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RESEARCH PAPER

Chitosan hydrogel containing tacrolimus-loaded nanoliposome for ocular drug delivery: Physicochemical analysis and stability evaluation

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ABSTRACT

Objective(s): Recently, the use of tacrolimus in treating eye diseases has received much attention. Although this drug is powerful in treating eye diseases, however for various reasons, it lacks the necessary efficacy for multiple reasons. This research investigated the development of Tacrolimus encapsulated liposomes, optimization, loading effectiveness, increasing drug efficiency through absorption, controlled release, drug targeting, and reducing drug side effects such as nephropathy.

Materials and Methods: Two agents, liposome and chitosan, have been chosen to transport the drugs used in this study. Nanoliposomes were synthesized through the heating method and chitosan nanoparticles were by reversing the micelle method. A field emission scanning electron microscope(FESEM) was used to prepare images and a zeta sizer was used to measure the average size and distribution of particles. Drug release for 18 days was checked by in vitro and ex-vivo(Franz diffusion) tests. The MTT method was used to evaluate the cytotoxic effect of nanoparticles loaded with tacrolimus drug.

Results: The molar ratio of the drug to liposome and chitosan was chosen to be 0.002. A drug loading effectiveness of (88-95%) was obtained. Tacrolimus drug loading efficiency in liposomes (EPC100, EPC80, DPPC60, DPPC100) value (88.95-95-74%) was obtained for its entrapment in liposome core with passive loading strategy. The difference in drug release rate for EPC 80/chitosan liposome and EPC 100/chitosan was 83.6% and 93.1%, respectively, and for DPPC60/chitosan and DPPC100/chitosan liposomes, 72.8% and 78.8%, respectively.

Conclusion: With this study, it can be concluded that DPPC liposome was good for drug loading. The results of the test (FT-IR) showed that the loading of the drug was successful. The results of electron microscope tests in both samples (EPC, DPPC) indicated the synthesis of drug delivery systems with a spherical morphology with a diameter of less than 100 nanometers. The release results showed that the highest release rate was related to EPC liposomes. In the MTT test, it was observed that nanocarriers without tacrolimus drugs do not show any toxic effect on cells.

Keywords: *Tacrolimus, Nanoparticles, Ophthalmic, Chitosan, Hydrogel*

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INTRODUCTION

Tacrolimus (FK 506) is a potent lactone macrolide that has a tremendous effect in stopping the immune response. This drug is obtained from the soil fungus Streptomyces tsukubaensis [1]. The use of FK506 is important in ophthalmology because it can be effective in the treatment of autoimmunities such as corneal transplant rejection, allergic ocular diseases, ocular pemphigoid, and uveitis [2]. Due to its potential range of efficacy, this drug has received a lot of attention in the field of research [3]. Recently, tacrolimus is of importance because this drug can be used in the treatment of diseases caused by high immune system reactions such as corneal transplant rejection in the recipient patient, ocular allergic diseases and seasonal allergies, ocular pemphigoid, and iris inflammation [4]. However, therapeutic applications of tacrolimus have been limited due to its significant side effects [5]. In addition, topical administration of eye drops is the most common method of using tacrolimus for the eye [6]. The maintenance of the inoculated drug administered on the surface of the eye was very

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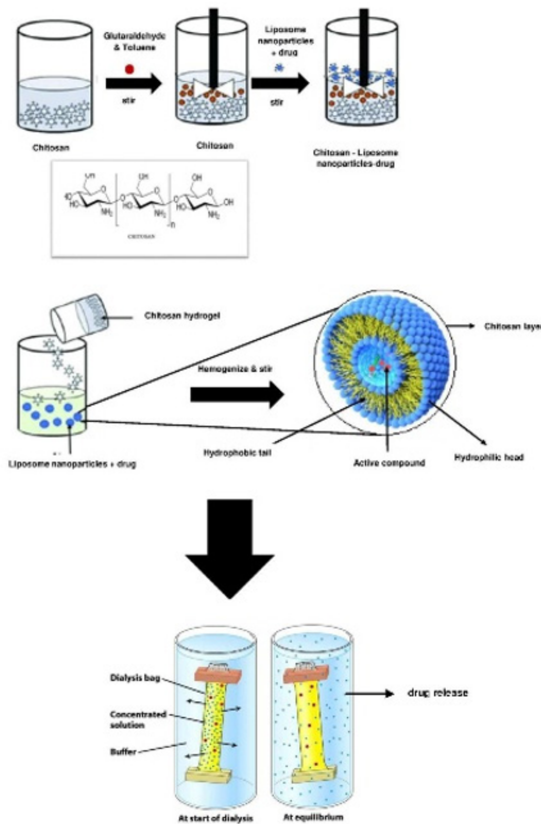

Graphical abstract

short and most of the drug disappeared quickly from the area before loading [7]. Therefore, increasing corneal permeability or prolonging the retention time of the drug on the ocular surface was the key factor in improving the effective ocular bioavailability of the drug which should be considered when designing the formulation [8,9]. Tacrolimus has a low solubility in water, ranging from 4-12 µg/mL, that researchers have used possible approaches to improve pharmacokinetic properties and increase the effectiveness of drug formulations. The basic mechanism of the various formulations is to increase the solubility of tacrolimus in water [10]. In one study, an attempt was made to improve the bioavailability of tacrolimus through employment of proliposomes [11]. Nano-drug delivery systems, such as nanoparticles [12], kebobs [13], nanoemulsions [14], and liposomes [15], have been shown to improve corneal penetration and increase retention time.

