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Tacrolimus phospholipid based nanomicelles as a potential local delivery system for corneal neovascularization therapy

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Abstract

Introduction:

Tacrolimus, an immunosuppressive agent, has been shown to be an effective treatment against corneal neovascularization (CNV). However, the poor solubility of this compound restricts its clinical application. The goal of this study was to incorporate tacrolimus into phospholipid-bile salt mixed micelles.

Methods and Results:

Tacrolimus loaded phospholipid-bile salt mixed micelles were prepared, employing three different methods of direct dispersion, thin film hydration, and remote film loading, and the effects of various formulation parameters (type of dispersion medium, phospholipid to bile salt molar ratio, lipid-to-drug (L/D) molar ratio, time of probe sonication, and type of bile salt) on the physicochemical characteristics of the mixed micelles were assessed. Remote film loading method indicated higher efficacy for drug entrapment in comparison to the other methods. Encapsulation of tacrolimus within the micelles increased remarkably by the use of sodium taurocholate (NaTC) as bile salt, higher phospholipid percentage, and increasing the total lipid level. Atomic force microscopy (AFM) studies confirmed the size and size distribution of the mixed micelles and their spherical morphology. It was observed that release of tacrolimus from the micelles was in a controlled manner, without an initial burst.

Conclusion:

By adjusting process and formulation factors, phospholipid-bile salt mixed micelles with high entrapment efficiency of (99.5%) and controlled release behavior were achieved, which possess great potential to be valuable carriers for ocular delivery of tacrolimus for the treatment of CNV.

Keywords: Mixed micelles, Phosphatidylcholine, Bile salt, Tacrolimus, Corneal neovascularization

1. Introduction

The cornea of the eye is avascular, clear, and transparent for light to pass into the retina. Under pathological conditions, such as trauma, inflammation, infection, and hypoxia, angiogenic factors, such as vascular endothelial growth factor (VEGF), bind to their receptor and result in an invasion of vessels from the limbal arcade to normally avascular cornea, leading to corneal neovascularization (CNV) [1-3]. CNV is a leading

cause of irreversible visual loss in a number of eye diseases, such as proliferative diabetic retinopathy, corneal graft rejection, age-related macular degeneration, retinopathy of prematurity, and traumatic corneal injury [2, 4]. Treatments for CNV, including topical corticosteroids, methotrexate, low-molecular-weight heparin sulfate, topical bevacizumab, nonsteroidal anti-inflammatory agents, and laser photocoagulation have met limited success due to the poor understanding of CNV pathogenesis and low drug ocular bioavailability [5, 6].

Tacrolimus is a macrolide antibiotic that has been successfully used to prevent allograft rejection in kidney, liver, lung, and heart transplantation. Recently, systemic and topical tacrolimus administrations have been found to be efficacious for CNV inhibition [7, 8].

However, drug bioavailability to the cornea is always challenging due to continuous clearance by the tear fluid. Furthermore, ocular barriers lead to poor drug permeation. Therefore, intravitreal injection and/or frequent use of eye drops throughout the day may be used, which are accompanied by various problems, such as cataract, endophthalmitis, vitreous hemorrhage, and blurred vision [9]. On the other hand, promising beneficial activity of tacrolimus has been limited mainly due to its poor water solubility. Nanoparticulate delivery systems have been successfully applied to improve ocular penetration, to sustain drug release, and to increase drug ocular residence time [10, 11].

Among various nanoparticles, micelles may be particularly useful for ocular delivery of poorly soluble drugs due to their unique combination of excellent properties, such as the solubilizing ability for lipophilic compounds, controlled release properties, fine particle size, biocompatibility, and enhanced permeability [12-14]. Mixed phospholipid-bile salt nanomicelles have attracted great attention in recent years due to their solubilizing capacity and physiological biocompatibility [9, 15-17] and could also be good candidates for ocular delivery of tacrolimus.

Therefore, the aim of the present study was to incorporate tacrolimus into phospholipid-bile salt mixed micelles. The effects of various parameters, such as preparation method, phospholipid percentage, lipid-to-drug (L/D) molar ratio, and type of bile salt on the physicochemical properties were then studied.

