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Solid lipid nanoparticles prepared by coacervation method as vehicles for ocular cyclosporine

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Abstract: Cyclosporine-loaded solid lipid nanoparticles were prepared according to the coacervation method: various stabilizers were used in order to obtain nanoparticles with different surface charge. These formulations were tested on rabbit corneas. Corneal irritation was measured according to opacity and permeability test; the interaction of labeled solid lipid nanoparticles with the cornea was studied by fluorescence microscopy; drug accumulation and permeation through the cornea were studied after labeling cyclosporine with a fluorescent probe. Nanoparticles were not irritant to the cornea, according to the opacity and permeability test. Cationic (chitosan coated) solid lipid nanoparticles enhanced drug accumulation and permeation through rabbit cornea, compared to anionic and non-ionic solid lipid nanoparticles and other reference formulations, probably due to the interaction of chitosan coated nanoparticles with corneal epithelial cells.

The 2007 International Dry Eye Workshop (DEWS) defined dry eye as “a multi-factorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface” [1].

The main mechanisms resulting in dry eye disease are tear hyperosmolarity and tear film instability: tear hyperosmolarity, produced by either the decreased production of tears or the increased tear evaporation rate, results in inflammatory events: inflammatory mediators cause damage to the superficial epithelium and apoptosis of goblet cells, thereby decreasing mucin in the tears, causing tear film instability [1]. Since inflammation can be a key component of dry eye, many anti-inflammatory drugs have been investigated for its treatment.

Cyclosporine A, a water insoluble molecule, has been used systemically as an immunomodulatory drug in solid tissue transplantation, and, more recently, to treat other systemic inflammatory diseases. Its mechanism of action has been very extensively studied: initially it focused on the molecule’s ability to inhibit activation of T lymphocytes. Later it was appreciated that cyclosporine A had other activities in different cell types, such as inhibition of apoptosis [2].

Cyclosporine A was investigated as a treatment for prevention of corneal allograft reaction in the 1980s, but the presently marketed topical emulsion was not approved until the end of 2002 for the treat- ment of inflammatory aqueous tear deficiency. Cyclosporine A 0.05 % ophthalmic emulsion (Restasis; Allergan, Inc., Irvine, United States) has been available as a FDA-approved treatment for dry eye disease since 2003 [3]. Given its efficacy for treatment of dry eye, a number of clinicians and researchers have investigated the effectiveness of topical cyclosporine A emulsion for treatment of other ocular surface conditions. Such disorders include those with signs and symptoms that overlap those of dry eye disease, and those that appear likely to respond to immunomodulatory therapy.

Many off-label applications of ocular cyclosporine A have been found: atopic keratoconjunctivitis, blepharitis, contact lens intolerance, graft-versus-host disease, herpetic stromal keratitis, laser-assisted *in situ* keratomileusis (Lasik), meibomian gland disease, ocular rosacea [1]. Nanotechnology-based drug delivery systems, like nanosuspensions, solid lipid nanoparticles and liposomes have been widely used for the delivery of poorly soluble drugs, like cyclosporine A.

Depending on their surface charge and relative hydrophobicity, nanoparticles can be designed to be successfully used in overcoming corneal barrier [4]. Presently, pharmaceutical scientists are concentrating on investigations about nanoparticulate-loaded drugs, genes and proteins as modified eye drops. Nanoparticles have the potential to target ocular tissues at minimum cost and high therapeutic value [5]. Solid lipid nanoparticles (SLN) are disperse systems with mean diameters ranging between 50 and 1000 nm and represent an alternative to polymeric nanoparticulate carriers. The main advantage of lipid carriers in drug delivery is the use of physiological lipids or lipid molecules with a history of safe use in therapy [6]. SLN have already been proposed as drug delivery systems for ocular therapy [7, 8].

Recently, a new, solvent-free SLN production technique was developed. Briefly, when the pH of a fatty acid alkaline salt micellar solution is lowered by acidification, fatty acid precipitates, owing to proton exchange between the acid solution and the soap: this process was defined as “coacervation”

[9]. Myristic, palmitic, stearic, arachidic and behenic acid were used as lipid matrixes and partially hydrolyzed polyvinyl alcohols of various molecular weights, modified dextrans and hydroxypropylmethyl cellulose were investigated as stabilizers [10]. Despite coacervation being a well known technique for polymeric nanoparticle preparation [11, 12], its application to lipid nanoparticles is quite innovative [9, 10].