Liposomes are sac-like structures that have a soluble nucleus surrounded by two hydrophobic phospholipid layers. They are biocompatible compounds that cause the least immune response in the body. Liposome-forming phospholipids have a phosphate hydrophilic head, a small polar molecule, and a long non-polar hydrophobic head that forms a micelle structure in aquatic environments [16]. Liposomes can gradually release the drug at a specific lesion site and are suitable for intrauterine administration and accumulate at sites of inflammation for long periods of time [17, 18]. The liposomal loading system has become one of the significant methods to increase attention dissolution due to its biocompatibility and ability to encapsulate hydrophobic molecules in the field of lipids [19]. The *in-vivo* behavior of liposomes changes as one of the most effective release systems with the different physicochemical properties [20]. Some researchers have used liposomes to encapsulate tacrolimus and continuous release. Reduced drug toxicity and its side effects have been reported in the built-in systems [21]. Nanostructured carriers appear as minimally invasive drug delivery systems; they can maintain the concentration of therapeutic drugs in the posterior part of the eye for a long time and prevent the need for repeated injections and also, their small size prevents eye irritation [22]. Nanoliposomes increase the precise targeting of trapped material and its controlled release. These lipid vesicles have been used in the fields of healthcare, cosmetics, and food industries as a carrier to protect and transport bioactive agents [23]. Compared to liposomes, nanoliposomes provide more space and have the potential to increase solubility and bioavailability, improve controlled release, and accurately target enclosed materials to a greater extent [24].

Different methods for making liposomes have been proposed, as follows.

In the thin layer method, lipids are dissolved in an organic solvent (chloroform or a mixture of methanol), and then, the solvent is removed from the reaction medium by a strong vacuum to form a thin layer of lipids on the wall of the reaction vessel [25]. In another method, hydrated vesicles are exposed to ultrasound waves (either through a proboscis sonicitis or through an ultrasound bath) for a few minutes at a controlled temperature. In this method, the dispersion and size of the vesicles are affected by the lipid composition, concentration, temperature, duration of sonication, and the amount and volume of the sample. In the extrusion method, micro liposomes are transformed into large single-walled vesicles or nanoliposomes based on the size of the filter

holes used. Microscopic liposomes are physically under pressure and passed through polycarbonate filters with specified pore sizes. Another method is called microfluidization, in which nanoliposomes are made using a microfluidic system without the use of solvents having the potential to cause toxicity. The basis of this system is to separate the pressure flow into two parts and transfer each part to a small hole and finally direct the flow to the microfluidic cavity [26]. In another method called the heating method, phosphate buffer is as a solvent. First, phospholipids and cholesterol are hydrated under a nitrogen atmosphere, added to the glycerol mixture in phosphate buffer, and heated at high temperature in the presence of nitrogen to form liposomes [27]. Mozaffari method as one of the simplest methods for synthesis of nanoliposomes, is a revised version of the heating method. In this method, the components of the liposome react with each other in a short time in contact with an aqueous solution of glycerol and causes the production of liposomes [28].

The use of mucosal polymers as viscosity enhancers is essential to prolong contact time between formulations and corneal/conjunctival epithelium [29]. Polymer-coated liposomes gained long-term in-vitro drug release profiles due to diffusion barriers [30]. Amphiphilic polymers have shown more interest in the pharmaceutical field due to their unique structure and polymer nanoparticles can be used to make a hydrophobic core and a hydrophobic shell, thereby enabling the nanoparticles to act as a repository for hydrophobic drugs [31]. Properties such as biodegradability, low toxicity, and suitable environmental compatibility make it suitable for use in biomedical and pharmaceutical formulations [32].

Studies show that it has not only the positive charge but also the specific nature of chitosan that was responsible for achieving the advanced bioavailability of the drug contained in chitosan-coated nanocapsules and the effect of chitosan coating on ocular retention of liposomes provided good results [33]. Chitosan is one of the most common natural polymers in the production of nanopharmaceuticals, because it possesses very attractive properties for drug delivery and is very effective in the formulation of nanoparticles [34].

Chitosan nanoparticles have attracted a lot of attention in the recent decades due to thier great potential as nanocarriers to carry substances such as drugs or active compoundsfor controlled drug release [35].

So far, various methods have been proposed for the synthesis of chitosan, the most important of which are emulsion method and cross-linking of reverse micelles. The preparation of chitosan nanoparticles in this way was first done by Ohyo et al, (1994) [36]. The basic approaches used to form chitosan nanoparticles revolve around emulsion, precipitation, ionic or covalent crosslinking, or a combination thereof. Emulsion and crosslinking were the first methods described for the preparation of chitosan nanoparticles using the amino chitosan group and crosslinking of aldehyde group [39]. An emulsion consisting of an aqueous solution of chitosan and an oily phase is made using Span 80 as a stabilizer, and toluene and glutaraldehyde as a binder [36, 37]. Another method for chitosan synthesis is microemulsion method based on covalent bonding. At the present, the reverse micelle-water structures in oil contribute to the production of chitosan nanoparticles. The aqueous phase, consisting of chitosan and glutar aldehyde, is mixed with the organic phase, contaning lipophilic surfactant and organic solvent. The chitosan-containing micelle nucleus acts as a nanoreactor in which chitosan nanoparticles are formed by crosslinking [38, 39]. Phase inversion precipitation method is based on emulsification with precipitation, along with precipitation of oil-in-water emulsion with an organic phase (dichloromethane and acetone) and an aqueous solution of chitosan in the presence of a stabilizer (poloxamer). High-pressure homogenization is applied to obtain nanometer-sized emulsified droplets. Methylene chloride is then separated from the emulsion by evaporation at low pressure and room temperature, leading to the release of acetone from the droplets and the simultaneous precipitation of chitosan nanoparticles [40]. Another method named the emulsion-droplet coalescence or dehydration approach has been invented based on the combination of two water-in-oil emulsions, which precipitates chitosan nanoparticles because Sodium chloride (NaOH) acts as a precipitation agent in one of the emulsions. High-speed homogenization is used to prepare chitosan-containing emulsions. After combining the two emulsions, NaOH is dispersed in ultra-fine droplets, which reduces the solubility of chitosan and leads to the formation and deposition of nanoparticles. Chitosan nanoparticles are

obtained in three stages: centrifugation, washing with different solvents: toluene, ethanol, and water, and ice drying, respectively [41, 42]. The ionic gelation method was first investigated by Calvo et al. (1997) [43]. The basis of this method is ion cross-linking, which occurs in the presence of reverse-charged groups. Anionic molecules are dispersed in a mixture of positively charged chitosan molecules and crosslinking leads to the formation of nanoparticles [44].

Badawi et al. worked on chitosan nanoparticles with indomethacin and concluded thatchitosan nanocarriers were able to make close contact with the cornea, causing gradual release with long-term drug levels and thus, increasing delivery to the external and internal tissues of the eye [45].

