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Spanlastics—A novel nanovesicular carrier system for ocular delivery

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ABSTRACT

The work describes usefulness of a novel, surfactants based elastic vesicular drug carrier system (spanlastics), for targeting topically applied drug(s) to the posterior segment of the eye. The system constituted span 60 and a edge activator (tween 80).

Ketoconazole, a lipophilic drug with a large molecular weight of 531.44 Da and a limiting solubility of 0.04 mg/ml is expected to show a poor transport across the cornea; hence no ocular formulations are available. Developed spanlastics were of nanosize and elastic in nature. They showed 2 times better corneal permeation ($p \leq 0.001$) in comparison to correspondingly prepared niosomal formulation. The system was tested for stability for 2 months under refrigerated conditions. It was found to be safe in terms of genotoxicity (Ames test), cytotoxicity (MTT assay; Normal human gingival fibroblast), acute dermal/eye irritation/corrosion and chronic eye irritation/corrosion tests (OECD guidelines). Safety was an important issue considering that the system is novel (Indian Patent Application 2390/DEL/2008; 1447/DEL/2010) and is totally surfactant based (spans plus edge activators). Fluorescent vesicles labeled with 6-carboxyfluorescein when applied topically to the rabbit eye were observed intact in vitreous and the internal eye tissues 2 h post application. Results confirm that spanlastics can be used to deliver drugs to the posterior segment of the eye.

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1. Introduction

Drug delivery to the posterior ocular segment poses significant drug delivery challenges due to its unique anatomical and physiological barriers. The posterior segments of eye require site-specific drug delivery systems to target the vitreous cavity, retinal pigment epithelium and choroid. Diseases affecting the posterior segment, such as age-related macular degeneration, cytomegalovirus, uveitis and fungal infections can cause irreparable vision loss due to inadequate drug levels arising from poor delivery. Ocular fungal infections may involve the cornea (keratitis), the interior of the eye (endophthalmitis), retina (retinitis) or the orbit and may occur following trauma (including surgery) or upon systemic disseminated infection. Fungal infections of the retina are among the most devastating ocular infections (Pettit et al., 1996). The most common among these infections is candidal chorioretinitis, usually caused by *Candida albicans* (Ahuja et al., 2008). *Aspergillus* species is the second most common fungal group that infects the choroid and retina (Riddell et al., 2002).

Present approaches available to deliver drugs to posterior eye like intravitreal injections, periocular routes, systemic administration and topical application are associated with either serious side effects or are not able to deliver drug to posterior eye in therapeutic levels (Geroski and Edelhauser, 2000).

Topical route is preferred route of drug administration, primarily for reasons of better patient compliance and cost affordability. However, topical application is associated with many other complications, such as extensive precorneal drug loss by high tear fluid turnover, non-productive absorption, drainage through the nasolacrimal duct, impermeability of the corneal epithelium, transient precorneal residence time and metabolism of the drug by anterior segment enzymes. The use of colloidal drug delivery systems, such as nanoparticles, nanoemulsions and liposomes, has received much attention as a way to enhance the bioavailability of drugs administered both systemically and topically. Hironaka et al. (2009) showed that liposomes, as a drug carrier system, can target the retina when administered topically as eye drops. They found that submicron-sized liposomes are able to deliver ocular drugs to the eye's posterior segment. Fluorescence emission of coumarin-6 formulated into submicron-sized liposomes was seen in that segment in mice after eyedrop administration of the liposomal suspension.

In 1992, Cevc and Blume introduced first generation of the highly deformable, elastic liposomes, referred to as Transfersomes®. A second generation of elastic vesicles, mainly consisting of non-ionic surfactants, was introduced in 1999 by Van den Bergh et al. In 1997, Touitou et al. developed ethosomes, soft vesicular carriers mainly consisting of phospholipids and ethanol. These elastic vesicles have been found to deeply and

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easily penetrate through the skin (Cevc et al., 1996; Touitou et al., 2000; Vaibhav et al., 2007).

Shen and Tu (2007) developed ganciclovir elastic liposomes which showed significant trans-ocular absorption as compared to the drug solution. The results were attributed to the particle size (200 nm) and the elasticity of liposomes. They proposed that deformable elastic liposomes can enter the corneal structure which is similar to the stratum corneum.

Ketoconazole (KTZ) is a broad spectrum anti-fungal agent, with high lipo-solubility ($\log P = 4$) and tendency to cause side effects upon oral administration (nausea, vomiting, gastrointestinal disturbance, hepatitis, gynecomastia and adrenal cortex suppression) (O'Brien, 1999) and a short ocular half life (elimination half life 19 min in aqueous humor and 43 min in cornea) (Zhang et al., 2008). Also, its absorption is heavily dependent on the gastric pH and possible drug–drug interactions on oral administration. On one side the lipophilicity may help in its permeation across the biological membranes, its large molecular weight (531.44 Da) impedes its transport across the biological membrane. Further, it may be noted that high lipid solubility can ensure its passage across corneal epithelium but further passage through corneal stroma of highly lipophilic molecules is hampered, this indicates a need for a suitable carrier system for such molecules (Barar et al., 2008). Further, the limiting water solubility of 0.04 mg/ml makes it difficult to present it in a solubilised form on the corneal surface, later being a prerequisite for ocular formulations.

Due to physiological and anatomical constraints (Le Bourlais et al., 1998) only a small fraction of the administered drug, effectively 1% or even less of the instilled dose is ocularly absorbed (Shell, 1984). This forces the clinician to recommend a frequent dosing at an extremely high concentration resulting in pulsed bioavailability profiles. This is all the more important with antifungal treatment which involves an aggressive and chronic therapy.