2. Materials and Methods

2.1. Materials

Tacrolimus was kindly donated by Aburaihan Pharmaceutical Co. (Iran). Purified egg phosphatidylcholine (EPC) was purchased from Lipoid GmbH (Germany), and sodium cholate (NaC), sodium deoxycholate (NaDC), and sodium taurocholate (NaTC) were all obtained from Sigma-Aldrich (Germany). Ethanol, HPLC-grade acetonitrile and methanol were supplied by Merck (Germany). Cellulose dialysis bag (molecular weight cutoff 12,000 Da) was procured from Sigma-Aldrich (Germany). All other chemicals were of analytical grade or of the highest purity available.

2.2. Micelle preparation

To develop mixed micellar solutions, three methods of direct dispersion, thin film hydration, and remote film loading were investigated.

2.2.1. Direct dispersion method

For the preparation of micelles using direct dispersion method [18], EPC, NaC (40:60, molar ratio), and tacrolimus with a L/D molar ratio of 10:1, were dispersed together in phosphate buffer (PB; 0.067 M; pH 7.4) at 37 °C for 24 h by use of a thermostated magnetic stirrer.

2.2.2. Thin film hydration method

In thin film hydration method [19], EPC, NaC (40:60, molar ratio), and drug with a L/D molar ratio of 10:1 were dissolved in methanol in a round-bottomed flask. The solution was subsequently evaporated under reduced pressure using a rotary evaporator (Heidolph, Germany) at 65 °C for 30 min to yield a thin film on the wall of the flask. Evaporation was continued for at least 2 h after the dry residue appeared, to remove all

traces of the organic solvent. Micelles were prepared by a vigorous vortexing of the film with PB (0.067 M; pH 7.4), which was followed by sonication (Powersonic 405, Hwashin Technology Co., Korea) at 30 °C for 10 min.

2.2.3. Remote film loading method

Remote film loading method involves the development of drug film and its subsequent addition to empty micelles.

In order to prepare empty micelles, a mixture of EPC and bile salt (NaC, NaDC, or NaTC)

in desired molar ratio was dispersed in an aqueous medium at 60 °C for 1 h and then at 37 °C for 47 h by use of a thermostated magnetic stirrer.

After that, methanolic solutions of tacrolimus were prepared and then evaporated under reduced pressure using the rotary evaporator to develop a thin-layer film. To prepare loaded micelles, the empty micelles were added to the dried drug film and the mixture was then sonicated for 30 min at 30 °C [20].

2.3. Micelle characterization

The prepared micelles were characterized in terms of particle size and size distribution, zeta potential, morphology, entrapment efficiency, *in-vitro* release profile, and stability.

2.3.1. Size distribution and zeta potential

Size, population distribution, and zeta potential of the micelles were determined by dynamic light scattering measurements carried out on a Malvern Zetasizer Nano (Malvern Instruments Ltd, UK). The analysis was conducted at 25 °C after the samples had been diluted with deionized water. The measurements were performed in triplicate and the results were presented as the mean ± standard deviation (SD).

2.3.2. Morphology

The morphology of the micelles was visualized via atomic force microscopy (AFM). For this purpose, the micelles were diluted with deionized water and an aliquot (20 µL) of the diluted micelles was immediately mounted onto the freshly cleaved mica surface, spread thin, and then dried at ambient temperature (25 °C). Contact mode was carried out, employing a Nanowizard II atomic force microscope (JPK Instruments, Germany) through the use of low-stress silicon nitride cantilevers (AppNano, USA).

2.3.3. Entrapment efficiency

To determine tacrolimus content in the micelles, the non-incorporated drug was separated from the drug encapsulated in micelles using centrifugation at 14,000 rpm for 15 min. Afterwards, small aliquots of the micelles (50 µL) were lysed with methanol (950 µL), bath-sonicated until micelles disruption, and then analyzed for tacrolimus content by HPLC. The percent of entrapment efficiency (% EE) was calculated from the amount of entrapped tacrolimus divided by the total amount of drug used initially for preparation multiplied by 100.