In 2016, Kalam et al. conducted studies on chitosan nanoparticles with dexamethasone-sodium (DEX) and the stability study on nanoparticles showed that significant changes in particle size, encapsulation, drug release, and physicochemical properties were not maintained at 25 °C for 3 months [46].

According to studies, chitosan can be a good carrier for liposome loading with tacrolimus. In the present study, the release of tacrolimus over a long period of time by drug loading on liposomes and chitosan was investigated. The liposome/chitosan tacrolimus formulation was evaluated by saturated lipids (DPPC) and unsaturated lipids (EPC) with the different drug/lipid cholesterol and chitosan weight ratios. Loading efficiency and final drug/lipid and chitosan ratio were measured and formulations were tested to measure in-vitro release and size stability under load and release conditions.

Tacrolimus drug is a proven drug used in cornea transplant surgery. According to the conditions of retention of the drug in the eye and considering that tacrolimus is a hydrophobic drug, biocompatible nanocarriers were chosen to solve this problem. Nanoliposomes are hydrophilic and hydrophobic carriers and are highly biocompatible. Therefore, tacrolimus drug was loaded on these nanocarriers so that this useful drug could be released into the eye with better conditions.

One of the methods of drug release is the in-vitro method, performed outside the body of a living organism, and tests are performed in a controlled environment, such as a test tube or dialysis bag, etc. Considering that the *in-vivo* method is performed outside the body, researchers can perform more tests and analyze for drug release than *in-vivo* method [47]. The in-vitro method is one of the most widely used methods for studying drug release. One of the *in-vivo* simulation methods for drug release is the *x-vivo* method, which has many advantages, such as more analysis of the drug and functional development of the product [48,49]. One of the devices developed over the years is the use of vertical diffusion cells [50]. This test can be easily used with different changes in different eye products and by using Franz diffusion cells [51]. Considering that the observance of animal rights is important and the experiment can be repeated many times, more accurate analyses can be achieved.

MATERIALS AND METHODS

Tacrolimus was purchased from the Sigma-Aldrich Company (Argentina country). 1,2-Dipalmitoyl-sn-glycero- phosphocholine (EPC), Dipalmitoylphosphatidylcholine (DPPC), and chitosan (27 Centipoise) were obtained from Sigma-Aldrich Company (from India). Methanol, sodium perchlorate, monohydrate, perchloric acid, calcium chloride, acetonitrile, ethanol, cholesterol, and glycerol were obtained from Merck Company (from Germany). A dialysis bag (100 kDa) and a pre-sterilized nylon 66 membrane with a pore diameter of 45 µm were supplied by Sigma Company (from England). Salts and other chemicals were of analytical or spectroscopic grade.

The DLS (VASCO / CORDOUAN TECHNOLOGIES / FRANCE (device was used to determine the average particle size and distribution (Dynamic Light Scattering, DLS**)** of nanoliposomes.

Fesem field emission scanning electron microscope (FESEM) system (MIRA III model Country of manufacture: Czech Republic) was used to determine the exact size of nanoliposome and chitosan hydrogel. UV-visible spectrophotometer PG INSTRUMENT was applied to analyze the samples.

Preparation of nanoliposome

According to the heating method [48], different weights of EPC and DPPC lipids, listed in

Table 1. Formula of liposomes investigated in this research

Types of liposomes	Additive	Molar ratio	Abbreviation
DPPC	Cholesterol	6:4	DPPC/ CHOL
DPPC	-	10	DPPC
Egg pc	Cholesterol	8:2	EPC / CHOL
Egg pc	-	10	EPC

Table 2. Chitosan hydrogel are prepared with the ratio of drug to polymer ratio

Medicine	Polymer	Molar ratio
Tacrolimus	chitosan	1:1
Tacrolimus	chitosan	1:1.5
Tacrolimus	chitosan	1:2
Tacrolimus	chitosan	1:2.5
Tacrolimus	chitosan	1:3

Table 1, were added to the preheated cholesterol mixture along with glycerol and stirred for 15 min in a nitrogen atmosphere at 70 ° C by a heater (approximately 500-1000 rpm). Medium size and particle distribution characteristics were measured employing DLS test, and fesem test was applied for accurate measurement of nanoparticles,.

Liposomes were made using different lipids and cholesterol with the formulas are shown in Table 2.

Preparation of chitosan hydrogel containing tacrolimus-loaded nanoliposome

1 g of chitosan (27 Centipoise) was dissolved in 100 mL of a solution containing 2% by volume of NaCl and 3% volume of acetic acid. The mixture was stirred continuously for 2 hr to obtain chitosan gel. The drug-loaded nanoliposomes were then dissolved separately in 5 mL of chitosan gel (drug to chitosan ratio 1.5:1, 2:1, 2.5:1, and 3:1) under a magnetic stirrer. 6 g of chitosan gel was added dropwise to 10 mL of flaxseed oil containing 2% (v/v) span 80 under magnetic stirrer. Then, 5 mL of acetone dropwise (2 mL per min) was added to the mixture. The system was kept under a stirrer for 1 hr while being covered with aluminum foil, and then, 5 mL of glutaraldehyde-saturated toluene was slowly added to the system and stirred continuously for 2 hr. The obtained nanoparticle suspension was centrifuged at 5000 reps per min, then, it was washed with toluene and dried.

Chitosan hydrogel was made with the different drug ratios to achieve the optimal ratio, given in Table 2. A ratio of 1:2 was chosen for this purpose.

The amount of the initial drug, loaded drug, loading efficiency, and loading rate were calculated from the following equation.

LE% = ((weight of the feeding drug - weight of the free drug)/weight of the feeding drug)×100

Statistical method

OVAT (one variable at a time)

First, the molar ratio of drug to polymer (varied between 1:1, 1:1.5, 1:2, 1:2.5, and 1:3) was optimized based on the maximum amount of drug loading using a single variable at any time method. Then, the amount of the loading on nanoliposomes was optimized with appplying the different molar ratios of liposome to cholesterol for DPPC in the range of 6:4 and 1. Finally, the EPC was optimized considering it in the range of 8:2 and 1, according to Table 1. It is worth to mention that the best amount of the drug loading was reported based on the results of FESEM, DLS, and in-vitro and *x-vivo* release tests. The experiments were repeated three times with the same conditions for drug release, and the standard deviation was calculated usinng the following formula.