The purpose of this study was to increase the corneal permeability of drugs using a surfactant based novel vesicular system (spanlastic) (IPA no. 2390/DEL/2008; 1447/DEL/2010), taking KTZ as a model drug. Another intent of the study was to evaluate the suitability of developed system to reach the posterior segment of the eye (including the vitreous humor and retina) upon topical administration. The behavior of vesicles labeled with 6-carboxyfluorescein as a fluorescence reagent was investigated using fluorescent microscope after its instillation into the rabbit eye. The safety of the elastic vesicles was also tested *in vivo* as per the OECD guidelines considering the novelty of the system.

2. Methods

2.1. Materials

Ketoconazole was a kind gift from Torrent Pharmaceuticals Pvt. Ltd., H.P., India; cholesterol was obtained from Sigma Aldrich Chemie GmbH and tween 80 from S.D. Fine Chemicals Ltd., India. Caroxyfluorescein as a fluorescent marker for vesicles was purchased from Acros Organics (Geel, Belgium). All other reagents used in the study were of analytical grade.

2.2. Preparation of spanlastics (SVs), control niosomes and 6-carboxyfluorescein loaded vesicles

SVs containing span 60 and edge activator (EA) tween 80 at a ratio of 80:20 (by weight) were prepared by ethanol injection method. Precisely, span 60 and KTZ were dissolved in ethanol and injected into preheated aqueous phase (consisting of tween 80 for SVs) which was stirred continuously on a magnetic stirrer. KTZ was used at a concentration of 10 mg/ml for the preparation of vesicles. Corresponding niosomal formulation was developed with span 60 and cholesterol, for comparison.

SVs loaded with 6-carboxyfluorescein (0.1%, w/v) (Cf SVs) were also prepared similarly. Free carboxyfluorescein was removed from the system by dialysis prior to application.

2.3. Preparation of drug suspension

KTZ was added to water containing 1% (w/v) tween 80 as a dispersing agent and stirred for 3 h. The final suspension contained 1% (w/v) of KTZ and was used as a control for the niosomal and SV formulation of KTZ.

2.4. Characterization of spanlastics

2.4.1. Morphology and vesicle size

SVs were characterized by using optical microscope (Nikon eclipse i90) and transmission electron microscope (TEM) for structural attributes such as lamellarity, uniformity of size, shape and physical stability characteristics i.e. aggregation and/or irregularity. The size of vesicles was determined by Zetasizer (Malvern instrument, UK). Before measurements, the vesicular suspension was diluted with water.

2.4.2. No. of vesicles per cubic millimeter

Vesicles were suitably diluted with water, and the number of vesicles per cubic mm were counted by microscopy using haemocytometer. The vesicles in 80 small squares were counted and no. of vesicles/mm^3 were calculated using the following formula (Chatterjee, 1985):

$$\text{total no. of vesicles per cubic mm} = \frac{\text{total no. of vesicles counted} \times \text{dilution factor} \times 4000}{\text{total number of squares counted}}$$

2.4.3. Total drug content

Isopropyl alcohol was chosen as a suitable solvent for disrupting the prepared vesicles. Aqueous dispersion (1 ml) was disrupted using sufficient quantity of isopropyl alcohol and the absorbance was recorded at 298 nm.

2.4.4. Entrapment efficiency

KTZ SVs were purified by using dialysis bag according to literature (Immordina et al., 2003). Dialysis membrane-70 (Himedia Laboratories Ltd., Mumbai, India) was soaked in 50% ethanol for 1 h before dialysis to ensure complete wetting of the membrane; 1 ml of the drug loaded vesicles were placed into the dialysis bag which was then transferred into 200 ml of 50% ethanol in water. The receiver medium was stirred with a magnetic stirrer. After extensive dialysis, the vesicles were disrupted by isopropyl alcohol and amount of drug was determined spectrophotometrically.

2.4.5. Elasticity measurement

Elasticity was measured (Van den Bergh et al., 2001) by extruding vesicles through the polycarbonate filter (Millipore, USA) of 50 nm pore diameter and measuring the change in size.

2.5. Differential scanning calorimetry (DSC)

For thermal analysis, samples were scanned using DSC and the thermograms so generated were observed/evaluated for any significant shift or disappearance/appearance of new peaks. DSC was performed with a DSC 821^e (Mettler Toledo, Switzerland). The calorimeter was calibrated for temperature and heat flow accuracy using the melting of pure indium (m.p. 156.6 °C and ΔH of

25.45 J gm^{-1}). The temperature range was from 0 to 400 °C with a heating rate of 10 °C/min. The gas used was nitrogen with a purging rate of 50 ml/min. The weight of each sample was 5–9 mg.

2.6. Corneal permeability studies

Ex vivo corneal permeability studies were performed using the membrane diffusion technique. The studies were conducted within a jacketed cell, maintained at a constant temperature (37 ± 0.2 °C), under mixing conditions using a magnetic stirrer. The cell used was a two-limbed reservoir (Aggarwal et al., 2004); on one limb of which cornea was mounted and the other limb was used as the sampling port (volume = 20 ml). The preparation (0.5 ml) to be studied was placed on the cornea. Porcine cornea was used for the study and the cornea was mounted within half an hour of sacrifice of the animals. Diffusion medium used was freshly prepared phosphate buffer pH 7.4 containing 40% ethanol. Aliquots of the medium were withdrawn after a fixed time interval from the sampling port and were replaced with equal quantity of fresh media to maintain a constant volume. Sink conditions were maintained throughout the study. Samples were analyzed spectrophotometrically.

The apparent corneal permeability coefficient (P_{app}) of different formulations was determined as reported previously (Aggarwal et al., 2004).

2.7. Stability studies

Stability studies were carried out to investigate the leaching of drug from SVs during storage. The ability of vesicles to retain the drug was assessed by keeping the selected elastic vesicular suspension in sealed glass ampoules (15 ml capacity) at 25 ± 2 °C, and 4–8 °C for 2 months. Samples were withdrawn periodically and analyzed for aggregation, drug entrapment and residual drug content. The initial entrapment and drug content was considered as 100%.

