

Study of ocular drug delivery system using drug-loaded liposomes

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Abstract

Background: This project aims to formulate and characterize a drug-eluting contact lens to provide controlled release of drug for a longer period of time, using liposomes as drug delivery system. **Materials and Methods:** Drug delivering contact lenses were created by coating disposable soft contact lenses with ciprofloxacin entrapped in the liposomes. Reverse phase evaporation and lipid film hydration methods were used for the preparation of ciprofloxacin trapping reverse phase evaporation vesicles, that is, unilamellar vesicles (REV) and multilamellar vesicles (MLV), respectively. Soya lecithin and cholesterol (CH) were used in the molar ratios of 7:4 and 7:2. The spherical structure of the liposomes, the mean diameter, and their purity were determined by photomicroscopic, transmission electron microscope, and chromatographic analysis, respectively. The prepared liposomes were evaluated for their entrapment efficiency, *in vitro* drug release, stability, and toxicity. **Results:** MLVs were larger than REV with their mean diameter 338.32 nm and also entrapped greater amount of ciprofloxacin. Drug loading and its release from the liposomal vesicles was dependent on CH content. Ciprofloxacin released from the liposomes coated on the contact lenses not only inhibited both *Staphylococcus aureus* and *Pseudomonas aeruginosa* on an agar plate but also showed an enhanced antibacterial effect as determined by minimal inhibitory concentrations. Approximately 40% of ciprofloxacin was retained up to a period of 3 months at 4°C. Furthermore, the formulation was found to be nontoxic and also a reduction in toxicity of ciprofloxacin was observed after entrapment when assessed by lymphocyte toxicity assay and chick embryo inoculation. **Conclusions:** An innovative drug delivery system consisting of drug-loaded liposomes coated onto the surface of contact lenses has been developed. This system is highly specific in terms of localized and sustained application of the drug.

Key words: Ciprofloxacin, disposable soft contact lenses, ocular infection, targeted drug delivery

INTRODUCTION

A major issue in ocular drug delivery is the maintenance of an adequate concentration of drug in the precorneal area. About 90% of eye medications are applied in the form of drops. This may lead to overdose and side effects,^[1] followed by a period of subtherapeutic drug levels before the next dose is administered.^[2] This indicates the need for a continuous slow release drug delivery system. Drug-loaded liposomes coated on hydrogel soft

contact lens may fulfil the need for an ophthalmic drug delivery device that is convenient and at the same time will localize and maintain drug delivery at the site of application.^[3] Liposomes are typically composed of natural, nontoxic lipids, so they are expected to be biocompatible and biodegradable.^[4] The release characteristics of the drug from the liposome trapped in the hydrogel matrix shows that liposomes might evolve into effective drug carriers.

The overall aim of this project is to develop a disposable soft contact lens that can be used to deliver drugs to the eye by entrapping an ophthalmic drug inside a liposome–nanoparticle and then coating the drug-laden particles into the contact lens.

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MATERIALS AND METHODS

Materials

Soya lecithin, cholesterol (CH), acetone, absolute alcohol, chloroform, methanol, diethyl ether, peptone, beef extract, and sodium chloride, potassium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from HiMedia Laboratories, Mumbai, India. Ciprofloxacin injectible, Ranbaxy

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200 mg/100 mL and disposable contact lenses. For transmission electron microscope (TEM), 300-mesh copper grids coated with 1% formvar were obtained from ACTREC, Navi Mumbai, India. TEM was carried out in TIFR, Mumbai, India.

Organisms: *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Methods

Liposome preparation and drug entrapment using soya lecithin and cholesterol in the molar ratio 7:2 and 7:4 Preparation of oligolamellar and unilamellar liposomes by reverse phase evaporation

Ciprofloxacin-containing unilamellar and oligolamellar (REVs) liposomes were prepared using the reverse-phase evaporation technique. Soya lecithin and CH (200 mg), in the molar ratios 7:2 and 7:4 were accurately weighed and transferred into a round-bottom flask and dissolved in chloroform:methanol mixture (2:1, vol/vol). A thin lipid film was formed on the inner side of the flask by evaporating the organic solvents under vacuum using a rotary evaporator (model Universal, R/160) at 40°C. The lipid film was redissolved in ether, in which the reversed-phase vesicles would be formed. The drug solution (20 mg) in acetone together with 6 mL phosphate-buffered saline (PBS, pH 7.4) was added. The system was sonicated for 4 min in a bath-type sonicator. The mixture was then placed on the rotary evaporator and the organic solvent was removed. The liposomes were allowed to equilibrate at room temperature, and 10 mL PBS was added to the liposomal suspension, which was kept in the refrigerator overnight. All the above-mentioned steps were performed under aseptic conditions.^[5]

