Hybrid ocular delivery system of cyclosporine-A comprising nanomicelle-laden polymeric insert with improved efficacy and tolerability

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ABSTRACT. We report on hybrid nanomicelle-polymer insert for improved delivery of cyclosporine A (CyA) to the surface of the eye. Hybrid inserts containing the nanomicellar formulation were prepared by the solvent casting method; their characteristics, in *vitro* release of CyA, eye irritation potential, nanomicelles distribution inside the insert, and *in vivo* pharmacokinetics of the most promising solid formulation (F3) were investigated. Nanomicelles capable of accommodating a therapeutically relevant amount of CyA (57.22 ± 5.90 - 68.52 ± 1.4 μg) were incorporated into five different polymeric formulations (F1-F5). The developed inserts displayed promising characteristics (size, weight, surface pH, and contact angle) that fulfill ocular tolerability requirements. Considering the technological properties and CyA *in-vitro* release, F3 and F5 were the most promising formulations. SEM analysis suggested F3 formulation as the potential prototype for CyA ocular delivery. F3 formulation (CyA: 60.08 ± 2.85 μg) did not induce conjunctival irritation when HET-CAM assay was performed, hence considered suitable for further study in a rabbit eye. The AUC value for CyA loaded in F3 insert was about 2-folds greater than that obtained with the Ikervis® used as a control formulation. F3 produced a significant reduction (of about 7-folds) in the rate of CyA elimination from the tear fluid relative to that of Ikervis® and about 4-fold greater than Nano-CyA ($p=0.0187$). The ability of F3 to delay the elimination of the drug from the precorneal area is particularly desirable when treating dry eye syndrome. Furthermore, F3 did not induce ocular discomfort, a typical characteristic of solid ocular inserts, including commercially available ones.

KEYWORDS: Nanomicelles, Cyclosporine-A, Mucoadhesive Inserts, Dry Eye Syndrome, Ocular Drug delivery.

INTRODUCTION

Cyclosporine-A (CyA) is a widely studied anti-inflammatory agent for the management of severe keratitis in adult patients with dry eye disease by virtue of its immunomodulatory activity that allows reducing the inflammation associated with subconjunctival and lacrimal glands, increasing the goblet cell density and tear production. Furthermore, CyA can block the mitochondrial permeability pores opening, inhibiting cell apoptosis; hence act on allergic inflammation due to its inhibitory action on eosinophil and mast cell activation and related mediators release [1-4].

The major obstacle in the ocular administration of cyclosporine concerns its chemical-physical characteristics, including relatively high molecular weight (1202.6 Da), neutral charge, high hydrophobicity (log P 2.92 at pH 7.4), and poor water solubility (27.67 μg/mL at 25°C) [5, 6]. These aspects have encouraged researchers to find a suitable formulation from the point of view of ocular tolerability and therapeutic effectiveness, considering that recent clinical evidence supports an effective CyA concentration of 0.05 to 0.1%w/v [2]. It is necessary to point out that some products like Restasis®, Ikervis®, and the most recent Cequa® have been introduced to the international market in the last few years. The first product Restasis® (Allergan Inc), an oil-in-water emulsion of CyA (0.05%), was approved by FDA in 2003 but not by the EMA due to its tendency to produce adverse effects at the site of administration, such as ocular burning, conjunctival hyperemia, discharge, epiphora, eye pain, foreign body sensation, pruritus, stinging, and blurred vision.

In 2015, Ikervis®, a cationic emulsion (Novasorb® technology) containing 0.1% CyA (Santen Oy, Tampere, Finland), received authorization by EMA. By virtue of the droplet's positive charge, this formulation exhibited prolonged residence time in the precorneal area of the eye [2,7,8], yet caused some adverse reactions such as eye pain, irritation, ocular hyperemia, and eyelid erythema.

More recently, the interest in developing a new aqueous-based formulation for the delivery of CyA has increased. Indeed, in 2018, the FDA approved a new nanomicellar-based eye drop containing 0.09% of CyA to treat dry eye disease, branded (Cequa®, Sun Pharmaceutical Industry Inc, NJ). The nano-micelles are composed of two nonionic surfactants of PEG-40 Hydrogenated Castor Oil and Octoxynol-40 and, based on the new NCELL™ technology, in which the nanomicellar system can entrap CyA, hence, promote better drug penetration into the ocular tissue. Cequa® ophthalmic micellar solution, which is administered twice a day to treat signs and symptoms of Dry Eye Disease (DED) in adults, is not free of side effects, including instillation-associated pain and conjunctival hyperemia [9]. As such, research continues to find new

formulations for a safer and more efficient CyA delivery to the eye, namely, to reduce ocular side effects and the frequency of administration by increasing ocular bioavailability.

Terreni et al. [10] have recently reported developing a new nanomicellar system for the ocular delivery of CyA based on two nonionic surfactants (Vitamin E-TPGS and IGEPAL® CA-630) combined with hyaluronic acid as a mucoadhesive polymer to prolong drug precorneal residence time and enhance ocular bioavailability. They demonstrated promising drug entrapment, reaching a CyA concentration of 0.105%wt, comparable to Ikervis®, and droplet size of 14.41 nm. Interestingly, the developed nanomicellar system showed a protective effect towards corneal epithelial cells with cell viability of more than 80% with the capacity of interaction with cellular barriers favoring the uptake and the accumulation of CyA in the apical cells of the corneal epithelium [10]. Moreover, pharmacokinetic studies on rabbits demonstrated the carrier's ability to prolong CyA precorneal retention for up to 30 minutes post-administration, mainly due to the presence of hyaluronic acid.

The present study aimed to take advantage of the combination of nonionic surfactant nanomicelles and a polymeric film that could be used as an insert for improved ocular delivery of CyA in terms of precorneal drug residence along with sustained drug release for better management of DED associated keratitis.

Mucoadhesive polymers including poly(vinyl alcohol) (PVA), sodium carboxymethylcellulose CMC), xanthan gum (XG), κ- Carrageenan (CAR), and sodium alginate (ALG) were investigated. The inserts containing the nanomicellar formulation were prepared by the solvent casting method, and the physicochemical characteristics, morphology, in *vitro* release of CyA, and nanomicelles distribution inside the insert were studied. Furthermore, the ocular pharmacokinetics of the most promising solid formulation was evaluated in New Zealand albino rabbits.