In this experimental study, the coacervation process was modified in order to obtain cyclosporine A-loaded stearic acid (SA) SLN with a different zeta potential. A series of *in vitro* studies on SLN were performed using rabbit cornea, [13]: interaction between labeled nanoparticles and rabbit cornea was investigated by fluorescence microscopy; cytotoxicity of nanoparticulate formulations on corneas was measured through the corneal opacity and permeation test on rabbit cornea. Cyclosporine A accumulation and permeation by rabbit cornea was studied after drug conjugation with a fluorescent probe. Although H^3-cyclosporine was already used for permeation studies [14], fluorescently labeled cyclosporine offers the possibility of larger scale production and easier handling compared to H^3-cyclosporine. Moreover, the chemico-physical properties of the native molecule are not extensively altered, because of its higher molecular weight than that of the chromophore; therefore, the conjugation strategy can be suitable for ocular studies.

I. MATERIALS AND METHODS

1. Materials

1.1. Chemicals

Lactic acid, phosphoric acid and citric acid were from A.C.E.F. (Fiorenzuola d'Arda, Italy), 80 % hydrolyzed PVA 9000-10000 MW (PVA), 85 % deacetylated (~ 800 mPas 1 % solution in 1 % acetic acid) chitosan, Alizarin Red S and sodium fluorescein were from Sigma (Dorset, United Kingdom); sodium stearate (SS) cyclosporine A, fluorescein isothiocianate (FITC), fluorenylmethyloxicarbonyl- chloride (FMOC), and Tween 80 were from Fluka (Buchs, Switzerland); Arabic gum was from Merck (Darmstadt, Germany); deionized water was obtained by a MilliQ system (Millipore, Bedford, MO, United States); glutathione bicarbonate ringer (GBR) buffer was prepared according to literature [15]; Steinhardt medium, a sterile medium for human cornea conservation, was prepared according to a patented formulation [16]; all other chemicals were of analytical grade and used without any further purification. Lipimix, a commercial ocular blank emulsion (phospholipids with medium chain triglycerides, soybean oil, glycerine, disodium edetate, α-tocopherol, distilled water), was purchased by Tubilux Pharma.

1.2. Corneas

Rabbits were sourced from the slaughter house. The corneas were explanted from rabbit eye-balls, excised within 2 h of the animal's death, according to a protocol currently used for human cornea transplantation [17]. A sclera ring of nearly 4 mm was maintained around the explanted corneas, which were kept in sterile Steinhardt medium for less than 1 week.

Before corneal opacity and permeation test, corneas were assayed for their opacity by using the holder described below in the text, and corneas whose opacity exceeded 0.1 units of absorbance (λ = 570 nm) were discarded [18].

1.3. Diffusion chambers and corneal holders

Corneal opacity and permeability test was performed on rabbit cornea by using a corneal holder as the one described in *Figure 1*. It consisted of a Plexiglas and glass structure, with a donor and a receiving compartment (0.65 mL volume), respectively, on the epithelial and on the endothelial side of the cornea, which has to be placed in the orifice (0.50 cm^2) which divides the two compartments.

To minimize the irritation caused to the cornea by the holder itself, according to literature [19], a Plexiglas cell was utilized so that its o-ring clamped the scleral ring all around the corneal circumference; moreover, the holder structure allowed to maintain the natural cornea curvature. The holder was then housed in a spectrophotometer (Lambda 2 UV/Vis Perkin-Elmer) for opacity readings with the help of a suitable support, so that the beam crossed precisely the donor, the receptor compartment and the cornea clamped in the holder.

Accumulation and permeation studies were performed by using a diffusion chamber, analogous to the one described in literature [15].

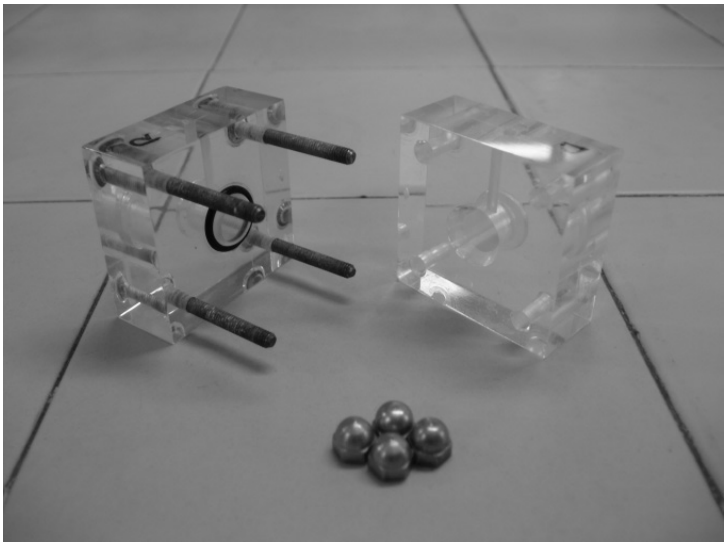

Figure 1 Corneal holder for corneal opacity and permeability test.