2.8. In vivo safety studies

Safety assessment for ocular application was approved by the Institutional Animals Ethics Committee, Panjab University, Chandigarh, India and performed as per the details below.

2.8.1. Dermal irritation/corrosion test as per OECD guideline 404 (ADI, 2002)

This test was performed to assess the safety of the sample for dermal application. The test is a preamble to the ocular toxicity studies (AEI, 2002). According to OECD guideline 405, before considering *in vivo* eye irritation/corrosion test, preferably a study of the *in vivo* dermal effects of the substance should be conducted and evaluated in accordance with OECD testing guideline 404.

2.8.2. Eye irritation/corrosion test as per OECD guideline 405 (AEI, 2002)

Three rabbits were used for the study. The right eye of each rabbit received elastic vesicular formulation. The test substance (0.1 ml) was instilled in the conjunctival sac once and also five times at 5 min intervals (*for repeat test*) of right eye of each animal after gently pulling the lower lid away from the eyeball. The left eye served as a control in all the experimental rabbits. The eyes were examined at 1, 24, 48, and 72 h after test substance application based on the described scale. *A chronic repeat dose study* in which five repetitive doses of the vesicular dispersion were instilled everyday, in the conjunctical sac at an interval of 5 min, for a period of one week were also performed. In order to evaluate whether the vesicular system(s) were safe for long term therapy or not. Animals were evaluated for ocular irritation/corrosion based on scale as per OECD guidelines (405).

2.9. Cytotoxicity test using normal human gingival fibroblast cell line

The cytotoxicity of SVs and free drug suspension was evaluated in triplicate using normal human gingival fibroblast cell line. Normal human gingival fibroblast cell line (CDRI, Lucknow, India) were cultured under standard conditions (5% CO_2, 98% humidity, 37 °C) in medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), pyruvic acid (0.11 mg/ml), 0.37% $NaHCO_3$ and 50 μM of 2-mercaptoethanol. The cells were plated in 96-well plates at a density of 0.6×10^6 in 200 μl of medium per well and incubated with different concentrations of test material (the SV dispersion or suspension containing 1% (w/v) of KTZ was diluted with cell suspension to achieve 1, 5 and 10% (v/v)) for 48 h. The medium was refreshed with fresh medium containing 100 μg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. Plates were centrifuged at 200 × *g* and supernatant was aspirated and violet color MTT-formazan crystals dissolved in 100 μl DMSO. Thereafter, plates were stirred for 20 min and OD measured at λ 570 nm (reference wavelength, λ 620 nm) on ELISA plate reader (Thermo Labs, USA). Cell growth was calculated by comparing the absorbance of treated versus untreated cells. The optical density of violet color formazan crystals was directly proportional to the cell viability (Mosmann, 1983; Bhushan et al., 2007). Plot was drawn between % cell viaibility and the different concentrations of tested materials.

2.10. Determination of mutagenic activity (Ames test)

The test was carried out as per details given by Maron and Ames (1983).

2.11. Animal studies

All the animal study protocols were approved by the Institutional Animals Ethics Committee, Panjab University, Chandigarh, India.

2.11.1. Observation of fluorescence in the aqueous and vitreous humor

Un-anesthetized female rabbits (Central Animal House, Panjab University, Chandigarh, India) weighing 1.4–1.7 kg and fed a regular diet were used for the study. A single drop of the Cf SVs was dropped onto the surface of the left eye of the rabbit. The contralateral eye served as control and received an aqueous solution of carboxyfluorescein (Cf S). The animals were sacrificed by an overdose of pentobarbital (100 mg/kg) into the marginal ear vein 4 h after instillation. The whole eye bulbus was enucleated from its socket immediately after sacrificing the rabbits. Aqueous humor and vitreous humor was separated and viewed under fluorescence microscope (Axiovert 200, Carl Zeiss, Germany). In a subsequent experiment, the Cf SVs and Cf S was instilled into the rabbit eye (five times at 5 min intervals) and animals were sacrificed at an interval of 1, 2, 4 and 6 h and the aqueous and vitreous humor were separated and viewed under fluorescence microscope as mentioned before.

2.11.2. Observation of fluorescence in eye tissues (cross-sections of rabbit eye)

The Cf SVs and Cf S were instilled in the rabbit eye and the animal was sacrificed as mentioned above after 2 h of administration of the formulation. Both eyes were enucleated

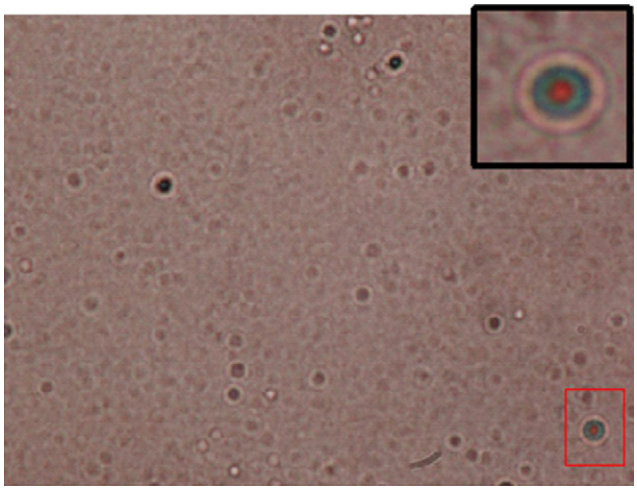

Fig. 1. Optical photomicrographs (400× magnification) of vesicles and inset shows multillamilarity of a single vesicle marked in red (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