Preparation of multilamellar liposomes by lipid film hydration

Multilamellar vesicles (MLVs) containing ciprofloxacin were prepared using the lipid film hydration technique. Neutral MLVs were composed of Phosphatidyl choline and CH mixed in different molar ratios, that is, PC:CH (7:2) and (7:4). The lipid components (200 mg) were dissolved in chloroform:methanol mixture (2:1, vol/vol) in a round-bottom flask. Then, 20 mg ciprofloxacin dissolved in acetone:methanol mixture (4:1, vol/vol) was added to the lipid solution. The organic solvents were slowly removed using rotary evaporator at 40°C such that a very thin film of dry lipid was formed on the inner surface of the flask. The dry lipid film was slowly hydrated with 10 mL of PBS (pH 7.4). The liposomal suspension was mechanically shaken for 1 h using a mechanical shaker. The liposomal suspension was left to mature overnight at 4°C, to ensure full lipid hydration. For sterility, all of the above-mentioned steps were done under aseptic conditions.^[5]

Separation of free drug

Free untrapped drug was separated from ciprofloxacin REVs and MLVs liposomes by centrifugation at 20,000g for 1 h at 4°C using a refrigerated centrifuge (model Compact, R-4C DX, Remi Electronixs, Mumbai, India). The pellets formed were washed twice with 10 mL PBS and recentrifuged again for 1 h.^[5]

Liposome characterization

Photomicroscopic observation

The liposomes were examined under a photomicroscope at an original magnification $\times 400$, using a fitted camera (model Olympus, DP 25), to determine their physical morphology.^[5,6]

Transmission electron microscopy observation, TIFR

The sizes of the REVs and MLVs liposomes were analyzed with a TEM (model LIBRA 120, Carl Zeiss, Bangalore, India) at an accelerated voltage of 120 kV. For sample preparation for TEM analysis, a drop of homogeneous liposome solution was placed on a 300-mesh copper grid precoated with a 0.1% formvar film.^[7]

Paper chromatography

With the help of this technique, qualitative analysis of the purity of liposome was carried out using soya lecithin as a standard.^[8] Application of the sample was done by spotting the extract after which the paper was dried. Separation of the components takes place in the glass tank that is already saturated and containing the developing solvent to about the length of 3–5 mm for 2–3 h.

Immobilization of the intact drug-loaded liposomes on soft contact lenses

For developing drug delivering disposable soft contact lenses, immobilization of the intact drug-loaded liposomes was carried out onto the soft contact lenses by soaking the contact lenses in the ciprofloxacin–liposomal formulation for about 1 h at 37°C before using.^[9,10]

Determination of trapping efficiency of liposomes by UV absorption at 265 nm

The percentage of drug entrapped was determined after lysis of the prepared liposomes with absolute alcohol and sonication for 10 min. The concentration of ciprofloxacin in absolute alcohol was determined spectrophotometrically at 265 nm using a UV–Vis spectrophotometer, (model UV-1601 PC, Shimadzu, Kyoto, Japan).^[5,11] The entrapment efficiency expressed as entrapment percentage was calculated through the following relationship:

$$\text{Entrapment efficiency percentage} = \frac{\text{entrapped drug} \times 100}{\text{total drug}}$$

MIC determination by broth dilution method

The antimicrobial activity of drug-loaded liposomes was determined in comparison with that of the free drug, with MICs determined by using the standard broth dilution assay. Nutrient broth was used and the test cultures used were *S. aureus* and *P. aeruginosa*.^[12–14] Stock solutions of drug-loaded liposomes and standard drug (10 $\mu\text{g}/\text{mL}$) were prepared and diluted as proposed by National Committee for Clinical Laboratory Standards. A total of 5 concentrations of each sample were prepared, and 0.1 mL of test suspension containing 10^7 cfu/mL was added to each tube. A positive control (growth) consisting of organisms in broth, a negative control (sterility) consisting of uninoculated broth, and

drug-free liposomes were included for both the bacterial strain tested. Tubes were sealed and incubated at 37°C for 24 h.

