Formulation and development of Liposomal tacrolimus for treatment of various ocular diseases

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Abstract— **Corneal transplantation is one of the most successful tissue/organ transplant procedures. While approximately 40,000 are performed each year. Tacrolimus (FK 506) is a potent macrolide lactone immunosuppressive agent that exhibit excellent therapeutic efficacy in suppressing abnormal immune responses and prevention of rejection in transplantation. In spite of its success in ensuring graft survival, therapeutic use of tacrolimus is restricted due to its narrow therapeutic index and a poor oral bioavailability because of its poor solubility and liver first pass effect. The purpose of this study was to develop tacrolimus encapsulated liposomes, to optimize the loading efficiency and to achieve a sustained release profile.**

Tacrolimus encapsulated liposomes were prepared by thin film hydration method, and extruded to large unilamellar vesicles (LUVs) with mean particle size (Z ave~ 100 nm) by high-pressure extrusion technique. Drug loading efficiencies (78 - 98%) achieved with high final drug/lipid ratio. the effects of liposomal structure and drug/ lipid ratio have been investigated on the loading efficiency. Developed formulations were found to have in vitro sustained drug release up to 14days. furthermore, the stability of liposome in size and shape was confirm for at least one month. The investigation provides a practical approach for direct delivery of tacrolimus encapsulated in liposomes for controlled and prolonged retention at the site of action.

Keywords: Liposome, Tacrolimus, sustained released, ocular therapy, Corneal graft.

I. INTRODUCTION

Tacrolimus (FK506) is an immunosuppressive agent that has been isolated from the fermentation broth of Streptomyces tsukubaensis. it has a mechanism of action similar to that of cyclosporin A, but is more potent. In vitro, FK506 inhibits the generation of cytotoxic T lymphocytes and the production of interleukin-2 and -3 and interferon at levels approximately 100 times lower than that of cyclosporin A [1]. In vivo, FK506 showed a strong immunosuppressive effect in a variety of animal models of transplantation and in the treatment of experimental autoimmune uveoretinitis. The use of FK506 is of special interest in ophthalmology, because it may be effective in the treatment of immune-mediated disease, such as corneal graft rejection, keratitis, scleritis, ocular pemphigoid, and uveitis [2].

The in vivo efficacy of tacrolimus might not be satisfactory due to its narrow therapeutic index (5 -15 ng/ml), low solubility in aqueous media (4□12 µg/mL in water), low bioavailability and limited half□life (8.7□11.3 h) [1,2]. Moreover, the drug dosage is limited when administered systemically via Eye drops (0.005%-1%) [3,4], Ointment (0.03%-0.1%) [5] or Intravenous injection due to adverse effects include nephrotoxicity, insomnia, photophobia, gastrointestinal symptoms, and central nervous system alteration [6]. In some cases, such as allergic ocular diseases, patients have to use Eye drops (0.005%) four times a day [3]. Various formulation approaches such as encapsulation of FK506 in biodegradable polymeric scleral plug (PLGA) [7] polymer implants [8] and liposomes [9] have been investigated for achieving sustained release, improving patient comfort, enhancing the efficacy of drug and decreasing its side effects [10,11]. Since their discovery, liposomes have become important drug delivery systems that offer unique properties. Liposomes are self-assembled spherical vesicles that consist of different types of phospholipids and cholesterol that have the most structural similarity with the cellular membrane These lipid vesicles can entrap both hydrophilic and hydrophobic drugs [9]. Improved therapeutic index of Fk506 and 60% reduction in drug nephrotoxicity were observed for liposomal encapsulated tacrolimus [10-12]. In the present research, the studies were focused on providing sustained release of tacrolimus over prolonged periods of time by entrapment of drug into liposomes. liposomal formulations of tacrolimus have been prepared by saturated lipids (DPPC) and unsaturated lipids (EPC) with various drug/ lipid weight ratio. The loading efficiency and final drug/lipid ratio were measured and formulations were further tested for in vitro release rates and size stability on storage and release conditions.