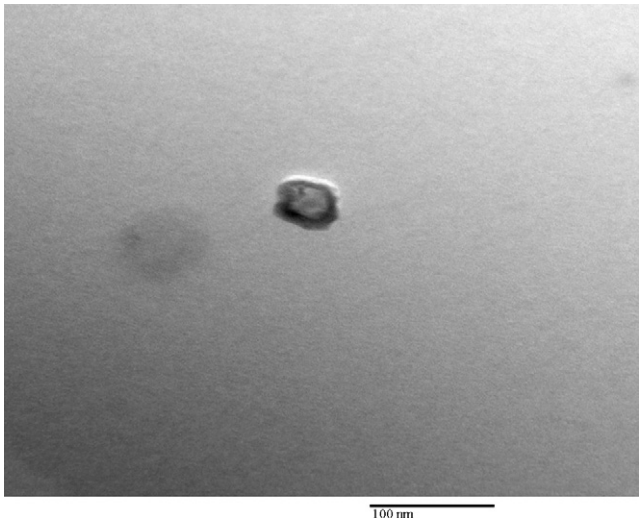

Fig. 2. TEM of vesicles at 2,00,000× magnification.

immediately and washed with excess amount of saline. The samples were sliced into 10 μm thick sections using a cryostat, and placed onto slides under a cover slip and various tissue sections were observed under fluorescence microscope (Nikon eclipse i80, Nikon, Japan).

3. Results

3.1. Microscopic evaluation of KTZ loaded novel SVs

Optical inspection indicated the vesicles to be small in size, round in shape, bilamellar/multilamellar (Fig. 1) and no aggregation irregularities were observed in the system. Similar results were obtained with TEM. SVs appeared as spherical structures, confirming the vesicular characteristics (Fig. 2).

3.2. Size and size distribution

Both SVs (126 nm) and niosomes (154 nm) showed average particle size (Table 1) in the nano range.

3.3. Drug content

Actual amount of KTZ added for all practical purposes into the vesicular dispersions was 10.0 mg/ml and the drug content of the developed formulations was not found to be significantly different ($p < 0.001$) from the added amount (Table 1).

3.4. Entrapment efficiency

The ability of the vesicles to entrap KTZ was investigated. Significant differences were observed between the niosomal (35.05%) and spanlastic (68.82%) formulations ($p < 0.001$). Niosomes though large in size, were invariably uni or bilamellar (SVs were multilamellar) in nature and less abundant than SVs (Table 1; Fig. 1).

3.5. Elasticity measurement

The extremely high flexibility of the membrane of SVs permits them to squeeze through pores much smaller than their own diameters. This deformability characteristic permits SVs to penetrate spontaneously through the biological membranes, minimising the risk of complete vesicle rupture while squeezing through. The developed SVs were tested for a possible deformable characteristic. Developed nanospanlastics showed a much smaller change (12%) in average particle size (Table 1) in comparison to niosomes showing a large decrease in size of 26.28% after passage through 50 nm polycarbonated membrane.

3.6. Differential scanning calorimetry (DSC)

DSC is a fast and reliable method to screen drug-excipient interactions as indicated by appearance of a new peak(s), change in the peak shape and its onset, peak temperature/melting point and relative peak area or enthalpy. Fig. 3 depicts various DSC thermograms obtained during the study. Pure ketoconazole showed a sharp endothermic peak at 149.05 °C (m.p. 148–150 °C). Thermogram of span 60 exhibits an endothermic peak with onset at 46.03 °C and maximum occurrence at 50.65 °C. Tween 80 is reported to show an exothermal peak at −43 °C and an endothermal peak is observed at −14 °C (Hillgren et al., 2002). It is evident that the original peaks of KTZ, span 60 and tween 80 disappear from the thermogram of SV dispersion. These observations confirm incorporation of KTZ into SVs.

Span 60 vesicles showed transition temperature (T_m) of 92.89 °C (Fig. 3d) while a decrease in T_m (61.94 °C, Fig. 3e) value was

Table 1
Particle size, extent of entrapment, and % deformation of spanlastics and niosomes.

Formulation	Lamellarity	No. of vesicles (mm^3)	Average particle size (nm)	Drug content (mg/ml) ($n = 6$)	% Entrapment ($n = 6$)	% Change in particle size
Niosomes	Bilamellar, unilamellar	40,500	154 ± 6.4	9.98 ± 0.01	35.05 ± 0.35	26.28
SVs	Multilamellar, bilamellar	51,500	126 ± 4.7	9.99 ± 0.01	68.82 ± 0.42	12.00