2. Methods

2.1. Synthesis of isocyclosporine A-FMOC

In order to study corneal permeation and accumulation, a fluorescent conjugate of cyclosporine A was synthesized according to a slightly modified literature method [20]: cyclosporine A was

converted to isocyclosporine A by heating (50 °C) a 10 % cyclosporine A solution in acid anhydrous methanol (1.2 % methanesulfonic acid) for 18 h [21], thus exposing a secondary amine group on the molecule backbone (*Figure 2A*). Unreacted cyclosporine A was precipitated by water addition and the resulting suspension was centrifuged (40000 g, Allegra R64 centrifuge, Beckmann Coulter, United States) and sodium carbonate was added to the supernatant in order to precipitate isocyclosporine A, owing to amino group deprotonation. The obtained suspension was centrifuged (40000 g), and the precipitated pure isocyclosporine A dried under vacuum. Every reaction step was checked by silica TLC. The mobile phase consisted of chloroform, methanol, acetic acid (90/4/6) [16].

The fluorescent probe was conjugated to isocyclosporinA by adding a molar excess of FMOC-Cl (1:2 w/w ratio) in hot acetonitrile solution (0.7 % isocyclosporin A, 70 °C), in the presence of sodium carbonate, in order to remove HCl forming during the reaction (*Figure 2B*). After 3 h of reaction, the mixture was filtered to remove sodium carbonate, ac- etonitrile was evaporated, and the residue dissolved in chloroform. Each reaction step was checked by silica TLC. The mobile phase consisted of chloroform, methanol, and acetic acid (92/2/6). Isocyclosporine- FMOC was separated from the reaction mixture by gradient column liquid chromatography (Kieselgel 60, Merck, Darmstadt, Germany). Mobile phase A was chloroform, mobile phase B was methanol The gradient was: eluent A for 30 min, then shifted from 100 % A to 100 % B in 10 min, finally eluent B for 30 min. Fractions were assayed by silica TLC-mobile phase chloroform/methanol/acetic acid (92/2/6). Fractions containing the conjugate were dried under vacuum, re- dissolved in methanol, from which the conjugate was re-crystallized by water addition, and finally dried under vacuum. Isocyclosporine A-FMOC was identified by mass spectrometry in chemical ionization (TSQ 700, Finnigan-Mat). Reaction yield was calculated by weighting the dried powders.

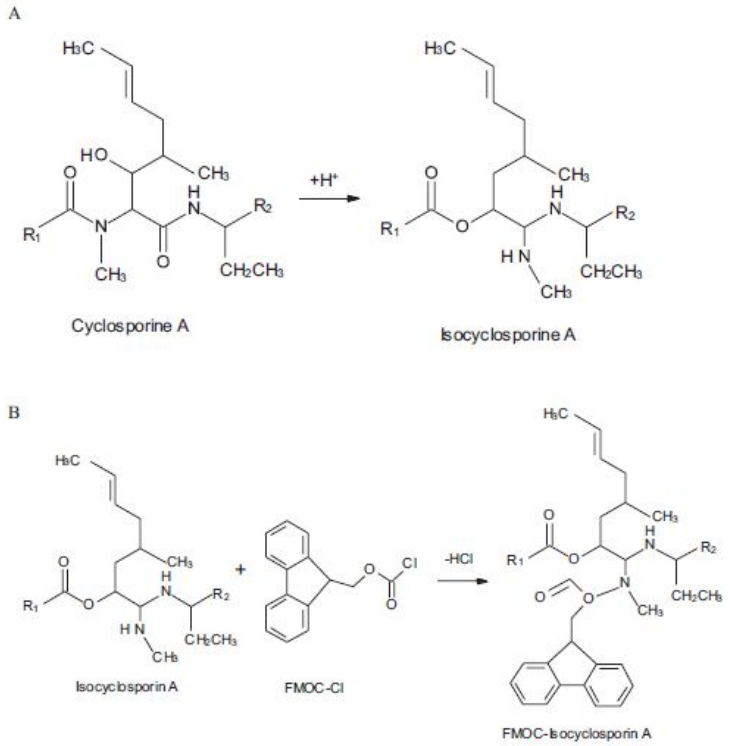

Figure 2 Synthesis of isocyclosporin A-FMOC. **A**: synthesis of isocyclosporin A. **B**: synthesis of isocyclosporin A-FMOC from isocyclosporin A.