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \ddot{X}^2)}{n-1}} = \sqrt{\frac{\sum_{i=1}^{n} x_i^2 - n\ddot{X}^2}{n-1}}$$

s is the standard deviation of the sample, xi is single values in the sample, 2 is the average of all x values in the sample, and n is the total number of x values in the sample.

Drug loading

An initial molar ratio of drug to lipid (0.002) was chosen for active drug loading on liposomes. First, liposomes were prepared, and then, glycerol was added to the mixture of cholesterol and liposomes. Cholesterol should be added after the complete dissolution of lipids, then, the drug was dissolved in 1 mL of acetonitrile. After that, this solution was added to the phospholipid mixture. When adding the medicine, the temperature, stirring speed, and employed time should be 50-60 0C, 1000 rpm, and 30-60 min, respectively. Using the initial drug amount, the loaded drug amount, loading efficiency, and loading rate can be calculated from the following equation.

Drug loading efficiency = 100 LUVs / MLVs) (1)

in which LUVs and MLVs are initial drug weight and weight of the remaining drug in the liposome after dialysis, respectively.

FTIR test

FTIR test: result of chitosan/tacrolimus

Fig. 1 shows the infrared spectrum (FTIR) of chitosan and tacrolimus drug. In Fig. 1 (a), the band at 3661 cm^{-1} is related to the free stretching group (O-H), and the hydrogen bond (O-H) is shown at 3452 cm^{-1}. The specified ranges of 2985 cm^{-1},

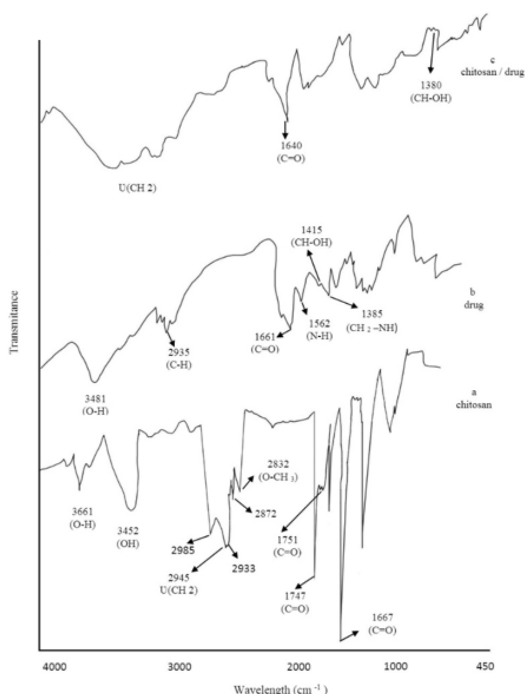

Fig. 1. FTIR of chitosan (a), tacrolimus drug (b), and chitosan loaded with drug (c)

2945 cm^{-1}, 2933 cm^{-1}, and 2872 cm^{-1} correspond to sp2 and sp3 hybrid carbon stretching vibrations. Stretching vibrations of (O-CH3) and C=O) groups have been identified at 2832 cm^{-1} and 1751 cm^{-1}, respectively. The absorption peaks related to the ketone group (C=O) are shown at 1747 cm^{-1} and 1667 cm^{-1}. Similar cases were also found in other articles [52-57].

In Fig. 1 (b), the stretching vibrations of the (O-H) group were shown in the range of 3481 cm^{-1}. The absorption peaks of 2935 cm^{-1} and 2935 cm^{-1} are attributed to the stretching vibration of the (C-H) group. The absorption peak at 1661 cm^{-1} is related to the carbonyl group (C=O). The bending vibrations of the (N-H) group are at 1562 cm^{-1}. The peak corresponding to the vibrations of the (CH_2-OH) group is shown at 1385 cm^{-1} .

Fig. 1 also shows the FTIR analysis of the sample containing chitosan loaded with tacrolimus drug. The stretching vibrations of the (N-H) group of chitosan interfere with the stretching vibrations of the (OH) group in both substances, obtaining a very wide band in the range of 2500 cm^{-1} to 3500 cm^{-1}.

By comparing the two spectra, we can conclude that there is an overlap in the range of 3600-3400 cm^{-1} related to the (OH) group, and the peaks that at 1640 cm^{-1} related to (C=O) vibrations become bigger and sharper. Drug loading on chitosan caused the peaks by the symmetric and asymmetric stretching vibrations of the (CH-OH) group in the range of 1382 cm^{-1} for the hydrogen bonds between the drug and chitosan, proving the successful formation of chitosan/tacrolimus complex.

Result of FTIR test of liposome synthesis and tacrolimus loading

Fig. 2 (a) indicates FTIR spectra of the lipid spectrum of DPPC alone, liposome/drug complex/chitosan, and DPPC/chitosan/Drug. Also, a graph was taken for a clearer comparison of the drug/liposome and DPPC/Drug sample to track the peaks caused by connections more accurately. According to Fig. 2, for DPPC, the peaks at 2845 cm^{-1}, 2950 cm^{-1}, and 3451 cm^{-1} are related to the vibrational group of (C-H3). The absorption peaks at 1640 cm^{-1} and 769 cm^{-1} are corresponding to the (C=O) and (P-O) groups . In Fig. 2 (b) forthe DPPC/chitosan/Drug complex, a large peak in the range of 3400-3500 cm^{-1} isrelated to chitosan binding and overlap of strong stretching vibrations of (N-H) group and stretching vibrations of (O-H) group as well as, the binding of chitosan caused the appearance of peaks related to stretching vibrations of the (CH_2-OH) group at 1382 cm^{-1}. Compared to Fig. 2 (c) related to the binding of liposome and tacrolimus drug, there is a strong band in the range of 1630