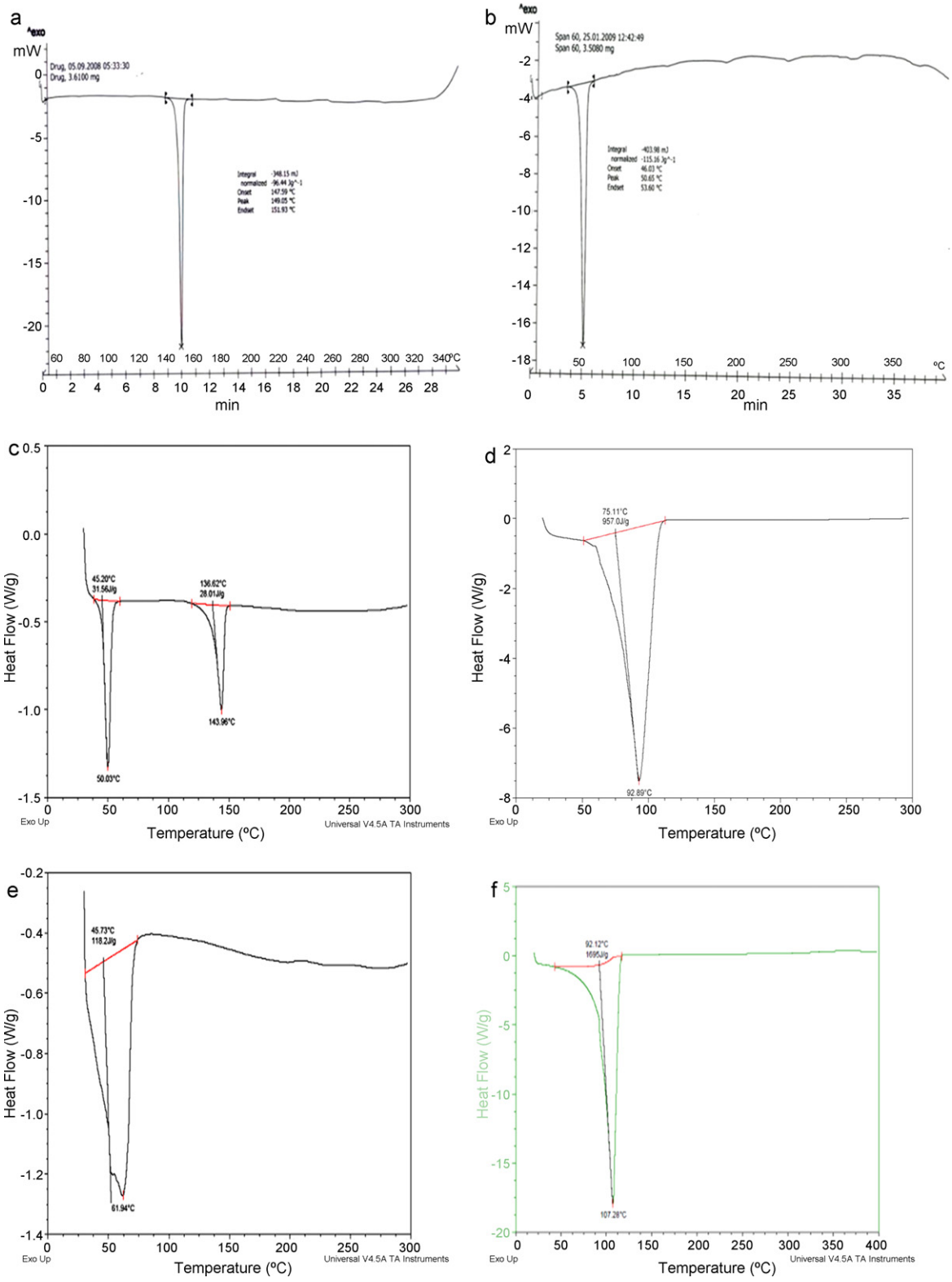

Fig. 3. Differential scanning calorimetry (DSC) thermograms of ketoconazole, Span-60, physical mixture, blank Span 60 vesicles, blank SV dispersion and drug loaded SV dispersion. (a) DSC of ketoconazole. (b) DSC of Span-60. (c) DSC of physical mixture. (d) DSC of blank Span 60 vesicles. (e) DSC of blank SV dispersion. (f) DSC of drug loaded SV dispersion.

observed for blank spanlastics (without drug). Further, the physical mixture (Fig. 3c) showed individual peaks for span 60 and the drug while a single peak was observed for drug loaded spanlastics (Fig. 3f), proving interaction of drug with the vesicle bilayers.

The DSC thermogram of drug-loaded spanlastic dispersion (Fig. 3f) interestingly showed transition at 107.35 °C, indicating an increase in the phase transition temperature of spanlastics upon loading with KTZ. Drug loaded spanlastic dispersion showed a broad endotherm showing good interaction of all components.

Table 2
Comparison of various formulations in terms of total amount permeated, % permeation and apparent permeability co-efficient P_{app} from ex vivo permeation studies using pig cornea ($n = 3$).

Formulation	Total amount permeated in 5 h (μg)*	Steady state flux (μg/min/cm^2)*	Apparent permeability coefficient P_{app} (cm/s)#
Niosomes	860.30 ± 9.7	2.04 ± 0.022	$5.92 \times 10^{-6} \pm 1.1 \times 10^{-6}$
SVs	1153.00 ± 13.4	2.56 ± 0.045	$7.47 \times 10^{-6} \pm 1.6 \times 10^{-6}$
Suspension	625.41 ± 8.6	1.53 ± 0.006	$4.46 \times 10^{-6} \pm 8.9 \times 10^{-7}$

All the values were significantly different from one another at $p \leq 0.001$ for * and at $p = 0.194$ for #.

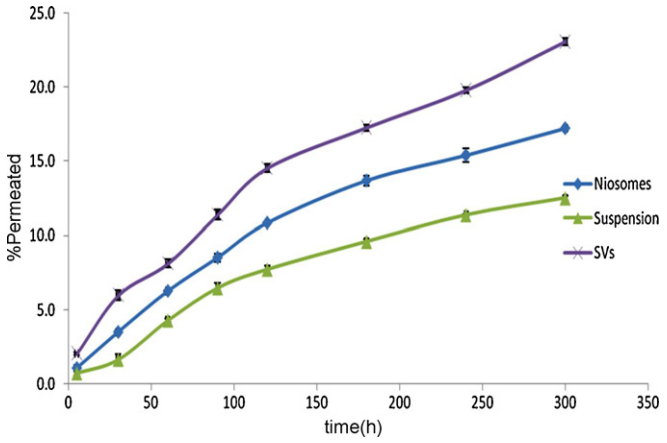

Fig. 4. % Permeated through porcine cornea vs. time (min) data of niosomes, spanlastics and suspension.

Table 3
Drug content and % leakiness of SVs upon storage at ambient and refrigerated conditions for 2 months.

Time	Total drug content (%)	Evaluation of SVs	
		% Leakiness	Aggregation
Refrigeration (4–8 °C)			
Day 0	100	0	×
1 month	99.30	2.23	×
2 month	97.69	5.67	×
Ambient room temperature (25 °C)			
Day 0	100	0	×
1 month	95.50	13.16	+
2 month	87.99	35.85	++

+: mild aggregation; ++: severe aggregation; ×: no aggregation.