2.3.4. In-vitro drug release

The *in-vitro* release of tacrolimus from the micelles was studied through cellulose dialysis bag. Tacrolimus-loaded micelles (0.5 mL) were transferred into the bag and dialyzed against releasing medium (PB (0.067 M; pH 7.4) containing 30% ethanol for achieving sink condition) under 100 rpm magnetic stirring, at 37 °C. At predetermined intervals (1, 2, 3, 4, 6, and 8 h), 1 mL aliquots of the release media were withdrawn and replenished with the same volume of fresh media to maintain sink condition. Each experiment was performed in triplicate and the collected samples were analyzed by HPLC.

2.3.5. Drug analysis

The amount of tacrolimus in micelles and in release medium was quantified through a validated HPLC method. The HPLC system (Knauer, Germany) employed composed of a WellChrom K-1001 solvent delivery pump with an online degasser, a column oven, and a K-2600 ultraviolet (UV) detector. Chromgate software (Version 317) was used to record and process the data obtained during chromatographic analysis. The separation was carried out through a PerfectSil ® C18 column, 150×4.6 mm, 3.5 µm particle size (MZ-Analysentechnik GmbH, Germany), with a mobile phase of water and acetonitrile (20:80,

v/v) at a flow rate of 1 mL/min. The eluent was monitored via UV detection at a wavelength of 210 nm. The injection volume was 100 µL and the column was maintained at 45 °C. Prior to the analysis, method validation was conducted in accordance with the International Conference on Harmonization (ICH) guidelines in terms of selectivity, accuracy, precision, linearity, limit of detection (LOD), and limit of quantification (LOQ) [21].

2.3.6. Stability study

The stability of the micelles was assessed at 4 °C for a period of 1 month. At specified time intervals, aliquots of samples were taken out, and the characteristics of EE (%), particle size, and its distribution were investigated.

2.4. Statistical analysis

All statistical analyses were conducted using SPSS version 17 (SPSS Inc., USA). The data were presented as the mean ± SD. The comparison of the formulations was performed through analysis of variance; p-value < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Preparation of micelles

Micelles can be developed using numerous preparation methods. The choice of an appropriate method depends on the physicochemical characteristics of each cargo. For this purpose, preliminary studies were performed, utilizing three methods of direct dispersion, thin film hydration, and remote film loading. A series of formulations were developed by the first two methods, altering type of dispersion medium (distilled water, normal saline (NaCl 0.9% w/v), phosphate buffered saline (PBS; pH 7.4), and PB (0.067 M, pH 7.4)) and time of hydration, while, keeping other parameters invariant. As shown in Table 1, both methods were not very effective, having large particle sizes, with average diameters in the range of about 150 to 300 nm, and broad size distribution. Remote film loading method clearly gave micelles with smaller particle sizes. In the next step, to optimize the size of the micelles prepared by remote film loading method, two methods of high frequency probe sonication at an amplitude of 150 W and bath sonication after hydration were investigated. Bath sonication after hydration gave significantly smaller size (93.4 ± 16.3 nm); however, with a highly disperse size distribution (PDI = 0.96 ± 5.06), which is considered not to be suitable for ocular delivery. Using probe sonication after hydration yielded micelles with a particle size of 88.3 ± 10.6 nm, narrower size distribution (PDI = 0.15 ± 0.00), and higher EE (%). Thus, remote film loading followed by probe sonication after hydration was utilized for tacrolimus micelle preparation.

Table 1 Effect of preparation method on particle size and % EE of tacrolimus-loaded micelles; data are represented as mean ± SD (n = 3).

Formulation	Preparation method	Size (nm)	EE (%)
F1	Direct dispersion	179.9 ± 6.1	10.3 ± 3.0
F2	Thin film hydration	301.3 ± 6.6	32.7 ± 4.3
F3	Remote film loading	93.4 ± 16.3	43.0 ± 6.6

3.2. Factors affecting physicochemical properties of micelles

To identify a micellar formulation with appropriate physicochemical properties, various factors were assessed and optimized, including the type of dispersion medium, EPC to bile salt molar ratio, L/D molar ratio, time of probe sonication, and type of bile salt surfactant.