2. EXPERIMENTAL SECTION

Material & Methods

Materials

The following materials were used: Octylphenoxy poly(ethyleneoxy) ethanol (OPPEE, IGEPAL® CA-630, Sigma-Aldrich, Milan, Italy); d-α-Tocopherol polyethylene glycol succinate (Kolliphor® TPGS, BASF, Ludwigshafen, Germany); Cyclosporine A (CyA, Poli Industria Chimica s.p.a, Milan, Italy); Poly(vinyl alcohol) (PVA, MW 146000-186000), Carboxymethylcellulose sodium salt high viscosity (CMC, high viscosity, CAS 9004-32-4

(1500-1300 cps), Xanthan Gum from Xanthomonas campestris (XG), Sodium Alginate (ALG) and Coumarin-6 obtained from Sigma-Aldrich, London, UK; k-Carrageenan (CAR, Tokyo Chemical Industry, London, UK); Glycerol (GLY, Fisher Scientific, Loughborough, UK). All other reagents were analytical grade.

Animals

Male New Zealand albino rabbits weighing 2.8-3.5 kg were purchased from Pampaloni Rabbitry (Pisa, Italy). They were housed in standard cages in a light-controlled room (10 h dark/14 h light cycle) at 19 ± 1°C and 50 ± 5% relative humidity and were given a standard pellet diet and water ad libitum. During the experiments, the rabbits were placed in restraining boxes to which they had been habituated in a room with dim lighting and were allowed to move their heads and eyes freely [11]. For *in vivo* studies, the rabbits were used and treated according to the "Guide for the Care and Use of Laboratory Animals". All experimental procedures were carried out following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Union guidelines for the use of animals in research and approved by the Ethical and Scientific Committee of University of Pisa and carried out under veterinary supervision (Authorization n.350/2018-PR).

Quantitative Analysis of CyA by HPLC Method: experimental conditions and validation

The amount of CyA in the nanomicelles dispersion was determined by HPLC adding acetonitrile. HPLC system (Shimadzu LC-20AD, Shimadzu Italia s.r.l., Milan, Italy) consisted of a quaternary pump, an automatic sampler, and a UV SPD-10A detector with data acquisition by lab solutions software version 586 (Shimadzu Corporation, Japan). The chromatographic separation was achieved using a Kinetex-C18 (150 mm x 4.6 mm, 5 µm, 100 Å, Phenomenex) heated to 80°C. The mobile phase was prepared from a 0.1%TFA solution (adjusted to pH 1.7 with acetic acid) (20%v/v) and acetonitrile (80%v/v). The isocratic flow rate was 1.0 ml/min, and the injection volume was fixed at 20 µL. Detection was carried out using a UV detector at a wavelength of 210 nm. Cyclosporine-A retention time was 3.5 min. The method was validated according to FDA guidelines by the following procedure. The stock solution of CyA was made by dissolving an appropriate amount of CyA in acetonitrile; aliquots were progressively diluted with the same solvent (standard solution). Accuracy, precision, linearity, lower limit of quantification (LLOQ), and lower limit of detection (LLOD) were determined. For accuracy, intra- and inter-day determination, three different quality control samples, quality control low (QCL), quality control medium (QCM), and quality control high (QCH)- were

selected such that they are representatively distributed over the linear range of the calibration curve. Six replicas of each quality control concentration were measured. The accuracy of the method was calculated by comparing the measured mean of the six replicates of each concentration with the respective nominal values. The calculated mean value for four of the six quality control samples should not deviate by more than ± 15%, with the exception of the LLOQ, for which the mean value should be within ± 20%. For the intra-day precision experiment, six replicas of the same concentration were measured on the same day, whereas the inter-day precision was determined by measuring six replicas of the same concentration on three consecutive days. The coefficient of variation percentage (CV%) was used to give an indication of the precision of the used method. A CV value of ± 15% was considered acceptable for all quality control samples apart from LLOQ, where CV% values of ± 20% were deemed acceptable. A blank sample was introduced between the analyses; the absence of any interference between the matrix peaks with the analyte peaks was considered an acceptable indication of method selectivity. Finally, linear regression analysis was used to test for method linearity [12]. The calibration curve in acetonitrile was linear (r^2=1) over the concentration range studied (0.05 – 10.0 μg/mL). The LLOQ was 0.05 μg/mL and LLOD was 0.02 μg/mL and were calculated as follows: LLOQ = (10*SD)/Slope and LLOD = (3.3*SD)/Slope. The accuracy and inter- and intra-day precision were found to be within limits set by the FDA guidelines. No interference between the analyte and blank (solvent) peaks was identified, which confirmed the method selectivity. A summary of intra- and inter-day precision and accuracy figures for this method are summarized in **Table 1.**

Table 1. Summary of intra- and inter-day precision and accuracy for the rapid detection of Cyclosporin A.

Analyte Concentration (μg/mL)	Intra-day (n=6)			Inter-day (n= 6+6+6)		
	Measured concentration (μg/mL ± SD)	Accuracy (%)	CV (%)	Measured concentration (μg/mL± SD)	Accuracy (%)	CV (%)
0.05 (LLOQ)	0.049 ± 0.002	97.68	3.37	0.048 ± 0.002	95.22	3.13
0.3 (QCL)	0.31 ± 0.001	103.23	0.19	0.29 ± 0.012	99.22	4.24
2.5 (QCM)	2.54 ± 0.006	101.60	0.26	2.49 ± 0.055	99.52	2.22
7.5 (QCH)	7.54 ± 0.009	100.50	0.13	7.43 ± 0.108	99.08	1.45

For pharmacokinetic studies in the tear fluid, 98 μL of the standard solutions in acetonitrile were added to rabbit rinsed tear fluid (2 μL), and the final solution was then centrifuged at

13000 rpm for 5 min, and 20 μL of supernatant were analyzed. LLOQ and LLOD calculated were 0.05 and 0.02 μg/mL, respectively, the same values previously obtained in the same solvent (acetonitrile).