2.2. Synthesis of FITC-chitosan

Chitosan was labeled with FITC according to a slightly modified literature method [22]. Chitosan hydrochloride was prepared by dis- solving chitosan in 0.1 M HCl. The solution was dialyzed by a Servapor cellulose membrane (MWCO 12000-14000 Da, Serva, Heidelberg, Germany) for one day and freeze-dried (Modulyo Freeze-Drier, Palo Alto, United States). Chitosan hydrochloride was dissolved in water at 1 % w/v. A 2 mg/mL FITC methanolic solution was added to the chitosan hydrochloride solution with an 1:2 volume ratio and the reac- tion mixed for 3 h at room temperature (25 °C). FITC-labeled chitosan was precipitated in 0.1 M NaOH solution. The precipitate was washed extensively with deionized distilled water to remove unreacted FITC. The labeled polymer was dissolved in 0.1 M HCl, then dialyzed and freeze-dried as described above. Labeling efficiency, calculated as the w/w ratio between reacted monomer and total polymer, was measured spectrophotometrically at 440 nm after dissolving the polymer in distilled water.

2.3. SLN preparation

SLN consisting of 1 % SA were prepared according to the co- acervation method described in a previous paper with slight modifica- tions [9]. Briefly, SS was dispersed in water and the mixture was then heated under stirring (300 rpm) up to 50 °C, to obtain a clear solution. Cyclosporine A or isocyclosporine A-FMOC ethanol solutions (25 mg/ mL) were added to SS solution and kept under stirring until complete dissolution.

Regarding to cationic and anionic SLN, aqueous solutions of the ionic polymers (arabic gum, chitosan hydrochloride) were prepared in half the volume of the total water of SLN suspension.

PVA was dissolved either in SS solution (non-ionic SLN) or in chitosan hydrochloride solution (cationic SLN).

Anionic SLN: arabic gum solution was added drop-wise to SS solution.

Cationic SLN: SS solution was added dropwise to chitosan hydrochloride-PVA solution.

In both systems the final amount of water corresponded to that usually employed in SLN preparation. Finally, different acidifying solutions (coacervating solutions) were added drop-wise to the mixtures until complete SA precipitation. The obtained suspensions were then cooled in a water bath under stirring at 300 rpm until a temperature of 15 °C was reached.

Cationic SLN for fluorescence microscopy studies were prepared by substituting chitosan hydrochloride with synthesized FITC-chitosan hydrochloride.

2.4. SLN characterization

SLN particle size and size distribution and zeta potential were determined by the laser light scattering technique (LLS - Brookhaven, New York, United States). The dispersions were diluted

with water (1:1000) and measurements were done at an angle of 90° with a laser beam of 675 nm; in the case of zeta potential, sample conductivity was adjusted by adding KCl.

Drug encapsulation efficiency (EE %) was calculated as the ratio between the amount of drug encapsulated within the lipid matrix and the amount used to prepare SLN. The encapsulated drug was deter- mined by centrifuging the SLN suspension at 55000 g and extracting the precipitate with methanol. The lipid fraction was selectively precipitated by adding a small volume of water and the supernatant was centrifuged and analyzed by HPLC.

The amount of drug adsorbed on the surface of SLN and that present in the lipid core were determined in a second series of experiments. The precipitate obtained after centrifugation at 55000 g was washed with 30/70 methanol/water solution, to remove surface-adsorbed drug. After washing the drug present in the lipid core of SLN remained in the precipitate and it was determined as described above.

Chitosan adsorption on the surface of cationic SLN was measured after centrifugation. The obtained precipitate was washed with water, then dried under vacuum overnight and dissolved in dichloromethane. Chitosan was extracted from the organic phase with an equal volume of 0.1 M HCl and the extraction was repeated three times. The pH of the obtained aqueous phase was then adjusted to 10 with 1 M NaOH in order to precipitate chitosan. The sample was then centrifuged and the pellet dissolved in 0.1 M HCl and injected in HPLC.

SLN suspension osmolarity was measured by a K-7400 osmometer (Knauer, Berlin, Germany) and corrected to physiological value with sodium chloride before use.

2.5. Fluorescence and optical microscopy

Fluorescence microscopy studies were performed with a DM2500 microscope (Leica, Germany) equipped with a Motic 480 camera. Fluorescent-labeled SLN were properly diluted with normal saline and incubated on cornea epithelial side for ten minutes, then the corneas were washed with normal saline. Before observation, the sclera was excised from the cornea, which was layered directly on the microscope slide (epithelial side up).

Microscopic observation in normal light was also performed on corneas to confirm their integrity. In this case, the corneas were stained (endothelial side up) with Alizarin Red S 0.2 % solution in normal saline for 10 min: they were then washed with normal saline before microscopic observation [23].