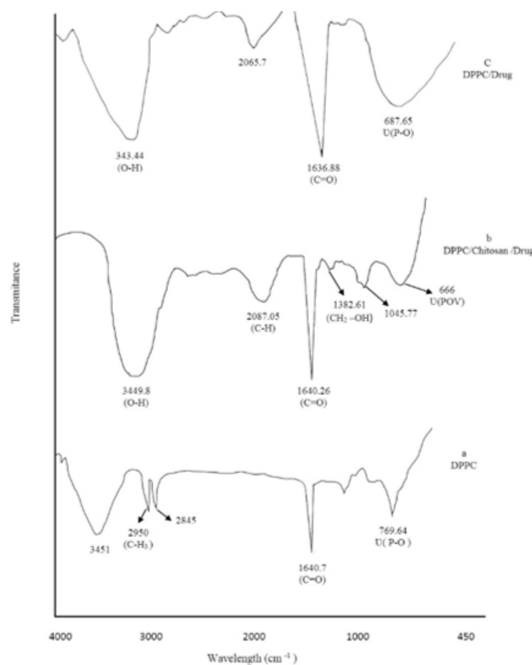

Fig. 2. FTIR spectrum related to DPPC (a), DPPC / chitosan / Drug (b), and DPPC / Drug (c)

cm^{-1} and 1640 cm^{-1} related to the overlap of the strong stretching vibrations of the (C=O) group , proving the synthesis of the liposome as well as the loading of the drug complex on this carrier. Tacrolimus peaks indicates the drug loading on the liposome. After drug loading, the peaks became weaker and slightly shifted to longer wavelengths, due to the interaction between tacrolimus and liposome.

Fig. 3 (a) indicates the FTIR spectrum of the EPC sample, including the (C-H) vibrational group in 3465 cm^{-1}. The stretching vibrations of the (C=O) carbonyl group were determined in 1637 cm^{-1}. The vibrations of the (C-O-H) group were at 1356 cm^{-1} and 1418 cm^{-1}. The vibration of the (C-O) and (C-C) groups were shown at 1232 cm^{-1} and 1097 cm^{-1}. The stretching vibration of the (CH_2) group was located at 711 cm^{-1}. In Fig. 3(b), related to the EPC/chitosan/Drug complex, a large peak was in the range of 3400-3500 cm^{-1} for chitosan binding, overlap of strong stretching vibrations of (N-H) group, and stretching vibrations of (O-H) group. Also, the binding of chitosan caused the appearance of peaks related to the stretching vibrations of the (CH_2-OH) group at 1229 cm^{-1}. Fig. 3 (c) shows the FTIR of EPC with drug EPC/Drug. There was a strong band in the range of 1630 cm^{-1} and 1640 cm^{-1}, corresponding to the overlap of the strong stretching vibrations of the (C=O) group and the liposome synthesis marker as well

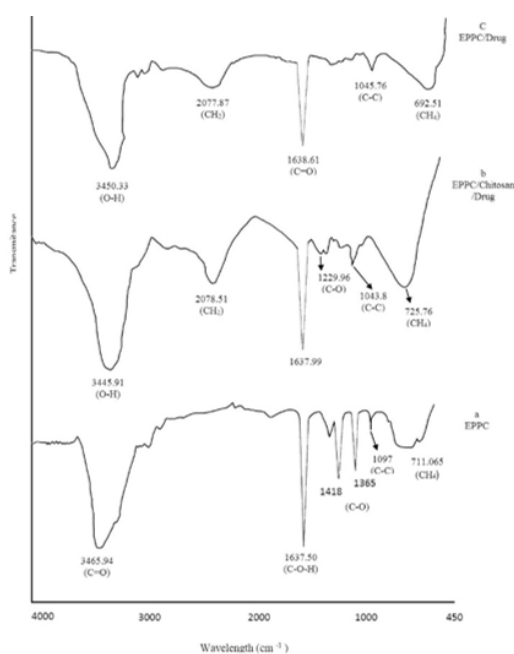

Fig. 3. FTIR spectrum (FTIR) related to EPPC (a), EPPC / chitosan / Drug (b), and EPPC / Drug (c)

as the loading of the drug complex on this carrier. Besides, tacrolimus peaks were observed drug loading on liposome. After drug loading, the peaks became weaker and slightly shifted to longer wavelengths, due to the interaction between tacrolimus and liposome.

Drug release

1 mL of liposome/chitosan hydrogel-containing drug was poured into the dialysis bag (100 kDa) along with 2 mL of PBS/acetonitrile solution (20/80 v/v) with pH=7.4. The leak-proof clip and the dialysis bag were suspended in 8 mL of PBS/acetonitrile solution with pH=7.4. The dialysis bag was continuously placed on a shaker incubator for 18 days at a temperature of 37°C and a speed of 80 rpm. Sampling was done at certain times to control the concentration of the released drug. In the first half hour, 2 mL of the sample was removed from the buffer solution, and in the next 6 hr, 2 mL of the solution was taken every hour and replaced with the same volume of fresh buffer. In the next 3 hr, 2 mL of the solution was removed and in the following hours, this procedure was repeated every 6 hr, and the drug concentration was continuously monitored. Then, every 24 hr samples were taken from the solutions, the release medium completely changed, a new buffer was replaced, and it was released and measured for 18 days. The release rate was calculated by measuring the concentration of the free drug in the upper solution using visible-ultraviolet light spectroscopy and measuring the absorption rate at the maximum wavelength of the drug and the calibration curve.

Franz diffusion test

In *ex-vivo* experiments, every effort is made to simulate an environment exactly similar to that *in-vivo*. Therefore, an environment was used for the solvent, which is similar to eye tear fluid. The laboratory release studies were done for eyes [58]. In this experiment, the cells were placed vertically. The volume of the cells was 7 mL. The simulated liquid was created for artificial tears to release the environment. pH of 7.4, temperature of 37°C, and stirrer speed of 750 rpm were set. Sampling was done automatically, 1.5 mL of each samplein 18 days. A pre-sterilized nylon 66 membrane with a pore diameter of 45 µm was used. The samples were analyzed using a UV-visible spectrophotometer at a maximum wavelength λ max of 210 nm [59].