Preparation of Nanomicellar CyA-loaded Polymeric Inserts.

Assembling surfactant nanomicelles

Cyclosporine A-loaded surfactant nanomicelles were prepared by adding a known amount of CyA to a mixture of the two polymeric surfactants, Vitamin E-TPGS and OPPEE, in a 2.25:1.0 ratio by weight, following the procedure described by Terreni et al. [10]. Briefly, Vitamin E-TPGS was melted at 50°C; then, OPEE and CyA (0.1% w/w) were added and mixed together to obtain a homogeneous blend. In the end, deionized water at the same temperature was introduced. The final mixture was stirred overnight and filtered through sterile filters (0.2 μm RC Syringe filter, Phenomenex®, Torrance, CA, USA) to remove the unloaded drug, aggregates, and other foreign particulates. The nanomicellar formulation prepared (***Nano-CyA***) was characterized in terms of size, amount of drug encapsulated, and loading efficiency. The mean hydrodynamic diameter of the formulation was determined by dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS ZEN 3600, Malvern Instruments Ltd, Malvern, UK) immediately after preparation. Samples were adequately diluted and analyzed at 25 °C and at an angle of 90°. The entrapped drug in the nanomicelles was determined by the validated HPLC method. Percentage of drug entrapped (CyA-EE) and loading (CyA-LE) efficiency were calculated according to **Eqs. (1)** and **(2)**, respectively.

$$(1)\ CyA\text{-}EE = \frac{\text{mass of CyA in nanomicelles}}{\text{mass of CyA added in mixture}} * 100$$

$$(2)\ CyA\text{-}LE = \frac{\text{mass of CyA in nanomicelles}}{(\text{mass of CyA added} + \text{mass of the other excipients})} * 100$$

Polymer Dispersions

Stock aqueous dispersions of carboxymethyl cellulose (CMC, 2%w/w), xanthan gum (XG, 1% w/w), sodium alginate (ALG, 10 % w/w), carrageenan (CAR, 1% w/w), and polyvinyl alcohol (PVA, 4%w/w) were, one by one, prepared, maintaining them overnight under stirring at room temperature. In the case of PVA, the dissolution was completed by heating at 90°C for 1 h and by re-integrating, if necessary, the water lost by evaporation.

Nanomicellar CyA-loaded Inserts

Nanomicellar CyA-loaded inserts were prepared by the solvent casting method. An appropriate amount of the polymer dispersion, plasticizer (glycerine), and Nano-CyA colloidal dispersion (according to the composition shown in **Table 2**) were mixed together under stirring. The final blend was poured into a Petri dish of 120 mm diameter and allowed to dry for 72 h at room temperature. The resulting films (F1-F5, **Table 2**) were cut in the form of circular discs (diameter 7 mm), each containing approximately 60 μg of the drug (very close to the recommended daily therapeutic dose of CyA) and were stored in cool and dry conditions until use.

Table 2. Formulation code and composition of the prepared ocular films.

Formulation code Composition/weight	**F1**	**F2**	**F3**	**F4**	**F5**
PVA 4% w/w (g)	5	5	5	5	10
CMC 2%w/w (g)	-	-	30	10	-
XG 1% w/w(g)	5	5	5	15	20
ALG 10%w/w (g)	5	10	5	5	-
CAR 1%w/w (g)	-	-	-	-	10
Nano-CyA 0.1%w/w (g)	10	10	10	10	10
GLY (mg)	362	604	653	508	338

PVA: polyvinyl alcohol, CMC: carboxymethylcellulose, XG: xanthan gum, ALG: sodium alginate, and CAR: carrageenan. GLY: glycerol. Nano-CyA (Cyclosporin A) amount was calculated based on the solid weight of polymers.

Characterization of the ocular formulations (F1-F5)

Physical characteristics

Weight and thickness of ten inserts for each formulation were experimentally determined as reported by Abelkader et al. [13], calculating the mean and standard deviation (SD).

Drug content of five inserts from each formulation (F1-F5) was analyzed by HPLC after the dissolution of each inserts in water under stirring overnight. At the same time, in the case of F3, the regeneration of the nanomicellar structure was verified by DLS analysis.

The contact angle of six inserts was measured by DSA30S instrument (KRÜSS GmbH, Borsteler Chaussee, Germany) using the static sessile drop method where the liquid phase the artificial tear fluid (ATF) was prepared by dissolving sodium bicarbonate (0.2%w/w), calcium chloride (0.008%w/w), and sodium chloride (0.67%w/w) in water. Eight (8) μl of ATF (pH 7.4) were vertically dropped onto the surface of the insert; using a high-tech optical camera,

the angle created between the baseline of the drop (solid-liquid interface) and the tangent (liquid-air surface) was determined (see **Figure 1**). The contact angle of a liquid drop on the solid surface can be determined by Young's **Equation (3)**.

$$(3)\ \sigma s = Ysl + \sigma l \cdot \cos\theta$$

where θ, σs, Ysl, and σl represent contact angle, the surface free energy of the solid, interfacial tension between the liquid and the solid, and surface tension of the liquid, respectively.

After swelling in 500 µL of artificial tear fluid for 10 min, the surface pH of all inserts was measured using a pH meter (ETI 8100 Plus pH meter, Worthing, UK).

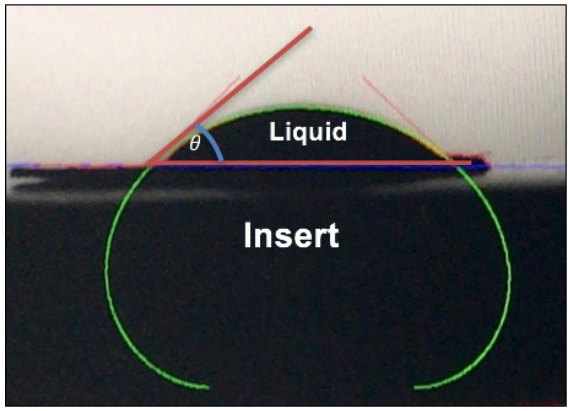


Figure 1. Example of measurement of contact angle.