3.2.1. Type of dispersion medium

For studying the effect of dispersion medium, four different mediums of distilled water, normal saline (NaCl 0.9% w/v), PBS (pH 7.4), and PB (0.067 M, pH 7.4) were investigated for the preparation of NaC-containing micelles with fixed molar ratios of EPC/NaC (40:60) and L/D (10:1) (Table 2, formulations F4 to F7). As presented in Table 2, of the different types of medium employed, the distilled water and normal saline resulted in micelles with

broad size distributions (PDI > 0.3), while, using PBS (pH 7.4) and PB (0.067 M, pH 7.4) both yielded micelles with significantly narrower size distributions (PDI ~ 0.1). However, PB caused a substantial and significant increase of EE (%), so it was selected as the best dispersion medium and used in formulations F7 to F23 to evaluate other formulation parameters.

Table 2 Effect of bile salt type, bile salt to phosphatidylcholine molar ratio, L/D ratio, medium type, and sonication time on particle size, size distribution, and % EE of tacrolimus-loaded NaC-containing micelles prepared using remote film loading method; data are represented as mean ± SD (n = 3).

Formulation	Molar ratios		L/D (molar ratio)	Medium type	Sonication time (min)	Size (nm)	PDI	EE (%)
	Bile salt surfactant	Phosphatidylcholine						
F4	60	40	10	Distilled water	10	143.4 ± 9.1	0.31 ± 0.04	-
F5	60	40	10	Normal saline	10	83.8 ± 10.6	0.41 ± 0.01	-
F6	60	40	10	PBS	10	84.0 ± 4.5	0.13 ± 0.00	12.3 ± 1.6
F7	60	40	10	PB	10	77.0 ± 5.0	0.11 ± 0.05	50.6 ± 3.1
F8	70	30	10	PB	10	-	-	51.7 ± 1.5
F9	30	70	10	PB	10	-	-	64.6 ± 3.5
F10	30	70	20	PB	10	-	-	72.0 ± 3.2
F11	30	70	20	PB	20	88.5 ± 3.6	0.42 ± 0.03	77.9 ± 0.3

3.2.2. EPC to bile salt molar ratio

Regarding the results, the EPC to bile salt molar ratio was found to have a considerable effect on the drug entrapment efficiency. Altering the EPC to bile salt molar ratio from (30:70) to (70:30), while keeping other parameters invariant, improved the EE (%) from 51.7 ± 1.5 to 64.6 ± 3.5 for NaC- (Table 2), from 53.3 ± 0.8 to 60.6 ± 1.1 for NaDC- (Table 3), and from 55.3 ± 1.7 to 63.4 ± 0.6 for NaTC- (Table 4) containing micelles, which all were statistically significant ($P < 0.001$).

Table 3 Effect of bile salt surfactant type, bile salt surfactant to phosphatidylcholine molar ratio, L/D ratio, medium type, and sonication time on particle size, size distribution, and % EE of tacrolimus-loaded NaDC-containing micelles prepared using remote film loading method; data are represented as mean ± SD (n = 3).

Formulation	Molar ratios		L/D (molar ratio)	Medium type	Sonication time (min)	Size (nm)	PDI	EE (%)
	Bile salt surfactant	Phosphatidylcholine						
F12	70	30	10	PB	10	-	-	53.3 ± 0.8
F13	30	70	10	PB	10	-	-	60.6 ± 1.1
F14	30	70	20	PB	10	-	-	76.9 ± 1.6
F15	30	70	20	PB	20	59.3 ± 4.8	0.15 ± 0.04	79.7 ± 3.7

3.2.3. L/D molar ratio

By keeping a constant EPC to bile salt molar ratio (70:30), the micelles with two L/D molar ratios (10:1 and 20:1) were prepared using all three different bile salts and investigated for tacrolimus entrapment. The results indicate that the increase of L/D molar ratio, from (10:1) to (20:1), gave rise to about 7.4 (Table 2), 16.3 (Table 3), and 22.0% (Table 4) enhancement of tacrolimus entrapment for micelles composed of NaC ($P < 0.05$), NaDC ($P < 0.01$), and NaTC ($P < 0.001$), respectively.