In vitro drug release study

Agar diffusion

Soft contact lenses coated with drug-loaded liposomes was placed on the surface of seeded plate to study the diffusion of the drug from the lenses. Soft contact lenses coated with drug and drug-free liposomes were also plated. Nutrient agar was used and the test cultures used were *S. aureus* and *P. aeruginosa*.^[15]

UV-Vis spectrophotometry at 265 nm

Drug release from liposomes was studied using a dialysis method. 10 mL of the drug-loaded liposomal formulations were placed in a dialysis bag (Spectra/Por dialysis membrane, 12,000–14,000 molecular weight), which was immersed in 40.0 mL phosphate buffer (pH 7.4). The temperature and stirring rate were 37°C and 50 rpm, respectively. Control bag containing only ciprofloxacin hydrochloride solution was prepared and tested along with the liposomal dispersions in a separate vessel at the same time. Aliquots of 2 mL of the release medium were withdrawn for analysis at 30-min intervals and replaced with fresh medium. Release runs were continued for 8 h. The absorbance of the collected samples was measured at 265 nm.^[16]

Physical stability and drug retention study in storage at 4°C for 3 months

Physical stability study was performed to investigate the leak out of the drug from liposomes during storage. For this liposomes were sealed in 20-mL glass vials and stored in refrigerator at 4°C for a period of 3 months. Samples from each liposomal formulation were withdrawn at definite time intervals and the residual amount of the drug in the vesicles was determined after separation from untrapped drug as described previously under the separation of free drug and entrapment efficiency.^[15,17,18]

Toxicity testing

Human lymphocyte toxicity assay using trypan blue dye exclusion method

Human lymphocytes were isolated from the peripheral blood using Ficoll hypaque density gradient centrifuge method. They were mixed with PBS (control), liposomes, drug-loaded liposomal formulations, and free drug and incubated for 3 h at 37°C. The percentage cell death was observed by the uptake of the dye trypan blue by dead cells or exclusion of the dye by viable cells by conventional microscopy.

Chick embryo inoculation

Fertilized eggs, after 5 days of incubation, were divided into 4 batches of 10 eggs each. The eggs were cleaned with absolute alcohol and it was punctured at the broad end with a sterile hypodermic needle. The 4 batches of the eggs were injected with 0.05 mL of PBS (control), liposomes, drug-loaded liposomal formulations, and free drug into the yolk sac and sealed with sterile coverslips. After incubation of the inoculated eggs at 37°C

for 24 h, the eggs were opened and the viability of the embryo was noted on the basis of their heart beats.^[19-21]

RESULTS AND DISCUSSION

Liposome preparation and drug entrapment

The prepared MLV formulations were clear, homogenized viscous solutions with bluish color, whereas the REVs were light-yellowish to brownish in color. It was found that homogeneous solution of REVs was prepared by ultrasonication, whereas agitation produced larger nonhomogeneous MLVs, which explain the color difference. The pH values of the prepared liposomal formulations were within the limits of 7.0–7.5.

Liposome characterization

Photomicroscopic observations [Figure 1] of the prepared liposomes reveal the presence of well-identified spheres with large internal aqueous space. It is noted that MLV liposomes are larger in size than REV liposomes. The sizes of prepared liposomal particles varied in the range of 40–400 nm, however, the mean diameter of MLVs and REVs was estimated to be 338.32 and 120.42 nm, respectively, by TEM analysis [Figure 2]. Figure 3 shows a chromatogram of standard phospholipid, that is, soya lecithin and prepared liposomal formulations that were applied as a single spot. Phosphatidyl compounds moved together with the corresponding phosphatidyl analog. No hydrolysis of phosphatidyl compounds was evident, as indicated by the clear well-defined spots matching with that of the standard soya lecithin.

Immobilization of the intact drug-loaded liposomes on soft contact lenses

The contact lenses coated with drug-loaded liposomes were used for drug release study. Since coating of drug was through physical adsorption, efficiency of immobilization was not determined.