II. EXPERIMENTAL

Materials and Methods

1,2-Dipalmitoyl-sn-glycero- phosphocholine (DPPC) and L-α-phosphatidylcholine (EPC) were purchased from NOF Corporation. Tacrolimus was obtained from Sigma- Aldrich and Chloroform, methanol, acetonitrile was prepared from Merck. Dialysis tubing's (100 kDa) were obtained from Spectrum laboratories. Analytical reagent grade salts and chemicals were used without further purification. A Malvern Zetasizer Nano ZS (UK) was used to study the average size

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and distribution (poly dispersity index, PDI) of liposomes. Tacrolimus concentration was estimated using a HPLC system (Agilent series 1200) and High-pressure lipid extruder (Northern Lipids Inc, Canada) was used for size reduction of liposomes.

Liposome preparation

According to the thin film hydration method, pre-determined weight of lipid and Drug solution, tacrolimus in acetonitrile (5 mg/ml stock solution), were dissolved in chloroform and methanol (1:2 v/v) solution and dried to a thin film in a round- bottom flask on a rotary evaporator under reduced pressure at 40oC until complete removal of solvents. The dried lipid mixture was then rehydrated in 2 ml PBS (pH 7.4, 150mM). The resulting suspension of multilamellar liposomes was extruded and 5 times passed through a 0.2 µm polycarbonate filter and 10 times through a 0.08 µm polycarbonate filter sequentially. The diameter of obtained liposomes was determined by a zetasizer apparatus [13].

Drug loading

For calculation the amount of drug retained in liposomes (LUVs), 40 µl of loaded liposomes were added to 160 µl acetonitrile and vortexed. acetonitrile was used to break the liposome structure through solving lipids completely. The broken lipids were ultra-centrifuged for 40 minutes at 13000 rpm. The amount of total tacrolimus in the supernatant was measured by HPLC. Drug loading efficiency (LE %) was calculated using equation (1):

LE % = 100× (Amount of drug retained in LUVs / Amount of drug taken initially in MLVs) (1)

Drug release

1 ml of drug-loaded liposome solution was suspended inside a dialysis bag (100 kDa) which was emerged in the 40 ml solutions of PBS/acetonitrile (90/10 v/v) with pH 7.4. Dialysis bag was continuously stirred in the release medium at 300 rpm in a shaker incubator at 37 °C. The PBS solution was completely exchanged every 24 hours and monitored for drug concentration continuously [9,13].

III. Results and Discussion

Liposome preparation

Liposomes (LUVs) of mean particle size (Z avg ~ 100 nm) with a low polydispersity index (PDI) were prepared by extrusion technique (Table 1). Tacrolimus as a hydrophobic molecule was entrapped in the bilayer, thus, a general increasing trend was observed for liposomes diameter with an increase in D/L initial weight ratios. DPPC liposomes (saturated lipids) are larger than EPCs (unsaturated lipids).

Table 1. Average particle size (Z avg) and PDI of preformed liposomes

Types of liposomes	D/L initial weight ratios	Zeta average size, (nm)	PDI
EPC	0.05	87.7±2	0.13±0.03
EPC	0.1	92.2±1.5	0.11±0.02
EPC	0.2	93.3±2.1	0.123±0.01
DPPC	0.05	100.2±1.06	0.15±0.03
DPPC	0.1	102.4±1.31	1.31±0.02
DPPC	0.2	106.37 ± 2.5	0.1 ± 0.02

Tacrolimus encapsulation

Drug loading efficiency, Initial and final drug/lipid weight ratios for liposomes are given in Table 2. The observed drug loading efficiencies were in the range of 78 - 98%.

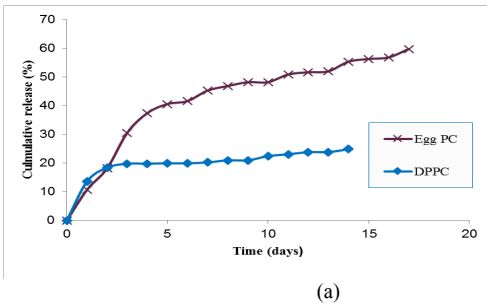
Table 2. Drug loading efficiency and final drug/lipid weight ratios of liposomes.