2.6. Corneal opacity and permeability test

Bovine corneal opacity and permeability (BCOP) test is an *in vitro* irritation test proposed in literature [18,24]. It measures the irritation caused by substances and formulations, after exposure to bovine corneas, by evaluating the increase in corneal opacity and sodium fluorescein permeability. The evaluation of common standard irritants provides an empirical irritation scale. In this

experimental work, this method has been adapted to rabbit cornea, widely used for the evaluation of corneal toxicity [18].

The opacity test was performed by clamping the cornea, through its scleral ring, in the appropriate holder, and filling both the compartments with GBR buffer. Then, in the donor compartment, GBR buffer was substituted by the irritation standards or by the formulations under study for 1 or 10 min. Then samples were withdrawn, and the donor compartment was washed three times with GBR buffer, and equilibrated with the same buffer for at least 15 min. Opacity was measured by determining the absorbance of the cornea (λ = 570 nm) clamped in the holder, before and after incubation with standards or formulations.

The permeability test was performed by incubating irritation standards and formulations in the donor compartment of the holder, as described for the opacity test. After removal of irritation standards and formulations, the donor compartment was filled with 0.04 % sodium fluorescein solution in GBR buffer. Permeability of the dye through the cornea was measured after 1 h, by spectrophometrical reading (λ =490 nm) of the content of the receiving compartment.

Irritant classification was done according to an empirical score [18]. Permeability classification was done adapting criteria of bovine holder [19] to rabbit holder, by comparison of the respective orifice surface areas (*Table I*).

Systems under study: blank and drug loaded SLN; irritation standards (30 % SDS, 1 M NaOH, 0,6 % Tween 80). GBR buffer was used as control.

Irritant classification	Opacity (λ = 570 nm)	Permeability (λ = 490 nm)
Mild	< 0.400	< 0.04
Moderate	0.400-1.300	0.04-0.14
Heavy	> 1.300	> 0.14

Table 1 Empirical classification of irritants [13, 14].

2.7. Accumulation and permeation studies

Corneas were mounted between the two halves of the diffusion cell: the area available for diffusion was 0.63 cm^2, the donor compartment (0.8 mL) was filled with isocyclosporine A-FMOC loaded formulations, while the receptor compartment (3 mL) was filled with GBR buffer. The diffusion chamber was kept at 37 ± 1 °C. After 1 h, drug permeation was determined by HPLC analysis of the receiving phase. The integrity of the epithelium after the experiment was checked by optical microscopy observation. Drug accumulation was also evaluated by HPLC analysis: the drug was extracted from corneas with methanol overnight, after careful removal of the corneas from diffusion cells and washing with tap water [25].

Systems under study: isocyclosporine-FMOC loaded SLN; isocyclosporine-FMOC suspension, prepared in normal saline, and Lipimix loaded with isocyclosporine-FMOC by simple mechanical dispersion, used as references. Drug concentration in all the samples was the same: 0.05 % w/v.

2.8. Cyclosporin A and isocyclosporin A-FMOC HPLC analysis

HPLC was performed on a Chromosystem 5 mm × 15 cm column by using a LC9 pump (Shimadzu, Kyoto, Japan), coupled either with a SPD10A (Shimadzu, Kyoto, Japan) UV detector (native cyclosporine λ = 210 nm), or a RF 551 (Shimadzu, Kyoto, Japan) fluorimeter (for FMOC-isocyclosporine, λexc = 265, λem = 315); mobile phase was made up of 75 % acetonitrile 25 % water, delivered at 1 mL/min. Retention times were 9 min for cyclosporine and 12 min for FMOC-isocyclosporine

2.9. Chitosan HPLC analysis

Chitosan hydrochloride was analyzed by size exclusion chromatography (TSK-GEL G2000SWXL 7.8 mm ID × 30.0 cm, Tosoh Bioscience), coupled with an evaporative light scattering detector (Sedex 75, Sedere, France). Mobile phase was made up of 0.025 % trifluoroacetic acid, 10 % methanol, 90 % water, delivered at 1 mL/ min with retention time of 5.5 min.

2.10. Data report

Each set of experiments described so far was repeated at least three times. Results were reported as mean ± standard deviation (SD) in text, and statistical analysis was performed using Student's t-test.

II. RESULTS AND DISCUSSION

1. Synthesis of isocyclosporine A-FMOC and of FITC-chitosan

Reaction yield for isocyclosporine A-FMOC was rather high (75 ± 5 %); moreover, unreacted cyclosporine A can be isolated by centrifugation, as described in the methods section. FITC-chitosan- labeling efficiency was 1.4 ± 0.2 %. Despite being quite a low yield, it was sufficient for fluorescence microscopy studies.