Mechanical properties: Tensile strength measurement

The strain and tensile strength of the polymeric films, cut into 30 x 10 mm (length x width) rectangles, were measured by TA-XT plus texture analyzer (Stable Micro Systems Ltd., England) after determining the thickness of the sample. The test was performed in tension mode at room temperature following the procedure described by Abdelkader et al. [11]. Tensile testing involved a sample held by two grips a set distance apart. The loading arm (attached to the top grip) moves up at a constant speed to deform the sample. If the force required to break the sample is within the limit of the load cell, a fracture will occur. The tensile stress (MPa) and elongation on break (strain, %) were calculated using Exponent Lite software version 6.1.4.0 (Stable Micro System Ltd., England). **Equation (4)** was used to calculate Young's modulus[14]. All measurements were carried out in triplicate.

$$(4)\ \text{Young's modulus} = \frac{Stress\ (MPa)}{Strain\ (\%)}$$

Folding endurance

Three samples from each formulation were cut (20x20 mm squares), and the folding endurance was determined by repeatedly folding the samples at the same place till the breaking point or up 300 times. The number of times the sample could be folded at the same place till breaking point or up 300 times without breaking gave the value of folding endurance [14].

Scanning electron microscopy (SEM)

The gold-sputtered inserts with and without (blank) CyA-loaded nanomicelles were examined using a Zeiss EVO 50 scanning electron microscope with an acceleration voltage of 10 kV.

In vitro drug release test

In vitro release experiment was performed using the apparatus that the Pharmacopoeia provides for the dissolution of the solid dosage forms, modified for testing small volumes. The inserts (F1-5) were put in the stainless-steel wire basket rotating at 35 rpm and immersed in 10 mL PBS maintained at 37°C. At predetermined intervals of time, 1.0 mL of receiving compartment dissolution medium was withdrawal and replaced with fresh dissolution medium to maintain sink conditions. The amount of drug released was determined by RP-HPLC. All experiments were performed in triplicate.

In parallel, the behavior of the insert, when in contact with the dissolution medium (phosphate buffer solution), was monitored and the diameter of the insert was measured at the different time points by a digital microscope (Dino-lite Pro, ANMO, Taipei, Taiwan) for as long as the insert was visible. The insert was placed in the stainless-steel wire basket and maintained under the same experimental conditions as the vitro release test.

The experiments were performed in two steps: preliminarily, the change in diameter of the inserts under study was carried out at only 2 times, 60 and 180 minutes after contact with release medium. In relation to the result obtained, a second set of experiments was performed at different measurement times: F1 and F4 formulations were no longer visible at 60 minutes, so the change in diameter was monitored at 5, 15, 30, 45 minutes. F3 and F5 inserts, on the other hand, were still clearly visible after 180 minutes of contact with the release medium, so the measurement of the insert diameter was carried out further at 300 and 360 minutes. In the case of F2 formulation, during the preliminary studies, after 60 minutes of contact with the release medium, the diameter of the insert remained almost unchanged with respect to time

zero while at 180 min, the insert was not well optically visible to determine the size. Therefore, in the second set of experiments, the measurement at 120 min was deemed necessary.

Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy was performed on F3 disc-shaped fluorescent insert (***F3/Nano*-C6**) prepared as described in Experimental Section but replacing the CyA-loaded nanomicelles with the same type of micelles but containing coumarin-6 (10 μg/ml) (***Nano-C6***) as a fluorescent probe. Nano-C6 was prepared as previously described in the Experimental Section and subjected to the size distribution analysis by DLS to verify nanomicelles formation. Then, the yellow F3/Nano-C6 insert was positioned on a microscope slide, fixed and covered with a coverslip with adequate thickness, and analyzed by a LEXT OLS4100 laser scanning confocal microscope (Olympus Corporation, Japan) at the excitation wavelength of 512 nm. Scans were performed on different zones of the insert to investigate the uniformity of distribution of the incorporated nanomicelles

Hens egg test-chorioallantoic membrane (HET-CAM)/ conjunctival irritation Modified HET-CAM test [15] was carried out to study the potential conjunctival irritation of the developed ocular inserts. Briefly, White Leghorn eggs (Henry Stewart & Co., Ltd., UK) were incubated as described before under conditions of 37 ± 0.5°C and 65% ± 5 relative humidity (RH) in an incubator (Termarks AS, Bergen, Norway). On day 10, testing was conducted by placing the formulations on the surface of the CAM and observing inflammatory responses. As positive controls, 0.1 M NaOH and propane 1,2 diol (propylene glycol) were used as strong and moderate irritants, respectively. PBS solution was used as a negative control. Examination of the CAM's blood vessels and the capillary system was performed after treatment to evaluate the vascular responses, i.e., the so-called irritant effects of hyperemia, hemorrhage, clotting, and/or coagulation at different times post-application.

Ocular inserts samples were dipped into PBS and then placed on the surface of the CAM. Hemorrhage, hyperemia, and clotting/coagulation of the blood vessels and capillaries of the CAM were visually recorded for 5 minutes. Time-dependent numerical scores were allocated; cumulative scores were interpreted as irritation potential. A cumulative score of ≤ 0.9 was considered as non-irritant; 1 < cumulative score < 4.9 was slight irritant; 5 < cumulative score <8.9 was moderately irritant and 9 < cumulative score < 21 was severe irritant.

In Vivo Ocular Pharmacokinetics Study

The selected formulation containing CyA-loaded nanomicelles (F3) was inserted into the lower conjunctival sac of one rabbit eye and, at predetermined time intervals (1, 3, 5, 10, 20, and 30 min), samples of tear fluid were withdrawn from the lower marginal tear strip, using a 1.0 µL disposable glass capillary (Microcaps, Drummond Scientific, NJ, USA) [16]. The capillary was rinsed with milliQ water (1.0 µL), and the resulting sample was further diluted with 98 µL of acetonitrile, obtaining a final sample volume of 100 µL. Samples were then centrifuged at 13000 rpm for 5 min (Micro CL 17, Thermo Fisher, Italy), and 50 µL of supernatant was analyzed by HPLC.