Types of liposomes	Initial Drug/Lipid weight ratio	Final Drug/Lipid weight ratio	Drug loading Efficiency (%)
EPC	0.05	0.044	0.13±0.03
EPC	0.1	0.08	0.11±0.02
EPC	0.2	0.156	0.123±0.01
DPPC	0.05	0.048	0.15±0.03
DPPC	0.1	0.095	1.31±0.02
DPPC	0.2	0.18	0.1 ± 0.02

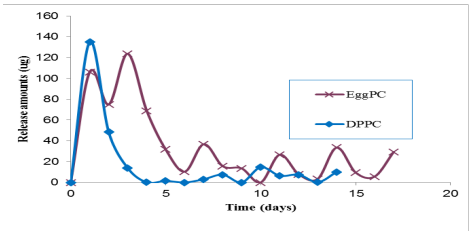
The loading efficiencies were higher for EPC liposomes and increased with decrease in D/L weight ratios for any formulation. This data suggests that high drug loading concentrations of Tacrolimus in nano sized liposomes were possible especially in EPC liposomes (> 90% loading efficiencies).

In-vitro Tacrolimus releasing

Tacrolimus release behavior for EPC and DPPC(D/L=0.2) at 37 °C, in a solution of PBS (pH 7.4) and acetonitrile (9/1 v/v) is expressed in term of cumulative drug release (%) and Release amount (µg) as shown in Fig 1 (a, b). Slow and sustained release of the drug was achieved with 60% of the drug being released for EPC and 20% for DPPC at the end of 14 days, that is a significant improvement compared to the release behavior reported for FK506 capsule (more than 90% released within 25 h) [14,15]. An initial burst (in terms of amount released (µg)) of tacrolimus was observed for liposomes in the first few days (Fig 1 b), beyond which the release was slow and sustained. A lower initial burst was observed for EPC.



(a)



(b)

Figure 1: Release of FK 506 from EPC and DPPC in 90% PBS (pH 7.4) and 10% ACN (a) cumulative release and (b) Release rate

Stability studies

Size changes of tacrolimus loaded DPPC and EPC liposomes with various D/L weight ratios over one month on storage at 4 °C and after in vitro release at 37 °C in PBS/ acetonitrile (9/1, v/v) were measured and results are given in Table3.

Table 3. Size measurement of Tacrolimus loaded liposomes during storage at 4 °C and after in vitro drug release

Size measurement, nm (PDI)				
Types of liposomes	D/L initial weight ratios	D_0	D_{30}	After in vitro drug release
EPC	0.2	93.3(0.11)	102.7(0.3)	110.5(0.1)
EPC	0.1	92.2 (0.12)	98.5(0.05)	100.7(0.06)
EPC	0.05	88.25(0.05)	90.67(0.01)	95.7(0.06)
DPPC	0.2	106.37(0.28)	112.8(0.22)	122.5(0.2)
DPPC	0.1	104(0.23)	115 (0.01)	120 (0.3)
DPPC	0.05	100.2(1.31)	107.5(0.02)	101.6 (0.23)

As seen from Table 3 vesicles were reasonably stable without aggregation on storage at 4 °C for 1 months without any evidence of micron sized particles, size changing increased with increase in D/L weight ratios. Tacrolimus loaded EPC (D/L 0.05) had minimal changes in size. minimal size changes were observed for liposomes at the end of drug release, suggesting that integrity of vesicles was maintained during dialysis in vitro. The liposome size for any formulation increased with increase in D/L weight ratios indicating probable destabilization of the vesicles during dialysis due to higher drug concentrations in the bilayer [10,17].

IV. Conclusion

Tacrolimus encapsulated liposomes (Z avg ~ 100 nm) were prepared with high drug loading efficiencies (78 - 98%) and drug/lipid weight ratio (0.05-0.2). Slow and sustained release of the drug was achieved with 60% of the drug being released for EPC and 20% for DPPC at the end of 14 days, that is a significant improvement compared to the release behavior reported for FK506 capsule (more than 90% released within 25 h). vesicles were reasonably stable without aggregation on storage at 4 °C for 1 months and after release. The results indicate the great potential for future applications of tacrolimus encapsulated Nano liposomes as rescue therapy in ocular disease.

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