2. SLN formulation and characterization

Table II shows the compositions, mean particle sizes, zeta potential and encapsulation efficiencies of the blank and cyclosporine loaded SLN. As it can be noted, drug encapsulation causes an increase in SLN mean particle size and polydispersity; zeta potential is highly influenced by the

polymer used, being negative with Arabic gum, neutral with PVA9000, and positive with PVA9000 plus chitosan. Probably, the polymer disposition on SLN surface influences the surface charge. In order to avoid SLN aggregation that occurred in anionic SLN when loaded with drug, it was necessary to increase the amount of Arabic gum from 1 to 2 %.

In literature, PVA9000 and chitosan have already been used to produce cationic polymeric nanoparticles [26], due to chitosan adsorption on nanoparticles surface. In this experimental study, the actual chitosan adsorption on the surface of SLN was confirmed after centrifugation; almost 34 ± 1 % of the total amount was adsorbed on SLN surface, determining the positive charge.

The positive charge of nanoparticles is very important for the *in vivo* behaviour of nanoparticles, in fact, it can enhance bioadhesion of the same to the negatively charged epithelium, favouring the persistence of the formulation in the site of administration [27].

	Blank			Cyclosporine A loaded			Isocyclosporine A-FMOC loaded		
	Neutral	Anionic	Cationic	Neutral	Anionic	Cationic	Neutral	Anionic	Cationic
SLN composition									
SS (mg)*	107	107	107	107	107	107	107	107	107
PVA9000 (mg)	100		100	100		100	100		100
Arabic gum (mg)		100			200			200	
Chitosan HCl (mg)			30			30			30
Cyclosporin (mg)				5	5	5			
Isocyclosporin-FMOC (mg)							5	5	5
IM phosphoric acid (mL)			0.5			0.5			0.5
IM citric acid (mL)		0.2			0.2			0.2	
IM lactic acid (mL)	0.5			0.5			0.5		
Water (mL)	9.5	9.8	9.5	9.5	9.8	9.5	9.5	9.8	9.5
SLN characterization									
Average size (nm)	286 ± 6	497 ± 50	320 ± 2	487 ± 45	442 ± 32	428 ± 23	382 ± 35	534 ± 24	355 ± 11
Polydispersity	0.008	0.014	0.018	0.141	0.119	0.104	0.125	0.139	0.126
Zeta potential (mV)	-1.11	-28.11	25.87	5.94	-48.34	24.23	-7.92	-32.1	19.17
Standard error (mV)**	4.05	1.85	3.05	1.44	5.38	2.22	1.14	2.21	2.5
EE %***	-	-	-	100 ± 1	99 ± 2	90 ± 1	82 ± 3	71 ± 1	89 ± 2
EE % (30 % methanol washing)	-	-	-	78 ± 1	72 ± 2	70 ± 1	ND	ND	ND

*Sodium stearate, corresponding to 100 mg stearic acid. **Calculated on zeta potential measurements (5 analyses). ***Encapsulation efficiency.

Table 2 Blank, cyclosporine A and isocyclosporine A-FMOC loaded SLN compositions, mean particle sizes, zeta potential and encapsulation efficiencies.

3. Corneal opacity and permeability test

The bovine opacity and permeability assay, from which our method was adapted, measures two important components of ocular irritation affecting the cornea, opacity and permeability. Irritant-induced opacity, which is experimentally determined by the amount of light transmission through the cornea, is an indicator of protein denaturation, swelling, vacuolization or damage in the epithelial and/or stromal layers.

Permeability is generally measured by the amount of sodium fluorescein dye that passes through all corneal cell layers [28]. In the present study, corneal opacity test was performed on rabbit corneas, owing to the ease of handling, economy, and availability of rabbit eyes. Moreover, they are generally more susceptible to irritating substances that the eyes of humans [13].

In *Table III*, opacity and permeability test results are shown, together with the classification of the different formulations under study. The higher irritation result obtained between opacity and permeability test was chosen as main result, since, in the toxicity evaluation, the entity of the damage is more important than its cause [18].

As it can be noted in the cumulative evaluation, blank SLN were only slightly irritant compared to irritation standards. Although further investigation on epithelium cell lines will be necessary to fully understand the behavior towards SLN, these preliminary results could probably make SLN promising for ophthalmic use. Drug loaded SLN showed very similar results to blank SLN (data not reported).