3.7. Corneal permeability studies

SVs (7.47×10^{-6} cm/s) showed a significant improvement (67%) in apparent permeation coefficient (P_{app}) of KTZ as compared to niosomal formulation (5.92×10^{-6} cm/s; 32%) and aqueous suspension of free drug (4.46×10^{-6} cm/s; Table 2) taken as control. There was a significant difference between SVs and niosomes in terms of total amount permeated and steady state flux; thus indicating SVs to be significantly better than niosomes. The % amount permeated was highest ($p \leq 0.001$) in case of SVs (23.1%) and lowest in case of suspension (12.5%) (Fig. 4).

3.8. Stability studies

The results of stability studies are compiled in Table 3. Stability of vesicles is referred to in terms of % leakiness and aggregation/irregularity of vesicles over a period of 1 and 2 month of storage. Extent of drug leakiness and aggregation upon storage in refrigerator was significantly low; while at room temperature there was an appreciable drug loss (12.01%). Hence the system needs to be refrigerated for use as is the case with all other vesicular systems (Plessis et al., 1996). Developed SVs were sufficiently stable under refrigerated condition and fulfill ICH guidelines showing 2.31% loss in drug content at 2 months. However, the formulations are not recommended to be stored at room temperature.

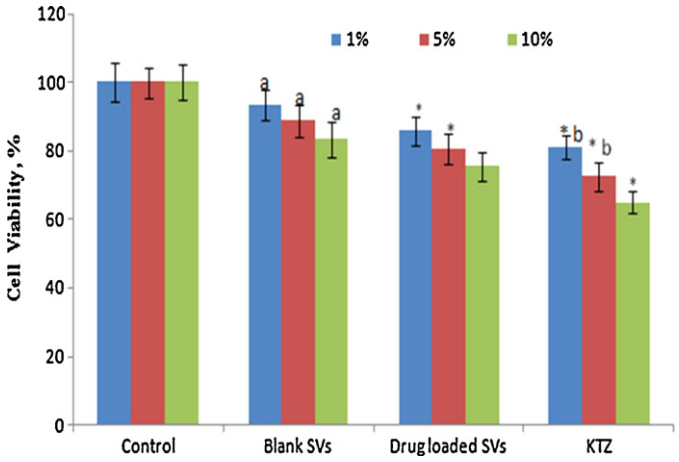

Fig. 5. % Cell viability vs. concentration ($n = 3$).

3.9. In vivo safety studies

3.9.1. Dermal irritation/corrosion test

Non-irritant/corrosive nature of developed SVs when applied to dermal tissues was confirmed as indicated by 0/40 score for erythema and oedema each. Hence they may be considered safe for dermal application.

3.9.2. Acute eye irritation/corrosion test

A score of 0/195 for single and 0/195 for repeat instillation of developed formulation(s) establishes that developed SVs are completely safe for ocular use.

Chronic repeat instillation irritation/corrosion test: The treatment of ocular fungal infection involves long term therapy, so we evaluated the developed formulation(s) for chronic repeat instillation. Again a zero score was observed for eye irritation/corrosion study which clearly depicted that SVs were non-irritant/corrosive even upon chronic use to all ocular tissues. In view of the zero score obtained in the study the developed SVs formulation may be considered safe for chronic ocular use.

3.10. Cytotoxicity studies

Both developed formulation and the corresponding empty vesicles did not show any significant toxic effect on cell proliferation of HGF as compared to the control ($p < 0.05$) when applied at lower concentrations of 1% (v/v) and 5% (v/v) in the cell culture suspension medium.

It may however be noted that free drug KTZ (1%, w/v suspension) at all the concentrations was sinificantly different from control ($p < 0.05$) which may be due to the fact that antifungal drugs due to a similarity between mammalian and fungal cells are unable to show selective toxicity against the latter and hence are in general also toxic to the mammalian cells (Barar, 2000). As seen from Fig. 5 there was higher cell viability in case of blank and drug loaded vesicles as compared to free drug suspension, hence it may be concluded that vesicles do not contribute to cell cytotoxicity. Also there was no significant difference in cell viabities of blank ($p = 0.099$) and

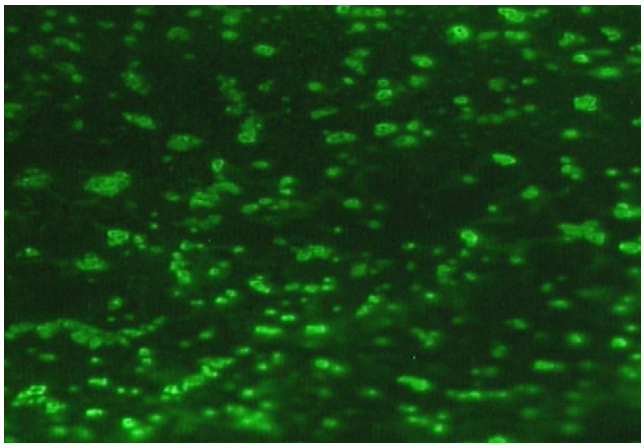

Fig. 6. Cf SVs under fluorescent microscope.

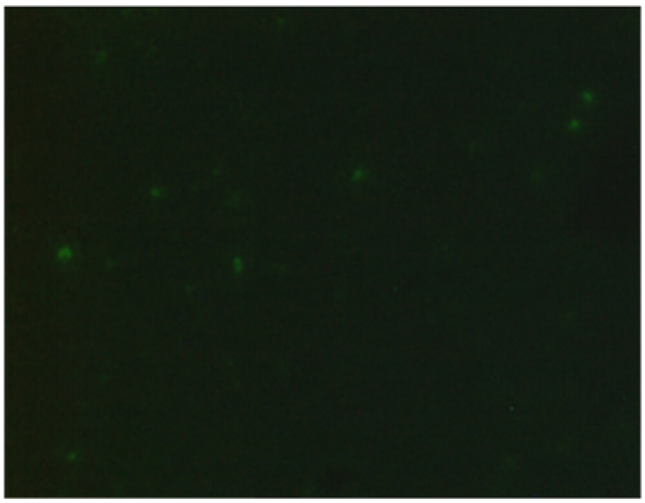

Fig. 7. Vesicles labeled with carboxyfluorescein seen in vitreous humor.

drug loaded vesicles ($p = 0.065$) with in different concentrations of 1%, 5% and 10% thus showing that there is no intrinsic toxicity associated with developed vesicular system, hence can be considered to be safe for use.