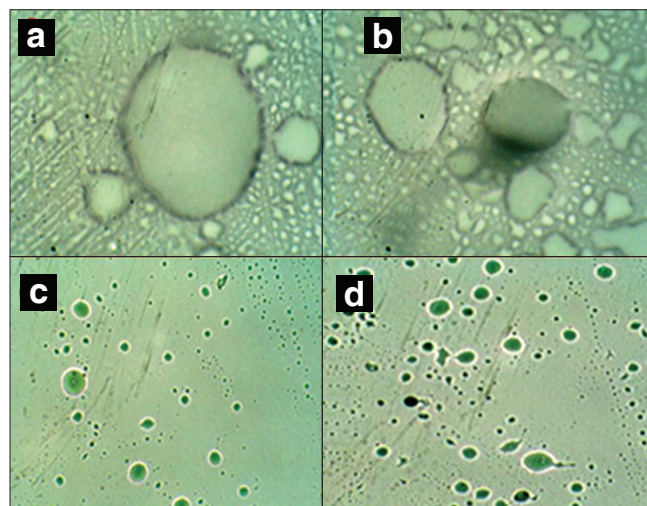


Figure 1: Photomicrograph of ciprofloxacin liposomes. (a) 7:4 MLVs; (b) 7:2 MLVs; (c) 7:4 REVs; (d) 7:2 REVs

Entrapment efficiency

The percentage entrapment efficiency of drug into reverse-phase evaporation liposomes was 27.5% and 15.625% for the molar ratios 7:4 and 7:2, respectively, and the values recorded for MLV liposomes were 58.75% and 41.875% for the molar ratios 7:4 and 7:2 (PC:CH), respectively [Figure 4]. Statistical analysis of the entrapment efficiencies shows significant difference between all the 4 types of liposomes by one-way analysis of variance (ANOVA) at $P < 0.001$. The results of one-tailed unpaired t test using 2 samples assuming equal variances indicate that a great difference exists between the MLVs and REVVs ($P < 0.001$). The entrapment efficiencies had higher values in case of MLV liposomes than in REV liposomes of the same composition and molar ratio. The MLVs are spherical in shape and contain internal granular structures. A lipid bilayer forms the outermost membrane and the internal space is divided into small compartments by bilayer septum. The REVVs are restricted in terms of the aqueous space for entrapment, and thus they have very low entrapment efficiency for water-soluble biologically active components. The MLVs, on the other hand, entrap a high percentage of the initial aqueous phase, and thus they can have

high entrapment efficiency. This may be because MLVs are larger in size. Also they contain multiple lamellae, which are capable of loading a drug of higher mass compared with reverse-phase evaporation vesicles.

MIC determination

It was found that drug encapsulated in liposomal formulations exhibited antibacterial activity against both *S. aureus* and *P. aeruginosa* [Figure 5]. The simple adsorption of liposomes on bacterial cells changes the surface charge from negative to positive, which leads to the death of the microbes.^[22,23] The one-way ANOVA indicates significant differences ($P < 0.001$) in MICs between free drug and liposomal formulations. Furthermore, one-tailed unpaired t test using 2 samples assuming equal variances showed that both *P. aeruginosa* and *S. aureus* are equally sensitive to the formulations ($P < 0.2$). Also, the 7:4 CH molar ratio of liposomes showed greater antimicrobial activity as compared with 7:2 ratio as well as with free drug at $P < 0.001$.

In vitro drug release study

Agar diffusion

When drug-loaded liposomes were placed onto a seeded agar

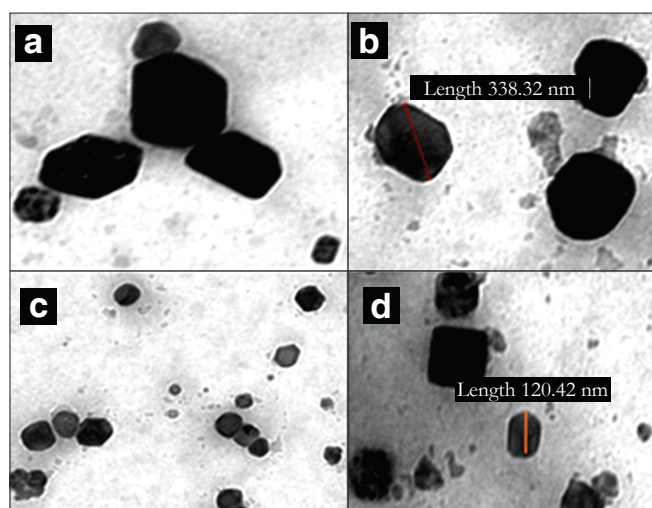


Figure 2: TEM images of ciprofloxacin liposomes. (a) 7:4 MLVs; (b) 7:2 MLVs; (c) 7:4 REVVs; (d) 7:2 REVVs