Table 4 Effect of bile salt surfactant type, bile salt surfactant to phosphatidylcholine molar ratio, L/D ratio, medium type, and sonication time on particle size, size distribution, and % EE of tacrolimus-loaded NaTC-containing micelles prepared using remote film loading method; data are represented as mean ± SD (n = 3).

Formulation	Molar ratios		L/D (molar ratio)	Medium type	Sonication time (min)	Size (nm)	PDI	EE (%)
	Bile salt surfactant	Phosphatidylcholine						
F16	70	30	10	PB	10	-	-	55.3 ± 1.7
F17	70	30	10	PB	20	-	-	21.8 ± 2.7
F18	30	70	10	PB	10	-	-	63.4 ± 0.6
F19	30	70	10	PB	20	-	-	39.3 ± 1.6
F20	70	30	20	PB	10	-	-	19.7 ± 0.6
F21	70	30	20	PB	20	-	-	31.8 ± 0.8
F22	30	70	20	PB	10	-	-	85.4 ± 1.8
F23	30	70	20	PB	20	84.1 ± 1.6	0.02 ± 0.03	99.5 ± 2.2

3.2.4. Time of sonication

For investigating the effect of sonication time on EE (%), NaTC-micelles with two levels for molar ratios of EPC to bile salt (30:70 and 70:30) and L/D (10:1 and 20:1) were prepared at two sonication times of 10 and 20 min. Regarding the results from Table 4, increasing the time of sonication from 10 to 20 min at L/D molar ratio of (10:1) led to reduction of EE (%) for micelles with both, low, and high EPC to bile salt molar ratios, whereas, at L/D molar ratio of (20:1) their EE (%) increased with increasing of time.

3.2.5. Type of bile salt

Bile salts, one of the major components of mixed micelle, can play a key role in the micelle properties. Thus, to assess the effects of bile salt structure on EE (%), a number of micellar formulations, containing NaC, NaDC, or NaTC with fixed molar ratios of EPC to bile salt (70:30) and L/D (20:1) were prepared by 20 min sonication. The results revealed that of the different types of bile salt used, NaTC yielded micelles with considerably narrower size distribution (PDI = 0.02 ± 0.03), and higher tacrolimus entrapment (99.5 ± 2.2%) (Table 4, F23).

3.3. Morphology

The surface morphology of the optimized micelles, consisting of NaTC and EPC (30:70) with L/D molar ratio of (20:1) prepared by remote film loading method followed by probe sonication for 20 min was represented in Figure. 1. The prepared micelles were uniform in size with an average particle size of about 90 nm and spherical in shape.

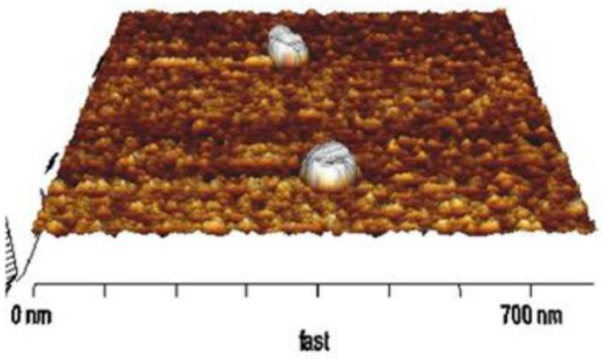

Figure. 1. AFM micrographs of the optimized formulation.

3.4. In-vitro drug release

The release profile of tacrolimus from the optimized micelles in PB (0.067 M; pH 7.4) containing 30% ethanol is depicted in Figure. 2. No significant burst effect was observed on the profile of the formulation, and it showed that 74.1 ± 8.6% of the drug was released within 8 h, indicating the usefulness of the proposed micellar delivery system for ocular delivery of tacrolimus.