These items were considered for conducting this experiment: increasing drug solubility [60,61], the temperature of 37 °C [62,63], stirring speed of

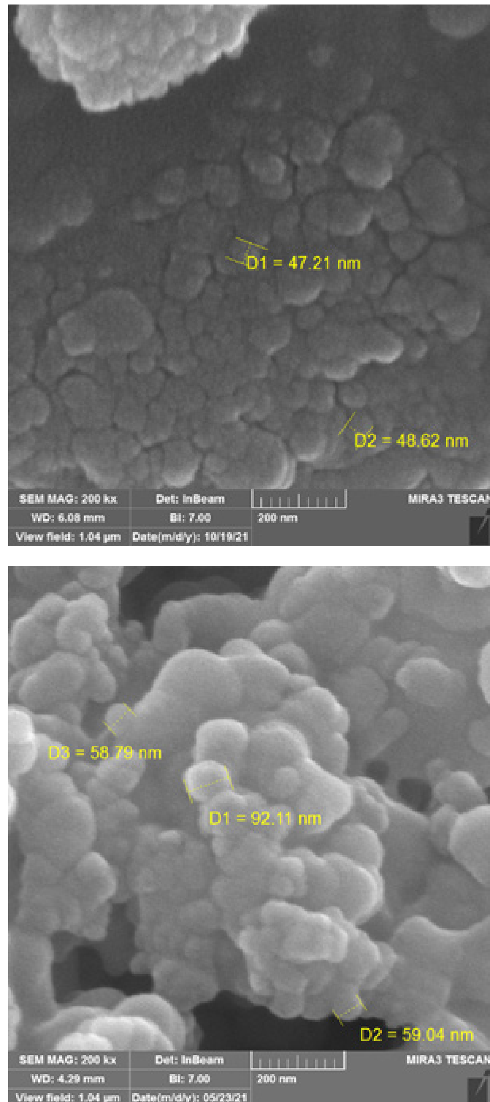

Fig. 4.Field emission scanning electron microscope image related to a) EPC, b) DPPC

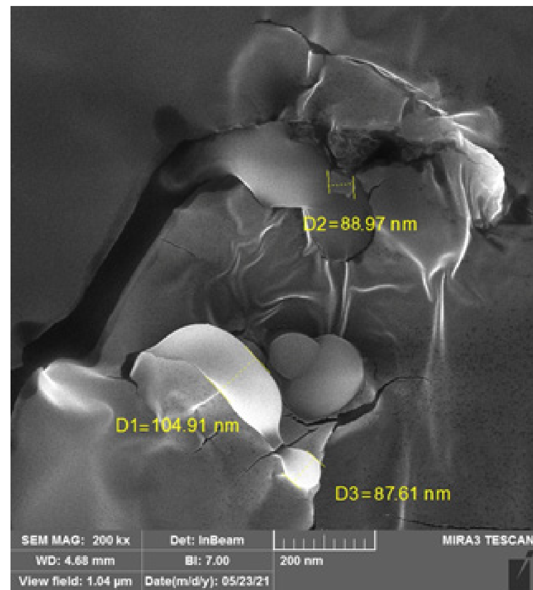

Fig. 5. Field emission scanning electron microscope image related to CS/DPPC100/TAC

200-900 rpm [64], pH of 7.14, and MDI membrane 45 µm [65].

RESULTS AND DISCUSSIONS

Nanoliposome morphology

The size of the liposome nanoparticles (LUVs) was measured by FESEM prepared by heating method (Fig. 4). Fig. 4 (a) shows a 100,000x magnification image of nanosized EPC liposome particles. The average size of EPC nanoparticles is 47.951 ± 75 nm. Fig. 4 (b) shows a 100,000x magnification image of the nanosized DPPC liposome particles. The FESEM images were analyzed using image software. The average size of DPPC liposome nanoparticles was 69.98 ± 75 nm. Tacrolimus was trapped in the bilayer as a hydrophobic molecule. Diameter of the DPPC/Tac complex particles was larger than that of EPC/Tac, due to the larger lipid size.

Considering that saturated liposomes perform much better than unsaturated ones in the release process. Therefore, DPPC was chosen for the final results of electron microscopy tests. Fig. 4 (a) shows a 100,000x magnification image of nanosized CS/DPPC100/TAC particles. The size of the formed nanoparticles was determined by zooming in 200 thousand times in Fig. 5. FESEM images were analyzed with the help of software. The average size of the nanoparticles is 98.75 ± 109 nm.

According to Fig. 5, diameter of the CS/DPPC100/TAC complex particles was larger than that of the EPC/Tac and DPPC/Tac particles.

Also, the elevations and depressions observed on the surface of the particles are due to the overlapping of the liposome surface with lipids and chitosan. These connections increase the chemical and physical stability of the liposome and also, drug wastage through eye protection mechanisms. They also prevent immediate drug leakage from the liposome.

DLS of tacrolimus-loaded nanoliposomes

Chitosan hydrogel along with monolayer nanoliposomes with an average diameter of 100 nm and a small dispersion index (by thermal method) were obtained and the resulting data

Table 3. Average particle size (Z avg) and PDI of the manufactured liposomes

Types of liposomes/poltmer	Z-average±SD(nm)	PDI±SD
CS/DPPC60/TAC	107±0.06	0.04±0.01
CS/DPPC100/TAC	97±0.5	0.11±0.05
CS/EPC80/TAC	93±1.4	0.07±0.02
CS/EPC100/TAC	103±1.03	0.04±0.01

are repeatable. Table 3 shows the average size of the manufactured liposomes and their dispersion index. The results prove that saturated liposomes are larger than unsaturated ones. Also, an increase in the size of the liposomes is observed with increasing cholesterol significantly.

In-vitro loading test

Tacrolimus drug loading efficiency of 88-95.74% was obtained for its entrapment in the liposome core by passive loading strategy, as shown in Table 4. The loading efficiency for EPC and DPPC was above 90%. However, tacrolimus forms a precipitate due to its limited solubility in aqueous medium and intra-structural foot-to-foot interactions. Also, the final lipid/drug molar ratio has been obtained for the different liposome compositions. Usually, the particles with a smaller size (in the nano-size range) have a high radius of curvature along with more bilayer membrane compression defects, which affects the amount of drug loading. Therefore, the smaller the liposomes, the lower the drug loading. The results of drug loading inside liposomes (EPC100, EPC80, DPPC60, and DPPC100) show that the presence of cholesterol in the bilayer reduces the loading efficiency. Since the mechanism of drug storage inside the liposome is permeation, It seems that the addition of cholesterol to the bilayer creates an additional barrier to the movement of tacrolimus, leading to a decrease in penetration speed and loading efficiency [49].