Fifty microliters of commercial 0.1% w/v CyA emulsion (Ikervis®) and starting CyA-loaded nanomicellar dispersion (0.1%$_{w/v}$) (***Nano-CyA***), as control, were instilled in the precorneal area. The experiment was performed at least six times.

The apparent first-order elimination rate constants (K_e) of CyA from the tear fluid and the corresponding half-lives ($t_{½}$= 0.693/K_e) were calculated from the linear phase of [log tear fluid concentration.] *vs.* [time] plots [17]. The Area Under Curve values (AUC) were calculated applying the linear trapezoidal method by considering the interval of time of 1- 30 min after instillation of Nano-CyA and Ikervis® and up to 180 minutes upon the application of F3-NanoCyA insert. In addition, maximum drug concentration in the precorneal area (C_{max}) and the time it is reached (t_{max}) were recorded.

The tear fluid concentration of CyA at the different time points for F3, Nano-CyA, and Ikervis® were compared for statistical significance ($p<0.05$) using Student's two-tailored unpaired *t*-test (Prism 8 software). The data point values were expressed as mean ± standard error (S.E.), N=6.

RESULTS AND DISCUSSION

Physical characteristics and mechanical properties of prepared inserts

The inserts were prepared in multiple steps, including the preparation of the nanomicelles capable of encapsulating a therapeutically relevant amount of CyA; those were then incorporated into the relevant polymer dispersion, which was subjected to casting to obtain the polymeric film (**Table 1)** that was cut to dimensions to form the hybrid ocular insert.

Nanomicelles dispersions prepared showed a small particle size of 10.80 ± 0.30 nm (SD) with a PDI of 0.08, indicating a uniform distribution, with drug entrapment and loading of approximately 89% and 10%, respectively (**Table 3-A)**, data comparable to that obtained by Cholkar et al. [3].

PDI values equal or below 0.2 are considered acceptable for drug delivery application of polymer-based nanoparticles [18]. What is particularly encouraging is the amount of CyA dissolved in an aqueous medium reaching 0.1% (1.11 μg/ml), a remarkable milestone considering this drug's very low solubility in water. These results indicate the potential of the nanomicelles preparation method, previously developed by the same research team [10].

A simple and scalable method was used to produce the nanomicelles-containing polymeric films employing water as solvent at mild preparation conditions (room temperature and atmospheric pressure). The physicochemical characteristics of all prepared ocular films are summarized in **Table 3-B**. The five polymeric films prepared were successfully cut, without any lamination to produce ocular inserts with a thickness between 92 ± 4.22 μm and 141 ± 17.92 μm (mean ± SD). The prepared inserts' average weight (± SD) ranged from 5.8 ± 0.30 mg to 12.6 ± 0.90 mg. Measured values of the insert's thickness and weight showed a low standard deviation in all cases, indicating good reproducibility of the method and good uniformity of the prepared inserts [11]. The surface pH of the prepared inserts was in the physiological range (6.3-7.3), reducing any possible alteration of the tear film's pH, which can induce ocular irritation, tearing, and reflex blinking causing a rapid drug loss via drainage. The developed inserts displayed features (size, weight, and surface pH) that fulfill the ocular tolerability requirements (e.g., lack of discomfort after application, minimized foreign body sensation, etc.), rendering them suitable for the ocular application.

Table 3. Formulation characteristics of A) nanomicelles and B) prepared ocular films. Results are expressed as mean ± standard deviation (SD).

A)

Formulation characteristic of Nano-CyA	
Size distribution (nm)	10.80 ± 0.30
PDI	0.08
Drug entrapment (%)	88.9 ± 1.45
Loading capacity (%)	9.85 ± 0.20
CyA (mg/mL)	1.11 ± 0.02

B)

Formulation code	**F1**	**F2**	**F3**	**F4**	**F5**
Thickness (μm)	94 ± 9.66	141 ± 17.92	126 ± 5.16	115 ± 5.27	92 ± 4.22

Weight (mg)	7.0 ± 0.90	12.6 ± 0.90	10.8 ± 0.30	9.4 ± 0.90	5.8 ± 0.30
Surface pH	6.3 ± 0.21	7.2 ± 0.12	7.3 ± 0.06	6.3 ± 0.14	7.3 ± 0.45
CyA content (μg/disc)	62.16 ± 3.03	68.52 ± 1.40	60.08 ± 2.85	59.44 ± 1.09	57.22 ± 5.90
Surface contact angle (°)	60 ± 1.90	57 ± 1.50	48 ± 1.70	41 ± 2.20	37 ± 2.70
Tensile strength (MPa)	9.6 ± 0.50	16.1 ± 0.80	8.7 ± 0.50	9.8 ± 0.10	10.9 ± 0.10
Strain (%)	6.6 ± 0.60	9.2 ± 0.70	7.2 ± 0.10	6.5 ± 0.50	9.3 ± 0.40
Young's modulus (MPa)	1.46 ± 0.21	1.75 ± 0.08	1.21 ± 0.09	1.51 ± 0.12	1.17 ± 0.05
Folding Endurance	115 ± 9.00	90 ± 8.80	> 300	259 ± 7.50	223 ± 5.70

The wettability of the inserts under study was determined by contact angle measurement, which demonstrates the degree of wetting when a solid comes in contact with a liquid; contact angle < 90° corresponds to a high wettability, while > 90° indicates low wettability [19]. In this context, the measurements were carried out using artificial tear fluid (ATF, pH 7.4) to simulate physiological conditions. All formulations tested possessed a good wettability (<90°). That is a desirable feature to not alter the spreading of the tear film following frequent eye blinking, especially in dry eye disease where tear film physiological characteristics are already compromised [20]. Accordingly, all polymeric materials used in this work have hydrophilic characteristics, hence may give rise to desirable wetting and spreading properties, despite their different properties, including molecular weight, number of hydroxyl groups, and solubility in water. The best wettability (Mean ± SD) obtained by formulation F5 was (37 ± 2.70°) followed by F4 (41 ± 2.20°) and F3 (48 ± 1.70°). The introduction of carrageenan (CAR), a higher amount of xanthan gum (XG), and polyvinyl alcohol (PVA) in formulation F5 seemed to help reduce the contact angle between the insert and the tear fluid. Replacing CAR with carboxymethylcellulose (CMC) and sodium alginate (ALG) while maintaining XG in formulation F4 decreased the wettability. CMC did not appear to improve wetting; on the contrary, a concentration increases from 10 to 30% produced a rise in the contact angle values from 41° to 48°, as demonstrated by the behavior of formulation F3. Ballesteros et al. [21] reported a contact angle of 54.80° for an insert consisting of only CMC. Formulations F1 and F2 showed the highest surface contact angle (60 ± 1.90° and 57 ± 1.50°, respectively), which could be ascribed to reducing the amount of the two more effective polymers (CAR and XG).