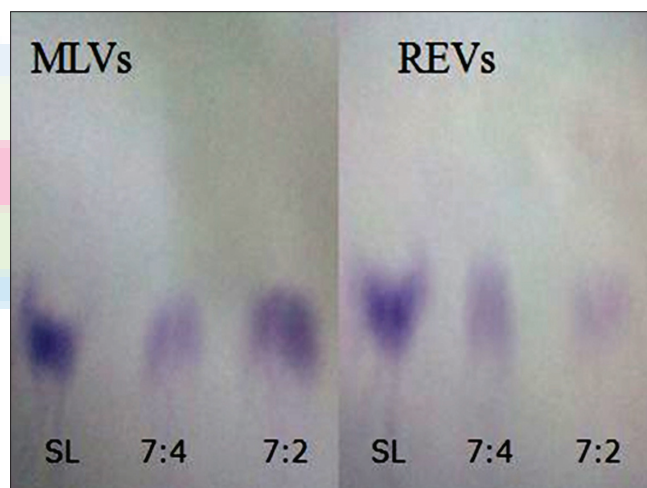


Figure 3: Paper chromatogram of liposomal formulations. (SL- soya lecithin)

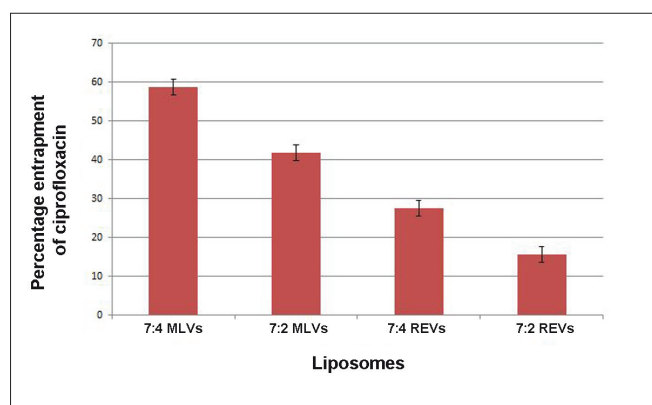


Figure 4: Percentage entrapment of ciprofloxacin in the prepared liposomal formulations (Error bars indicate standard deviation)

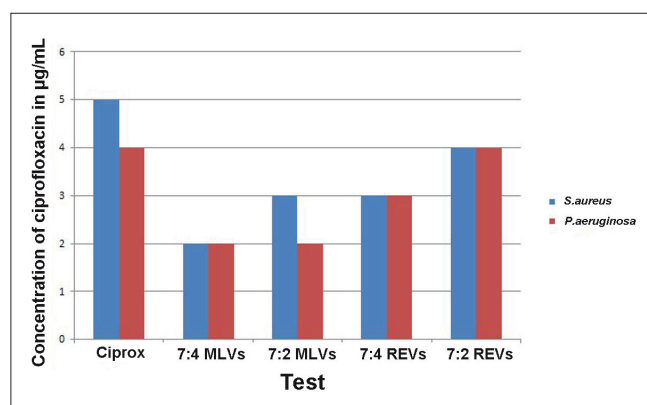


Figure 5: MIC determination of the prepared liposomal formulations. (Zero standard deviation)

plate, the zones of inhibition were much higher as compared with those obtained when only drug-coated lenses were used. No inhibition was observed using contact lenses coated with empty liposomes [Figure 6].

A significant difference in the diameter of zone inhibition has been recorded by one-way ANOVA ($P < 0.001$). According to one-tailed unpaired t test using 2 samples assuming equal variances, MLVs were found to be more effective as compared with the REVs in inhibiting both the test pathogens *P. aeruginosa* and *S. aureus* at $P < 0.001$. Both the CH molar ratios 7:4 and 7:2 had similar effect with both types of liposomes at $P < 0.1$. This shows that the CH does not impart antibacterial activity to the formulation. Furthermore, the diameters of the zones of inhibition were greater in case of *P. aeruginosa*, which showed that these are more sensitive to the drug as compared with *S. aureus*. These results were further supported by t test using 2 samples assuming equal variances at $P < 0.001$. The enhanced antimicrobial activity of these formulations is due to their fusion with the bacterial outer membrane.^[24]

UV-Vis spectrophotometry at 265 nm

By dialysis method, the concentrations of drug released from liposomal formulations after 8 h was determined. In case of the formulations, approximately 10%–30% of the drug is released at a relatively rapid rate during the first 2 h, followed by slower release rates over the next 10 h [Figure 7]. Initially the release of drug was fast and is due to detachment from liposomal surface, whereas later on there was slow release, which was due to sustained drug release from the inner lamellae. Complete release of drug was observed in less than 3 h. After 3 h, however, only about 35%–50% of the drug was released from the formulations. Thus, compared with free drug, its release from the liposomes is prolonged.