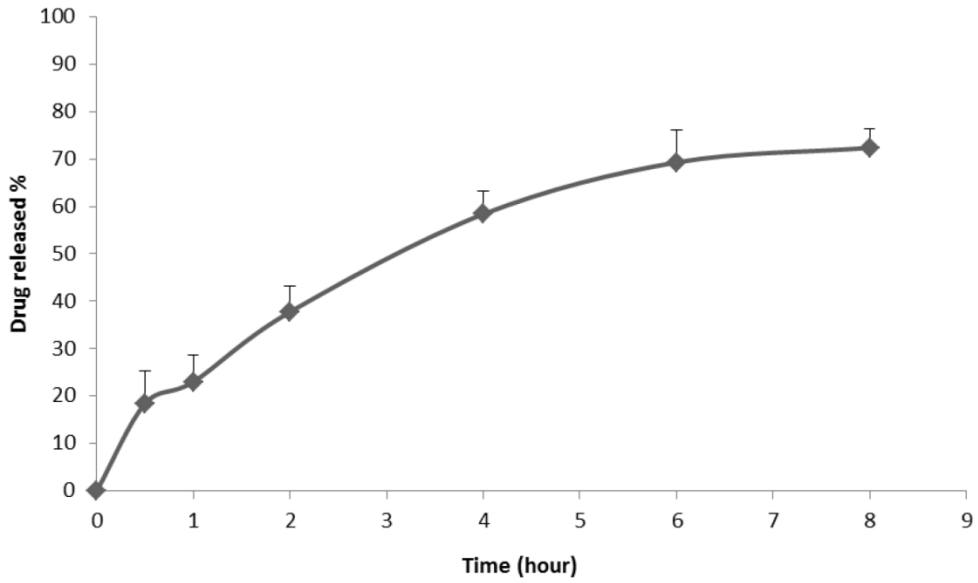

Figure. 2. *In-vitro* release profile of tacrolimus from the optimized formulation; data are represented as mean ± SD (n = 3).

3.5. Stability study

The physicochemical stability of the optimized micelles was investigated at 4 °C for a period of 1 month. The results of the stability study are summarized in Table 5, showing that the micelles did not suffer a significant change in their size characteristics; however, it should be noted that their entrapment efficiency decreased by about 24%.

Table 5 Stability of the optimized formulation stored at 4 °C for 1 month; data are represented as mean ± SD (n = 3).

Duration (day)	Size (nm)	PDI	Zeta-potential	EE (%)
0	84.1 ± 1.6	0.02 ± 0.03	-32.7	99.5 ± 2.2
7	84.9 ± 0.0	0.04 ± 0.00	-33.4	98.0 ± 0.1
14	86.6 ± 0.9	0.01 ± 0.00	-35.9	87.9 ± 3.9
21	86.3 ± 1.7	0.03 ± 0.01	-31.0	87.7 ± 3.1
30	86.4 ± 1.2	0.02 ± 0.10	-31.2	75.7 ± 3.9

4. Discussion and Conclusion

Mixed micelles have represented a milestone in the field of novel drug delivery with a considerable potential for encapsulation of poorly water-soluble drugs [18, 22, 23]. Their attraction lies in their composition, consisting of water-insoluble phospholipid molecules, located adjacent to a surfactant, which develop a structure with a hydrophobic core as a reservoir of hydrophobic compounds, and hydrophilic corona to provide stabilization against the external medium. Their unique characteristics, including nano-scale particle size, high thermodynamic stability, biocompatibility, cost-effectiveness, and ease of large-scale production make them interesting candidates for a variety of applications [18, 24]. Considering all these desirable features, we aimed to develop and optimize the mixed micelles, being composed of a phospholipid (EPC) and a bile salt (NaC, NaDC, or NaTC), for ocular delivery of tacrolimus to use against CNV.