Surface wettability is an important aspect of solid inserts intended for ocular application where a higher wettability is desirable to maintain a healthy ocular surface; on the contrary, a low

wettability could increase tear film's lipid deposition and promote proteins denaturation [20], taking into account that the inherent compromised hydration and lubrication of the ocular surface in case of dry eye disease.

The mechanical properties of the prepared solid inserts constitute impact both the manufacturing process and their method of application at the site of action. Polymeric films should possess enough tension to be easily retrieved and rolled up after casting, peeled from the release liner, but, at the same time, should not be too elastic since high elongation capacity during cutting and packaging might cause variation in drug content. Mechanical properties are affected by the manufacturing method, type of polymer used and their concentration, and plasticizers, such as glycerol. For this study, glycerol was used due to its well-known capacity to intercalate between polymer chains, disrupting polymer-polymer interactions, which gives a more flexible and porous tertiary polymeric structure. The plasticized polymer should be able to deform at lower tensile strength compared with a not plasticized polymer [22-24]. This would be particularly desirable for the intended use as an ocular insert. The Young's modulus of marketed ocular lenses is in the range of 0.3 to 1.4 MPa [25].Young's modulus was calculated for all formulations as an index of the stiffness or elasticity of the inserts, giving information related to the resistance of the solid formulation against deformation; the results of this experiment are summarized in **Table 3-B**. Young's modulus value of all formulations studied was under 2.0 MPa, with differences related to the different compositions. Young's modulus increased when the quantity of sodium alginate was doubled (F1: PVA / XG / ALG 5:5:5; F2: PVA / XG / ALG, 5:5:10). In contrast, the addition of 30% CMC (F3) markedly reduced the tensile strength with Young's modulus value of 1.2 MPa. Young's modulus rose again to values comparable to those obtained with F1 by replacing part of the CMC with XG (15%) as in the case of the formulation F4 (PVA / CMC / XG / ALG 5: 10: 15: 5). The combination of PVA/XG/CAR (F5) appeared to favor the strain to the detriment of tensile strength with Young's modulus of 1.17 MPa. Formulations F3 and F5 with the lowest modulus showed, therefore, the highest flexibility while the other formulations with greater Young's modulus proved to be harder and more brittle with a small elongation albeit in the acceptable range [26,27]. Formulations containing a high amount of CMC (F3) or CAR (F5) were more elastic than ALG-based formulations without CMC or CAR (F1 and F2).

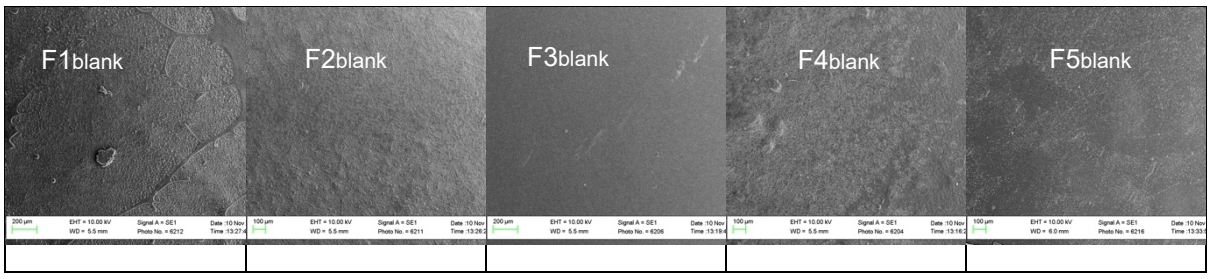
Moreover, the folding endurance of the formulations under study was determined since the ocular inserts, once administered, should be flexible enough not to break and to maintain its integrity during the normal blinking (10-12 blinks per min) [13,28]. Folding endurance data for all prepared CyA films were in the range of 90 and >300 (**Table 3-B**).

SEM Analysis

SEM analysis was performed on both blank (without nanomicelles) and Nano-CyA-loaded inserts in order to examine the effect on surface morphology. Phase separation and homogeneity of polymeric inserts were evaluated, since they are composed of more than one polymer [13,29]. **Figure 2** shows the surface morphology of the ocular inserts under study.

$F3_{blank}$ and $F5_{blank}$ (formulations without Nano-CyA, blank) showed a smooth and uniform insert surface. On the other hand, formulations $F1_{blank}$, $F2_{blank}$, and $F4_{blank}$ displayed a rough surface. A lack of homogeneity with apparent phase separation was observed only with the $F1_{blank}$ insert. Moreover, $F4_{blank}$ insert showed a coarse-grain surface with saliency. This behavior could depend mainly on the content of glycerol in the formulation; in fact, a concentration of 5-10% has been recommended as the best compromise to obtain a good mechanical resistance and flexibility of the polymeric film; concentrations under 3% produce brittle polymeric films, but a concentration higher than 12% might lead to phase separation on the film surface [30]. Nevertheless, others have successfully used a concentration of glycerol of more than 20%(w/w) for hydrophilic polymers [13,31-33].

The incorporation of *Nano-CyA* produced changes in the characteristics of F2 – F5 inserts, but not for F1. F2 had a smoother surface than that of blank, but little prominence of debris was evident on the surface. F4 insert surface presented an increase in the size and number of coarse grains, whereas, in F5, new saliencies appeared, suggesting a possible incompatibility between the components of inserts and those of the nanomicellar dispersion. On the other hand, F3 ocular insert showed a smooth surface, suggesting successful incorporation and uniform distribution of the nanomicelles. It has been previously reported that if the incorporation of the nanomicelles had been unsuccessful, the insert surface would show a rough surface [28], as in the case of F2, F4, and F5 inserts.