One-way ANOVA showed a highly significant difference in the drug release profile of the formulations and free drug, that is, ciprofloxacin ($P < 0.001$). From the P values obtained from one-tailed unpaired t test using 2 samples assuming equal variances [Table 1], it was found that the release of drug is slower from MLV liposomes than from REV liposomes of the same lipid

molar ratio. The MLVs consist of several concentric spheres of lipid bilayers separated by aqueous compartments. Therefore, MLVs would play a role as lipid reservoirs.

From the release profiles, it is found that increasing the CH molar ratio in the prepared liposomal formulations progressively decreased the release of drug from the vesicles. This is because CH modulates membrane fluidity and reduces bilayer permeability, and hence decreases the efflux of the encapsulated drug, resulting in prolonged drug retention.

Physical stability and drug retention study

After 90 days, the retention percentages of the drug in the liposomal formulations were 43.62%, 32.85%, 36%, and 15.9% for MLV and REV liposomes for the molar ratios 7:4 and 7:2, respectively [Figure 8]. One-way ANOVA showed that there is no obvious difference in physical stability between reverse-phase evaporation and MLV liposomes prepared with the same lipid molar ratio when stored in the refrigerator ($P < 0.1$). However, after 3 months there exists a significant difference in the percentage of the drug retained at $P < 0.005$. The one-tailed unpaired t test using 2 samples assuming equal variances indicate that the difference in the drug retention was not much significant between the MLV and REV liposomes ($P < 0.1$).

With respect to the effect of CH on drug retention, the liposomes prepared with the CH molar ratios 7:4 were found to retain more drug after 3 months as compared with those prepared using the CH molar ratios 7:2 ($P < 0.05$). This is because CH modulates membrane fluidity and reduces bilayer permeability, and hence

Table 1: P value: one-tailed t test: two-sample assuming equal variances

Liposomal Formulations →	7:4	7:4	7:4	7:2
	MLVs	REVs	MLVs	MLVs
Time in Hours ↓	7:2	7:2	7:4	7:2
	MLVs	REVs	REVs	REVs
8 h	0.041	0.0007	0.038	0.17
24 h	0.067	0.0001	0.0003	0.29

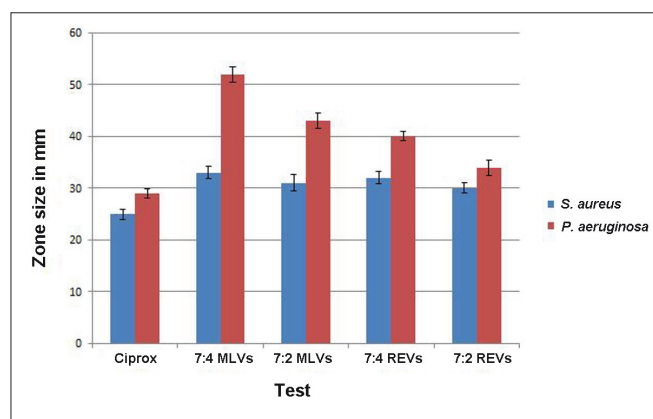


Figure 6: Zones of inhibition obtained by agar diffusion using coated lenses (Error bars indicate standard deviation)

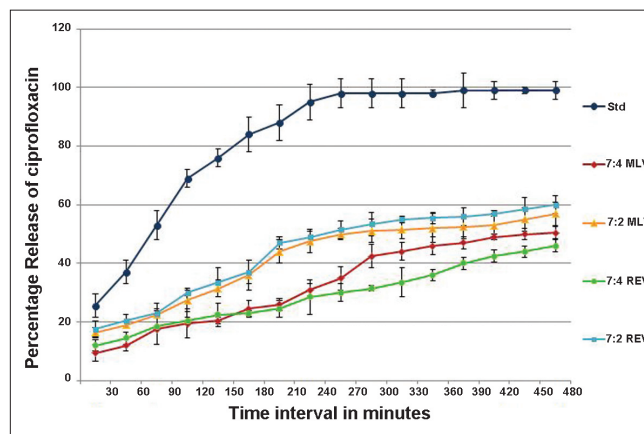


Figure 7: In vitro drug release study with respect to time. (Error bars indicate standard deviation)