3.11. Determination of mutagenic activity (Ames test)

Results of the Ames test indicated non mutagenic nature of the developed vesicles (19 ± 4 His^+ revertant colonies for TA 98 and 117 ± 6 His^+ revertant colonies for TA 100 respectively) when compared with negative (21 ± 4 His^+ revertant colonies for TA 98 and 120 ± 5 His^+ revertant colonies for TA 100 respectively) and positive controls (1237 ± 43 His^+ revertant colonies for TA 98 and 2045 ± 108 His^+ revertant colonies for TA 100 respectively). Since the revertant count produced by spanlastics were not more than the negative control, hence it was concluded that the formulation was non-genotoxic.

3.12. Animal studies

Absence of any generalised fluorescence in the vesicular dispersion with only the vesicles showing fluorescence confirmed that no free carboxyfluorescein was present in the developed Cf SVs (Fig. 6).

3.12.1. Observation of fluorescence in the aqueous and vitreous humor

The aqueous humor and vitreous samples obtained 4 h post instillation of a single drop of Cf SVs showed fluorescence with intact Cf SVs in vitreous samples (Fig. 7). While the aqueous humor and vitreous humor samples from the eye administered Cf S showed no fluorescence, confirming the potential of developed SVs to cross the cornea and reach internal eye.

The repeat instillations study also confirmed the prescence of fluorescence both in the aqueous and vitreous samples from 2 h onwards (Table 4). Samples at 1 h however did not show any fluorescence while aqueous and vitreous humor samples of eye receiving Cf S did not show any fluorescence at all times. Carboxyfluorescein being hydrophilic is unlikely to cross cornea or blood retinal barrier and reach retina (Laties, 1982).

3.12.2. Observation of fluorescence in eye tissues (cross-sections of rabbit eye)

Cryo-sections of rabbit eye, 2 h post single drop instillation of the Cf SVs showed fluorescence and fluorescent vesicles in different eye tissues (Fig. 8a–c). Further, the fluorescent vesicles were also observed in the retinal layer (Fig. 8c).

4. Discussion

Hironaka et al. (2009) have discussed the delivery of drugs to the posterior segment of the eye via topical administration. However, there has been no report in which an intact drug carrier system noninvasively targets the retina. For this we prepared non ionic surfactant based elastic vesicles (spanlastics) using span 60 and tween 80 and evaluated them for targeting posterior eye segment.

The presence of fluorescent vesicles in the internal eye tissues confirms that the intact vesicles are being transported across the biological membrane barriers. This is a direct evidence of the fact that the nanorange of elastic vesicular system developed presently by us, is capable of reaching the posterior segment of the eye upon topical application in the form of eyedrops. This establishes the elastic nature of the vesicles, such that they can squeeze through small spaces and retain their structure.

Particle size of the ophthalmic preparation should be less than 10 μm in order to avoid irritation to the eyes (Hecht, 2001). In ophthalmic drug delivery systems, nano-sized particles represent a state of matter characterized by higher bioadhesion (Yoncheva et al., 2005) and greater surface area available for association between the cornea and conjunctiva. Rao et al. (2008) have suggested a condensing ability of ethanol for lipid vesicles. It has been reported that with higher ethanol concentration, the membrane thickness of vesicles was found to reduce owing to the formation of a phase with interpenetrating hydrocarbon chains (Dubey et al., 2007; Barry et al., 1995). Also, ethanol may cause a modification of the net charge of the system resulting in some degree of steric stabilization that may finally lead to a decrease in the mean particle size (Lasic et al., 1998). A nano size of SVs indicates appropriate for ocular use. Furthermore, achievement of nanovesicles will help in passage across the anatomical constraints in the eye.

Kassem et al. (2007) reported that the mean residence time of drugs on the ocular surface increased as the particle size in the drug suspension decreased. Therefore, a nano size (126 nm) could be an added advantage for treatment of superficial fungal infections also. Even though the pore size of biomembranes is expected to be much smaller than the vesicle size but the surfactant nature of the vesicles may temporarily increase the pore size such that slightly bigger vesicles may also squeeze in. Latter being possible due to the elastic nature of the SVs. This elasticity of developed SVs, may be attributed to the presence of tween 80 as an EA. EAs are often a single chain surfactant, which destabilizes the vesicles and increases the deformability of the bilayer by lowering the interfacial tension. Probably the prescence of tween 80 reduced the cooperativity of

Table 4
Repeat instillation of Cf S or Cf SVs into left and right eye of rabbit respectively.

Time (h)	Aqueous humor		Vitreous humor	
	Solution (Cf S)	Vesicular dispersion (Cf SV)	Solution (Cf S)	Vesicular dispersion (Cf SV)
1	–	–	–	–
2	–	+	–	+
4	–	+	–	+
6	–	+	–	+

–: no fluorescence; +: fluorescence.