	Opacity (AU λ = 570 nm)	Opacity classification	Fluorescein permeability (AU λ = 490 nm)	Permeability classification	Total classification
GBR	0.008 ± 0.003	Mild	0.017 ± 0.003	Mild	Mild
Neutral blank SLN	0.212 ± 0.020	Mild	0.021 ± 0.002	Mild	Mild
Cationic blank SLN	0.089 ± 0.025	Mild	0.039 ± 0.004	Mild	Mild
Anionic blank SLN	0.154 ± 0.046	Mild	0.031 ± 0.005	Mild	Mild
Tween 80 0.6 %	0.278 ± 0.065	Mild	0.095 ± 0.037	Moderate	Moderate
SDS 30 %	0.237 ± 0.078	Mild	0.149 ± 0.046	Heavy	Heavy
NaOH 1M	1.866 ± 0.145	Heavy	0.111 ± 0.038	Moderate	Heavy

Table 3 Irritation classification of the formulations ($p < 0.1$ compared to GBR buffer).

4. Accumulation and permeation studies

In *Figure 3*, accumulation and permeation results of fluorescent isocyclosporin A-FMOC through rabbit cornea are shown. Drug- loaded SLN were compared, while drug suspension in normal saline, and Lipimix loaded with the drug, were used as references.

Drug permeation through and accumulation into the cornea were evaluated after 1 h: analyses at lower post-instillation times were not done (even if desirable to mimic the *in vivo* conditions), owing to the difficulty to determine the lower drug amounts with sufficient precision and accuracy.

Cationic SLN gave the highest drug accumulation and permeation compared to other formulations. These data confirmed disclosures cited in literature, which stated that chitosan coated nanoparticles can enhance corneal permeation of loaded drugs, probably because of a phagocytic uptake of chitosan coated nanoparticles by corneal epithelial cells [27].

All nanoparticulate systems showed greater drug permeation than the emulsion or drug suspension. On the contrary, in drug accumulation experiments, only cationic SLN revealed a drug accumulation higher than that obtained from suspension.

The greater extent of drug accumulation compared to total drug permeation through the cornea, especially for drug suspension formulation, should be noted. An analogous *in vitro* study for the delivery of norfloxacin loaded liposomes did not show such difference between accumulation and permeation in cornea [20]. Our data agree with an *in vivo* study regarding the administration of 2 % H3-cyclosporine solution in castor oil to rabbits: the amount of drug recovered in the cornea was much higher than the one found in the aqueous humour [14]. This can probably be attributed also to the high lipophilicity of cyclosporine, and consequently its higher affinity for the lipophilic epithelium of the cornea, which therefore inhibits drug dissolution in the aqueous receiving phase *in vitro* and in the aqueous humour *in vivo*.

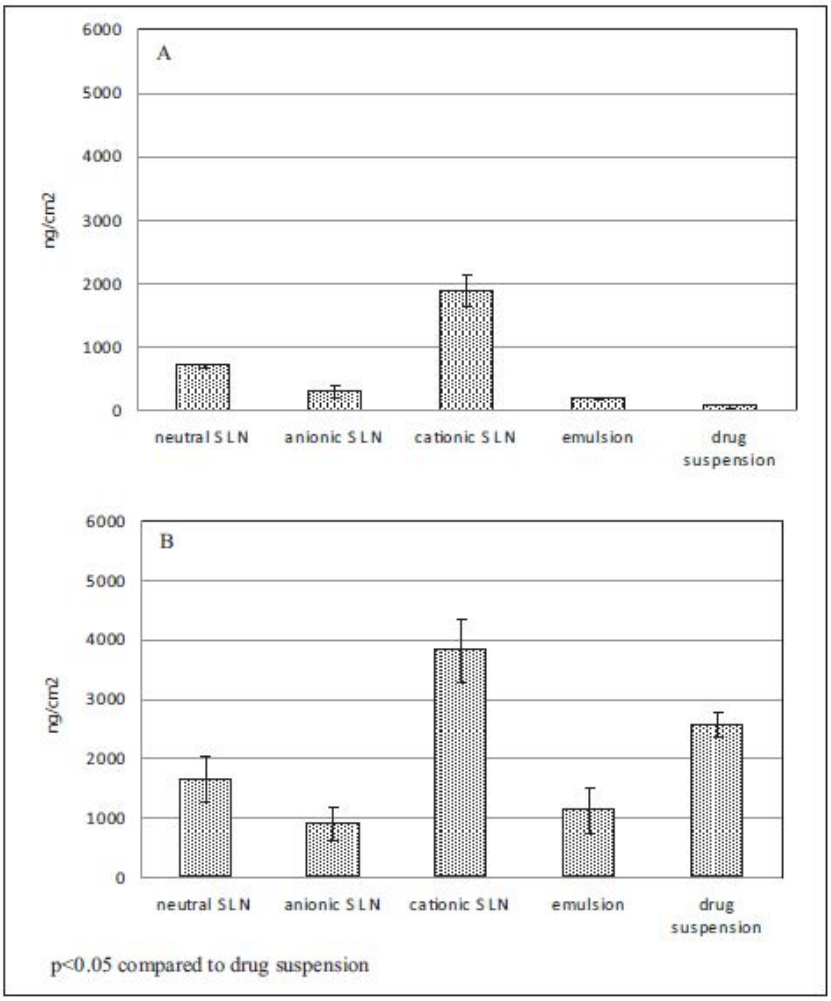

Figure 3 Isocyclosporine A-FMOC permeation and accumulation through rabbit cornea. **A**: drug permeation. **B**: drug accumulation.