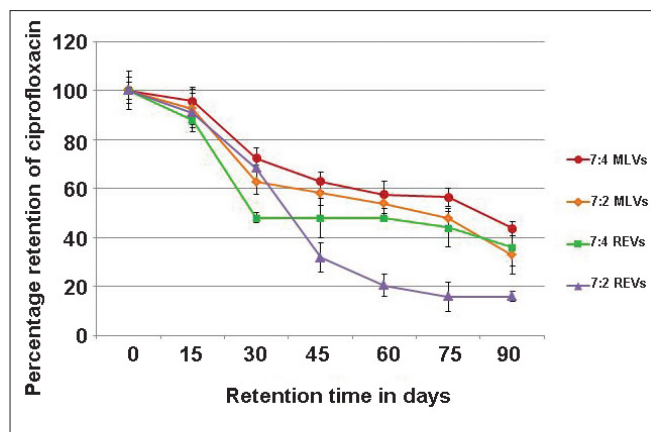


Figure 8: Percentage drug retention in the prepared liposomal formulations (Error bars indicate standard deviation)

decreases the efflux of the encapsulated drug, resulting in prolonged drug retention.

Despite the partial hydrolysis that would occur for the phosphatidylcholine and the leakage of the drug, the liposomes made from them are sufficiently stable under refrigerator storage and all the features of the lipid membrane were retained.

Toxicity testing

Toxicity testing of the liposomes by both lymphocyte toxicity assay and chick embryo inoculation method revealed that they are nontoxic and safe for use.

The percentage viability of lymphocytes was found as 62.61% and 72.44% with free ciprofloxacin and empty liposomes, respectively, whereas 77.1% when drug-loaded liposomes were used. In case of chick embryo inoculation method, only 40% of the chicks inoculated with the drug were viable, whereas 80% of chicks were found to be viable when they were inoculated with both empty liposomes and drug-loaded liposomes. Free drug was found to be toxic to both the human lymphocytes and the chick embryo. However, the toxicity of drug is found to be reduced when it is entrapped in the liposomes as compared with free drug^[25-27] [Figure 9].

CONCLUSIONS

From the comparison study between ciprofloxacin REV and MLV liposomal formulations, it could be concluded that the MLV liposomes are larger in size than REV and exhibited higher values of entrapment efficiencies. The release of ciprofloxacin is slower from MLV liposomes than from REV liposomes of the same lipid molar ratio. MLV liposomes produced a more sustained action than REV liposomes because of the presence of several lipid bilayers that release the drug slowly over a prolonged period of time.

The drug entrapped in liposomes when coated on lenses has a much longer residence time of about 24 h *in vitro* as compared

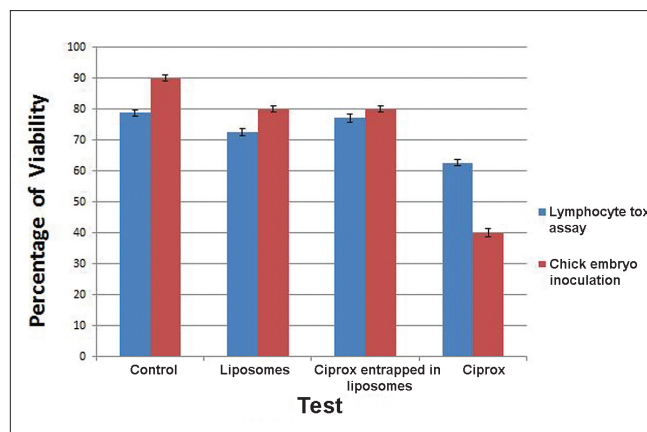


Figure 9: Toxicity assay of the liposomes (Error bars indicate standard deviation)

with 2–5 min residence time of the same drug when used for topical application as drops. The amount of drug entrapped and the release profile of the drug was found to be affected by CH content. The liposomes were found to be safe as a drug delivering agent. In fact it was found to reduce the toxicity of ciprofloxacin when entrapped in liposomes.

The present study was only a pilot study. More detailed studies must be conducted to develop methodologies to increase drug loading and its retention during storage, improve drug release profile, and factors affecting it and also to determine the toxicity of the formulations prepared before its scale up.

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