Table 4. Loading efficiency percentage and final lipid/drug molar ratio for the different liposomes

Types of liposomes/poltmer	Drug loading Efficiency (%)	Final Drug/Lipid weight ratio
CS/DPPC60/TAC	88.906	0.017543
CS/DPPC100/TAC	91.943	0.005236
CS/EPC80/TAC	90.945	0.006878
CS/EPC100/TAC	95.747	0.002638

In-vitro release study

Fig. 6 and 7 show the release rate of the total stored drug from the different liposomes and

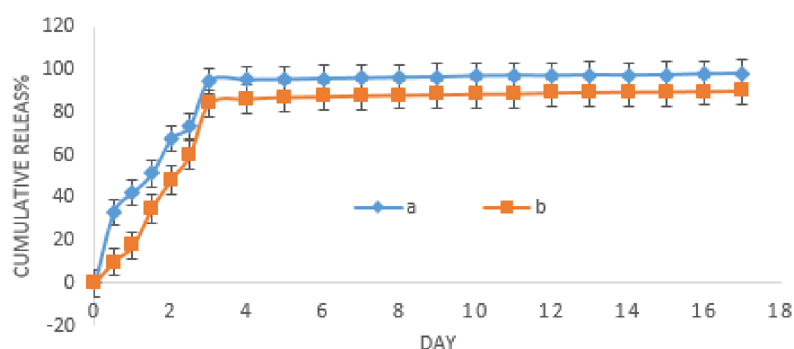

Fig. 6. Drug release of TOC from a) EPC100/chitosan and b) EPC80/chitosan (pH=7.4)

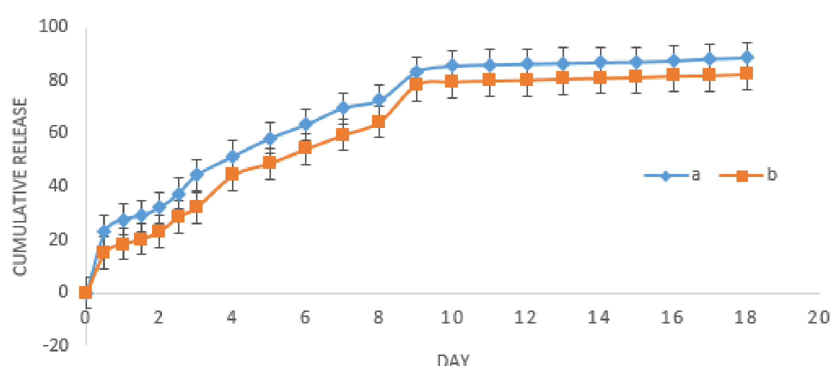

Fig. 7. Drug release of TOC drug from a) DPPC100/chitosan and b) DPPC60/chitosan (pH=7.4)

chitosan formulations loaded with tacrolimus at 37 °C in PBS solution with pH=7. A general trend can be observed in almost all graphs for the different liposome formulationsincluding an initial explosive release followed by an exponential process. This initial explosive release is probably due to the large difference in tacrolimus concentration on both sides of the bilayer. After this explosive release, the concentration gradient of the drug between the core and the outer environment decreased, leading to a decrease in the release rate. A lower release burst was observed for EPC. It should be noted that the release test was repeated three times and the results were similar.

In comparison to drug release from EPC liposomes/chitosan and DPPC liposomes/ chitosan, it was observed that the drug release in EPC liposome/chitosan was sudden and explosive. This sudden release was due to the difference in tacrolimus drug concentration on both sides of the layer. For EPC liposome/chitosan, 95% of the drug was released in the first 4 days and after this sudden release, the tacrolimus drug concentration gradient between the nucleus and the external environment decreased and slowed down the drug release rate. In the drug release from DPPC/ chitosan liposome, 83% of the drug was released in 9 days and after that, the release of the drug was on an upward trend and was exponential. It can be concluded liposome of EPC/chitosan is not a good choice for drug loading.

In-vitro release using Franz diffusion test

The results are shown in Fig. 8 and 9. The rate of drug release in the *ex-vivo* test was different compared to the *in-vitro* test. However, similarities were seen in the drug-release behavior.

The difference in the drug release rate for liposome EPC 80/chitosan and EPC 100/chitosan was measured as 83.6 and 93.1%, respectively and for liposomes DPPC60/chitosan and DPPC100/ chitosan were obtained 72.8% and 78.8%, respectively. However, the similarity of drug release is in the behavior of drug release. For EPC liposome/chitosan, drug release is still sudden and explosive so 70% was released in the first five days.

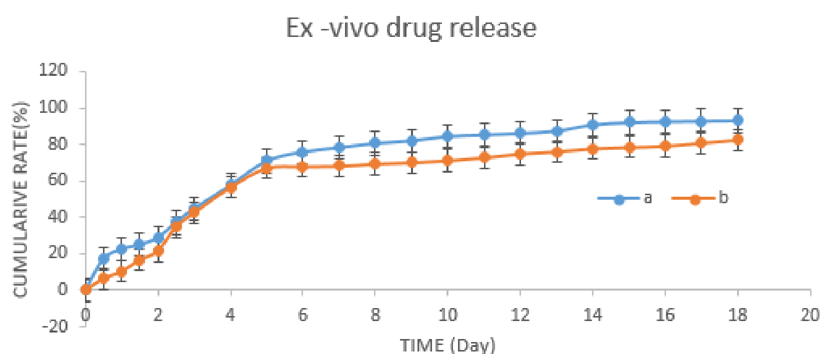

Fig. 8. Franz-diffusion of TOC from a) EPC100/chitosan and b) EPC80/chitosan (pH=7.4)