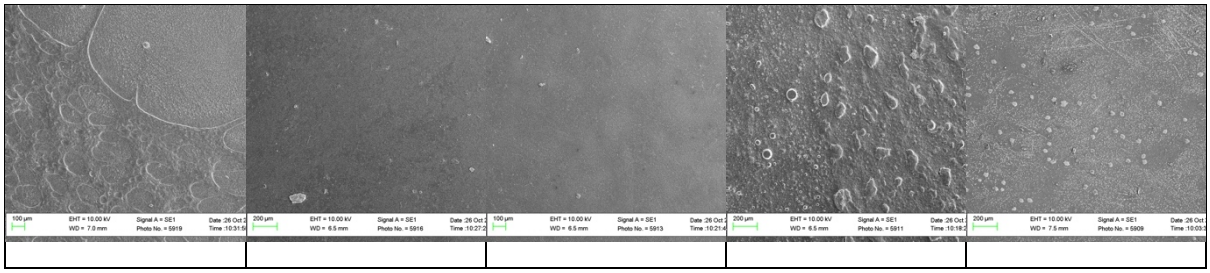


Figure 2. Scanning electron microscopy (SEM) of the surface of ocular inserts prepared from different polymers without Nano-CyA (blank formulation) and with Nano-CyA. 100x magnification.

In vitro drug release study

The results of this study account for the behavior of the formulations when in contact with the release medium and is essential to inform the future choice of an appropriate formulation for further investigation. In addition to *in vitro* drug release, the inserts were monitored for a change in diameter (mm) over time; the results are summarized in **Table 4** the relative images obtained by digital microscope are shown in **Figure 3.**

Table 4. Change in diameter of the inserts understudy when in contact with release medium. Results are expressed as mean ± standard error (SE, n=3).

Time, min	Diameter, mm				
	F1	**F2**	**F3**	**F4**	**F5**
0	6.95±0.05	7.01±0.01	6.98±0.03	7.02±0.02	7.01±0.01
5	6.6 ±0.15			6.63±0.13	
15	5.47 ±0.27	8.05±0.15		5.60±0.20	
30	4.32 ±0.32	8.04±0.04		3.79±0.21	
45	1.39 ±0.09	8.36±0.26		1.84±0.15	
60		8.33±0.17	11.58±0.28		7.12±0.08
120		8.38±0.18			
180			13.48±0.38		8.57±0.12
300			10.21±0.31		8.26±0.24
360			10.10±0.50		8.33±0.14

**** Blank spaces in the table represent no diameter measurement.***

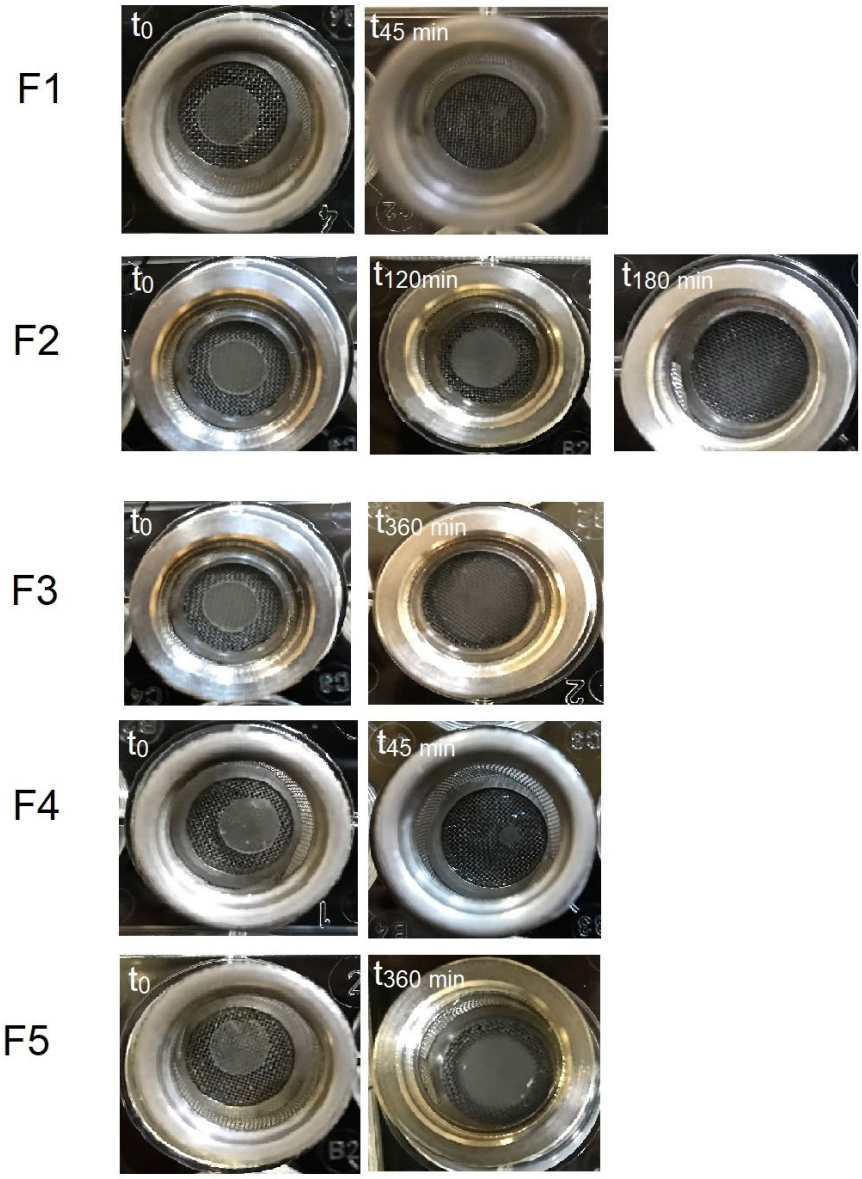


Figure 3 – Some images of the inserts under study when put in contact with phosphate buffer solution obtained by digital microscope.