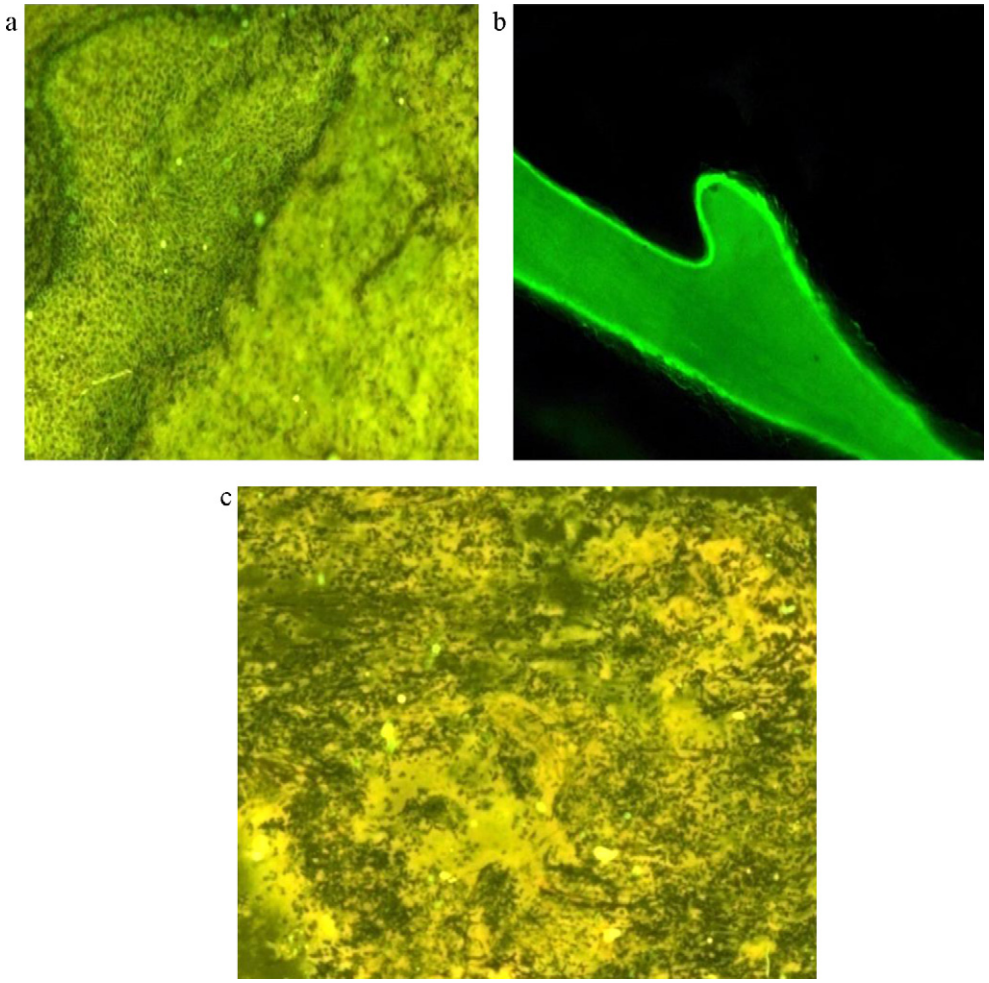

Fig. 8. Fluorescence seen in cornea (a), ciliary body (b), and retina (c) of the eye at 400× magnification.

the transition, which reflects the presence of a system having different degrees of disruption in packing characterstics (Trotta et al., 2002). The decrease in T_m value (from 92.89 °C for span 60 vesicles to 61.94 °C for span 60 and tween 80 vesicles) indicates that tween 80 acts as an edge activator and thus perturbs the packing characterstics and consequently fluidizes the vesicular bilayer.

While niosomes consist of cholesterol which is known to confer rigidity to the niosomal structure; this may explain the lower degree of deformation associated with these vesicles. Results obtained are in agreement with the reports published in the literature (Van den Bergh et al., 2001; Gompper and Kroll, 1995; Planas et al., 1992). Furthermore, SVs also show an approximately 2 times higher entrapment which may be attributed to their multilamellar nature (niosomes show uni- or bilamellar structure) and abundance (vesicle count/mm^3).

Corneal permeation data indicates that both spanlastics and niosomes are significantly better than KTZ suspension. SVs (2.56 μg/min/cm^2; 1153 μg) showed a significant improvement in flux and total amount of KTZ permeated as compared to niosomes (2.04 μg/min/cm^2; 860.3 μg) and aqueous suspension of KTZ (1.53 μg/min/cm^2; 625.41 μg). The developed SVs were found to be safe for ocular use.

Presence of vesicles in internal sections of eye provides a direct evidence that the nanorange of SV system developed presently by us, is capable of reaching the posterior segment of the eye in intact form, upon topical application in the form of eyedrops. This is a considerable achievement in the area of ocular therapeutics and will be of great significance in the treatment of internal eye diseases e.g. macular degeneration and diabetic retinopathy in addition to internal eye fungal infections like endophthalmitis and chorioretinitis for which presently only invasive intravitreal injections of the active therapeutic are available.

A mechanism similar to what is otherwise reported for penetration of elastic vesicles across the skin (Cevc and Blume, 1992)

is expected to operate when the SVs developed by us are applied on the corneal surface. Considering that ingredients (EAs) of these elastic vesicles are hydrophilic they are preferentially attracted toward area of high water content. Since aqueous humor and the vitreous of eye majorly constitutes of water (~90%) so it is expected that following the pore opening (due to the penetration enhancing effect of span and EAs of SVs) and squeezing through of the elastic vesicles across the cornea, these vesicles will pass through aqueous humor into vitreous in an intact form and will finally reach the posterior segment (iris, ciliary body) of the eye and elicit a suitable physiological response of the drug.

5. Conclusion

The fluorescence emission of 6-carboxyfluorescein, a fluorescent dye used as a hydrophilic model compound, was seen in the posterior segment of the eye after SVs containing 6-carboxyfluorescein were topically administered as eyedrops. These nanorange SVs could be potential carriers for targeting the posterior segment of the eye. Thus, the developed vesicles can be a breakthrough for effective delivery of agents (antifungal KTZ used as a model drug presently) into posterior eye. It can be extended to include antiviral agents, oligonucleotides, and newer antivascular endothelial growth factor and antiangiogenesis agents.

Conflict of interest

The authors report no conflict of interests.

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