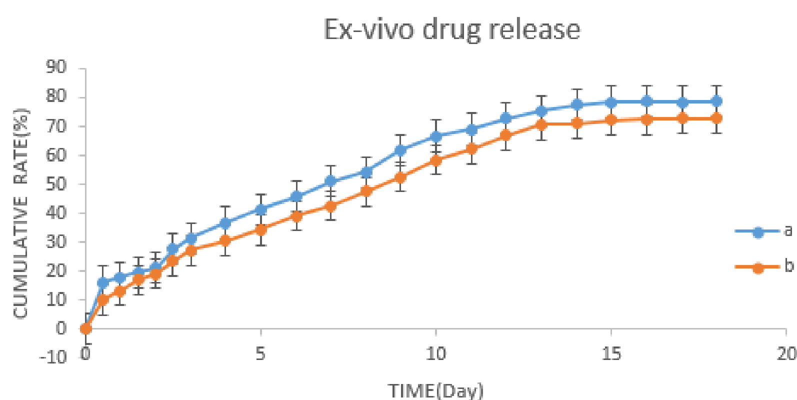

Fig. 9. Franz-diffusion of TOC drug from a) DPPC100/chitosan, b) DPPC60/chitosan (pH=7.4)

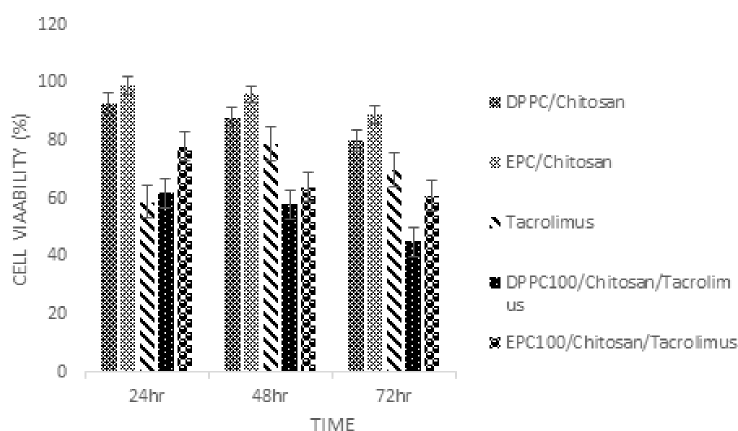

Fig. 10. The effect of nanocarriers, drug and drug-loaded with nanocarriers on the viability of Jarket cells

However, the drug release for DPPC liposome/chitosan was measured at 61.8% in nine days.

Cell studies

To evaluate the effect of cytotoxicity of nanoparticles loaded with tacrolimus drug, the MTT method was used. According to previous studies [66], the appropriate concentration for the tacrolimus drug was 0.2 mg/g. The effect of nanoparticles on the survival rate of NRK tubular epithelial cells was measured in three time periods of 24, 48, and 72 h after exposure by MTT test. The viability of cells were tested in the presence of DPPC 100/chitosan samples and EPC 100/chitosan samples with a concentration of 0.2 mg in distilled water, DPPC 100/chitosan/tacrolimus samples and EPC 100/chitosan/tacrolimus with a concentration of 0.2 mg/mL, and tacrolimus free drug with a concentration of 0.2 mg/mL. The results are shown in Fig. 10. Obviously, nanocarriers without tacrolimus drug did not show any toxic effect for cellsand the survival rate of cells after 72 hr was 92% for EPC/100 chitosan and 89% for DPPC/100 chitosan. In comparison with the cell viability in the presence of the free drug tacrolimus and drug-loaded nanocarriers with the same concentration, the cell viability after 72 hr decreased from 78% for the EPC 100/chitosan/tacrolimus system and from 62% to 45% for the DPPC 100/chitosan/tacrolimus system, which indicates the effectiveness of the drug delivery system.

Stability studies

Because liposomes may clump during storage or release and have an increase in size, the size of liposomes was measured one month after being stored at 4 °C and also after being left in the saline phosphate buffer at 37 °C, as shown in Table 5. Based on Table 5, formulations are almost reasonably stable. The maximum increase in the particle size was 24%, and no clumping behavior was observed after one month of storage and release. Also, micron-sized particles have not been seen. The diameter of all liposomes increased after release. However, this increase in diameter decreased with the increase in cholesterol percentage.

CONCLUSION

This research showed that the size and stability

Table 5. Size of the loaded liposomes after one month of storage at 4 °C and after release in the in-vitro environment

	Size measurement, nm (PDI)		
Types of liposomes	D0	Warehouse after one month (D30)	After *in vitro* drug release
CS/DPPC60/TAC	109±0.6	111±0.7	114±0.9
CS/DPPC100/TAC	99±0.5	121±0.3	110±0.4
CS/EPC80/TAC	95±1.4	99.5±0.5	98±0.05
CS/EPC100/TAC	106±1.03	109±1.5	108±2.2

of the liposome, the efficiency of the drug stored in the nucleus, and the speed of drug release depend on type of the lipid and cholesterol concentration. Liposomes made of saturated lipids (EPC) were larger than liposomes made of unsaturated lipids, DPPC. The size of the liposomes increased when the concentration of cholesterol enhanced, and the amount of cholesterol compression depends on the type of lipid and its concentration. A high amount of tacrolimus drug loading efficiency (88.95-95.74%) was obtained for its entrapment in the core of liposome and chitosan. The loading efficiency for EPC and DPPC was above 90%. The presence of cholesterol in the structure decreased the loading efficiency. Since the mechanism of drug storage inside the liposome was permeation, and addition of cholesterol to the bilayer was another barrier to tacrolimus movement, which led to a decrease in penetration speed and loading efficiency. The particles were completely stable at 4°C for 1 month.

Study highlights

What is the current knowledge?

- Nanostructured carriers are emerging as minimally invasive drug delivery systems.
- Nanostructured carriers can maintain the concentration of therapeutic drugs in the posterior part of the eye for a long time and avoid the need for frequent injections.
- The small size of nanostructured carriers prevents eye irritation.
-

What is new here?

- Development of a suitable structural formula for the preparation of liposome and chitosan
- Simultaneous achievement of high drug loading
- Controlled release and stability of liposome and chitosan
- The type and composition of the appropriate molar percentage of lipid, cholesterol, and chitosan to develop the optimal system
- Optimum initial ratio of lipid/drug/chitosan

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CONFLICTS OF INTEREST

All authors declare no conflict of interest.

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