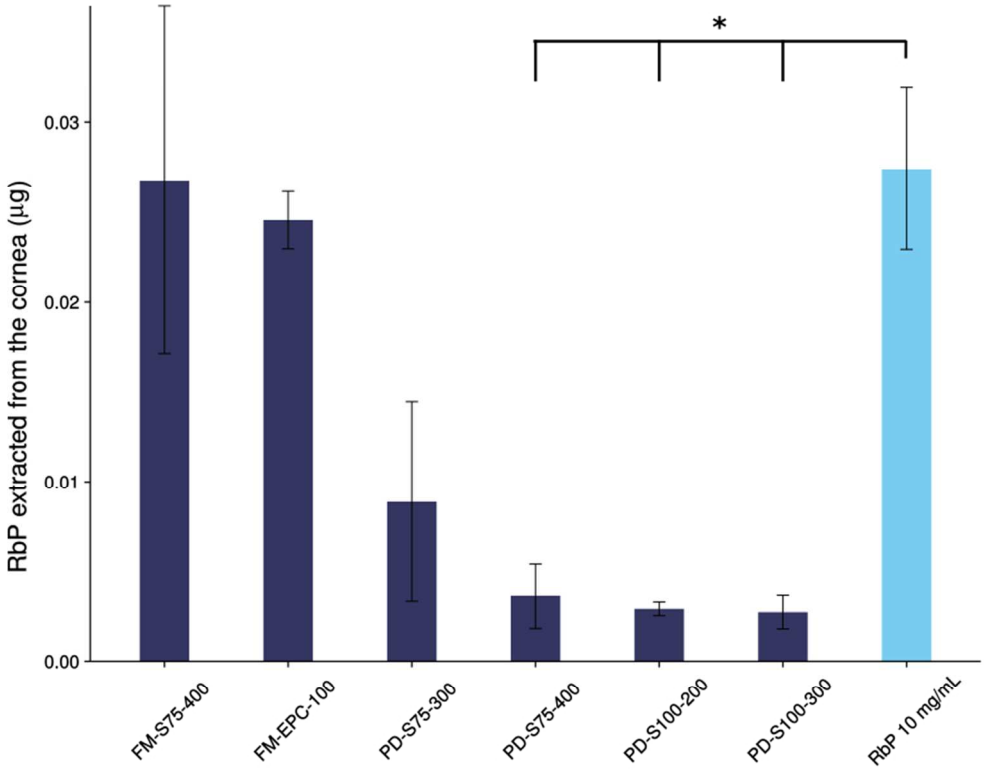

Fig. 5. Amount of RbP extracted from the bovine corneas treated with different liposomal formulations compared to RbP aqueous solution at 10mg/mL. *p<0.05, Student's t-test, n=3. FM, film method; PD, polyol dilution method; EPC, egg phosphatidylcholine

254x203mm (90 x 90 DPI)

Table 1. Composition and physicochemical properties of different liposomal formulations containing RbP. The total volume of liposomal suspensions was kept the same (5mL) in all preparations.

Sample Code	S100	S75	EPC	PG	RbP	Mean Diameter (nm)		Polydispersity Index		Zeta Potential
	(mg)	(mg)	(mg)	(g)	(mg)	Before extr.	After extr.	Before extr.	After extr.	(mV)
FM-S75-400	0	400	0	0	10	6836 ± 1602	189 ± 1	0.399±0.129	0.217±0.005	-47.5 ± 1.5
FM-EPC-100	0	0	100	0	10	2156 ± 345	187 ± 1	0.686±0.389	0.170±0.009	-15.1 ± 0.4
PD-S75-300	0	300	0	1	10	540 ± 9	142 ± 1	0.371±0.015	0.121±0.003	-24.8 ± 0.5
PD-S75-400	0	400	0	1	10	496 ± 13	142 ± 1	0.388±0.033	0.120±0.012	-26.2 ± 0.6
PD-S100-200	200	0	0	1	10	1838 ± 184	126 ± 1	1.000±0.000	0.167±0.008	-3.4 ± 0.3
PD-S100-300	300	0	0	1	10	1139 ± 121	132 ± 1	0.171±0.096	0.173±0.007	-3.6 ± 0.3

PDI, polydispersity index; FM, film method; PD, polyol dilution method; EPC, egg phosphatidylcholine; S75, soybean lecithin with 70 % phosphatidylcholine; S100, soybean lecithin, >94 % phosphatidylcholine.

Values shown are the mean ± standard deviation of three repeats. Note that some of the formulations have very high polydispersity index, which means that they are highly polydisperse and their real sizes could be different from the data determined using dynamic light scattering.