5. Fluorescence microscopy studies

Further investigation was performed on rabbit cornea with fluorescence microscopy; as shown in *Figure 4*, there was a preferential accumulation of the labeled polymer in correspondence to the epithelial cells. Some authors hypothesized a phagocytic uptake of chitosan coated nanoparticles by

corneal epithelial cells [27]. A similar mechanism could provide a possible explanation for the increased corneal accumulation and permeation of drug loaded in cationic SLN.

SLN with different surface charge were prepared according to the coacervation technique and cyclosporine A was encapsulated within the lipid matrix with good efficiency.

All the formulations showed good biocompatibility *in vitro* according to corneal opacity and permeability test.

Higher permeation of fluorescent labeled drug through rabbit cornea was demonstrated *in vitro* for all nanoparticulate system compared to emulsion and drug suspension. Cationic nanoparticles, obtained by coating SLN with chitosan showed a greater accumulation and permeation through rabbit cornea compared to neutral and anionic SLN. This could be due to the particular interaction of these nanoparticles with epithelial cells.

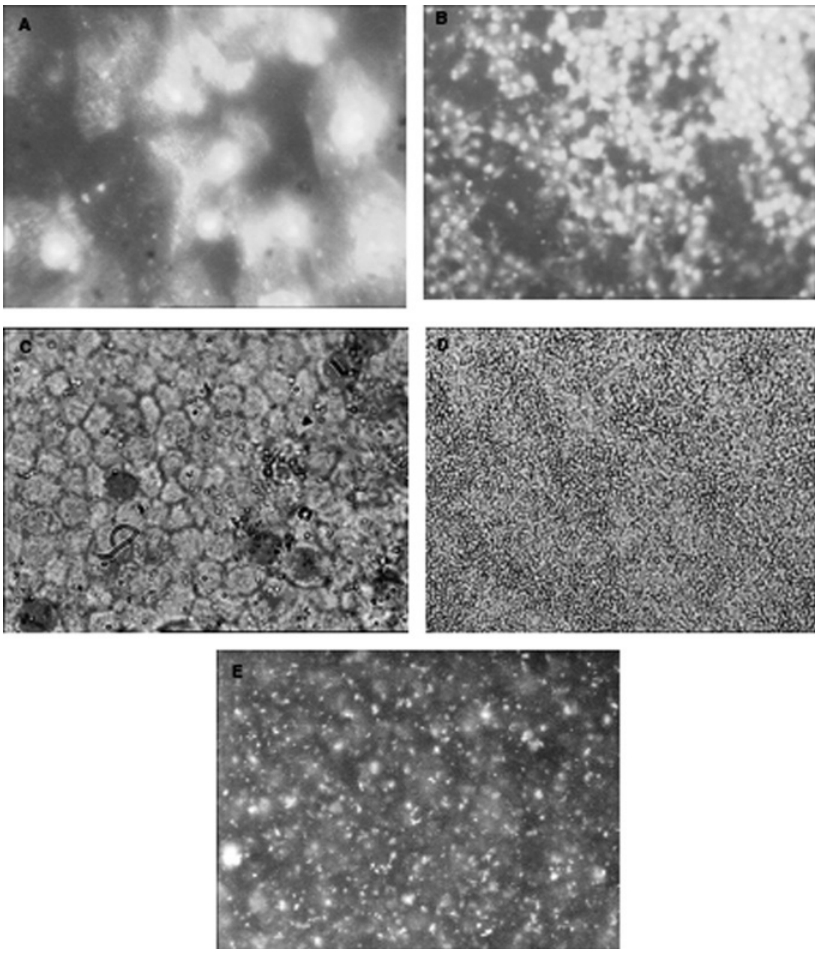

Figure 4 Corneas photomicrographs. **A**: cornea + SLN (fluorescence) (788X). **B**: cornea + SLN (fluorescence) (125X). **C**: non-treated cornea (normal light) (788X). **D:** non-treated cornea (normal light) (125X). **E**: SLN (fluorescence) (788X).

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