Since particle size exerts a significant effect on the performance of colloidal drug delivery systems, we first investigated the effect of preparation method on the particle size and its distribution. As represented in Table 1, the mixed micelles prepared by direct dispersion and thin film hydration methods were larger than 100 nm in size and indicated wide size distribution. While, the particle size and its distribution were dramatically decreased by remote film loading method, and the lowest diameter (88 nm) of micelles could be achieved when their preparation was followed by probe sonication. Owing to the presence of the water-insoluble phospholipids, the mixed micelles have been found to show a hydrodynamic diameter that can be larger than those for typical micelles [25-28]. In the absence of additional high energy input, such as probe sonication, large dispersed particles will be expected, which can be explained by inefficient solubilization of phospholipid molecules by surfactant molecules (bile salt), leading to an oversaturation of the system with respect to phospholipid and the subsequent formation of phospholipid aggregates dispersed into the saturated system.

In order to achieve an optimal formulation capable of being used in the ocular delivery of tacrolimus, different mixed micellar formulations were prepared and the effects of various factors, including type of dispersion medium, EPC to bile salt molar ratio, L/D molar ratio, time of probe sonication, and type of bile salt surfactant on the physicochemical characteristics of the micelles were extensively evaluated.

As shown in Tables 2-4, a significant increase in tacrolimus entrapment of the mixed micelles prepared using all three different bile salts was found with the increase in the ratio of EPC to bile salt. This enhancement may be explained by the fact that higher lipid content permits more hydrophobic interactions with lipophilic molecules of tacrolimus, leading to more incorporation of them into the micelles.

The results revealed that another factor, having a considerable effect on tacrolimus entrapment, is the L/D molar ratio. Increasing of this ratio augmented the % EE for micelles containing each of the three bile salts (Tables 2-4). It is clear that increasing the concentration of micelle membrane-forming materials, which increases the number of nanocarriers per unit volume, can lead to an increase in the amount of drug incorporated in the carriers [19, 29]. Although enhancement of this ratio results in higher EE (%), in some cases, it can cause increase the viscosity of the system, which is not desirable. An additional increase of this ratio was not studied since high amounts of lipid and surfactant may worsen the physical characteristics of the formulation, raise toxicity concerns, and negatively influence the economic feasibility of pharmaceutical scale production.

The structure of bile salt can be another factor, affecting the physicochemical characteristics of the mixed micelles. Thus, in this study, three types of bile salts were employed to prepare mixed micellar systems. The molar ratios of EPC to bile salt and L/D were (70:30) and (20:1), respectively, and kept constant in all three systems. As can be seen (Tables 2-4), among all three bile salts, NaTC combining with PC achieved the highest entrapment of tacrolimus compared to NaC and NaDC. Higher EE (%) of NaTC

containing micelles may be interpreted by higher hydrophilic property of conjugated NaTC, leading to reduction of the mobility of water molecules adjacent to the surface of the micelles thanks to hydrogen bonding interactions, which can enhance the stability of the micelles and prevent drug molecules from moving out into the external medium.

Regarding the obtained results, tacrolimus-loaded mixed micelles, consisting of EPC and NaTC (70:30) with L/D molar ratio of (20:1) prepared by remote film loading method (Table 4, F23), was found to be the optimized formulation, as it exhibited a narrow particle size range around 84.1 nm (PDI = 0.02 ± 0.03) with the highest EE (99.5%). This formulation was thus chosen as the optimal mixed micelle and was used for further studies.

In-vitro drug release studies indicated a controlled release of tacrolimus up to 74.1% for 8 h (Figure. 2), which is presumed to be ascribed to the high affinity of hydrophobic tacrolimus molecules for the hydrophobic materials in the system [30].

Assessment of the mixed micelles stability in terms of their physicochemical characteristics unveiled that they were relatively unstable in the case of EE (%) (Table 5), which could be attributed to the detergent action of the bile salt, increasing the solubilization of the phospholipid constituent of the mixed micelles with the pass of time, and thus leading to leakage of the encapsulated molecules to some extent.

According to these results, it can be deduced that the mixed micelle formulation proposed in this research may serve as a promising system for ocular delivery of tacrolimus. In order to confirm the efficacy and safety of this formulation, a series of *in-vivo* investigations, including pharmacokinetics, biodistribution, and inhibition effect on the CNV, and toxicity remain to be conducted in the future.

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