The release of the drug from the polymer inserts under study depends on the ease of matrix hydration, which weakens polymer-polymer interactions, favoring swelling that leads to the formation of a gel with subsequent polymer erosion. The drug dissolved/dispersed in the gel diffuses out at a rate that is dependent on the concentration gradient and diffusivity through the gel. Multiple processes such as hydration, dissolution, swelling, erosion, and diffusion can happen during the different phases of drug release. The release of CyA from our inserts is likely to be governed by a combination of these mechanisms with the possible prevalence of one or

the other depending on the composition. *In vitro* CyA release from formulations F1-F5 at different time points is shown in **Figure 4.**

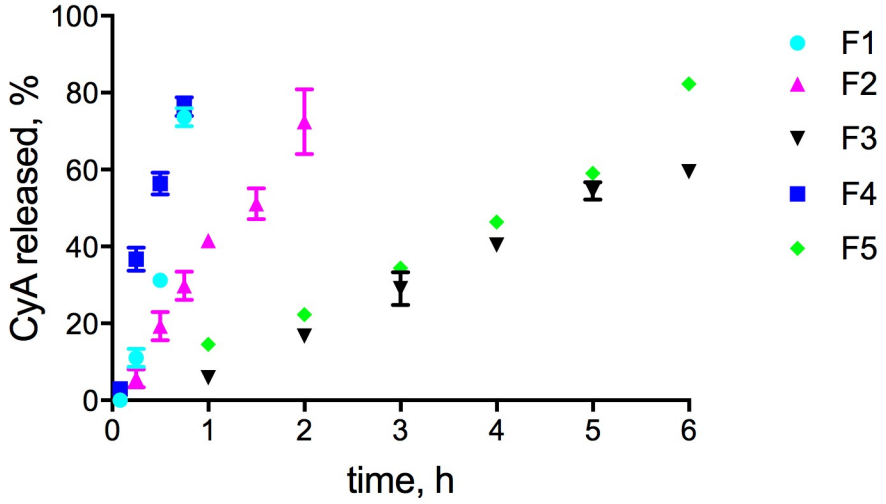


Figure 4. *In vitro* release of CyA from different polymeric inserts (mean ±SE, n= 3).

F1 (comprising a combination of PVA/XG/AG) and F4 (where 10% of CMC has been added to the previous components) showed the fastest CyA release (50µg/h). These formulations underwent a rapid erosion releasing the drug just as quickly with a close correlation between the percentage of drug released and the decrease in diameter as clearly evident from Figure 5. F1 produced a CyA release of 73.6 ± 2.36% in 45 min where its diameter had decreased by 80.0 ± 1.15%, and F4 yielded 76.34 ± 2.48% drug release at the same time combined with a reduction in diameter of 73.73 ± 2.3 %.

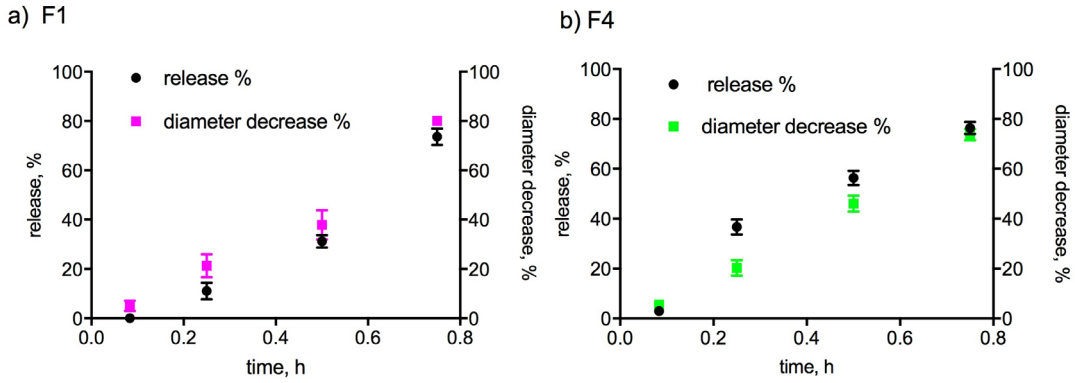


Figure 5. Percentage of drug released and decrease in insert diameter over time (mean ± SE, n= 3) for F1 (a) and F4 (b) formulations.

The apparent lack of homogeneity of the F1 and F4 inserts, highlighted by the SEM analysis, could be the basis behind these observations. The relatively high release rate and extent of CyA from F4 can be attributed to the visible presence of coarse grains of larger size and number after the addition of Nano-CyA, which suggests inefficient incorporation of the nanomicelles into the insert matrix.

The F2 inserts, on the other hand, exhibited inert monolith behavior towards the surrounding medium; in fact, their diameter remained almost unchanged (around 8 mm) over time during the experiment, not undergoing any visible swelling or erosion, and completely hydrated within 180 minutes. The release data were satisfactorily fitted to the Higuchi equation (obtained by plotting % cumulative release vs. square root of time), yielding an R^2 value of 0.9308 (modeling data not shown).

The increase in ALG concentration in F2 formulation relative to F1 seems to be responsible for the change in the behavior of the insert towards the release medium, basically avoiding erosion. Furthermore, F2 insert released CyA rather quickly, with 72.49 ± 8.48% of the drug released in 2 hours.

F3 comprised the single highest content of CMC, whereas F5 was the only CAR-containing insert along with the highest concentrations of PVA and XG. Both inserts experienced -albeit in different percentages- an increase in their diameter during the first 3 hours of contact with the release medium, hen, showed weak erosion over the following 3 hours. However, at the end of the release experiments, both F3 and F5 inserts were still visible in the medium despite their release of 59.46±1.890% and 82.31±0.894% of CyA, respectively. The relatively high concentration of CMC in the F3 formulation appeared to contribute to its characteristic swelling capacity and linear (zero-order release kinetics (R^2 = 0.9769). On the other hand, F5 had a lower swelling capacity (compared with F3), where its diameter remained constant after the initial increase producing a linear release over time (R^2 = 0.9665).

F2 and F5 formulations reached the same maximum value of diameters during the time, corresponding to a limited swelling degree without erosion (about 8 mm) but F5 maintained it for 6 hours while F2 completely hydrated within 180 minutes, producing a fast drug release. The different composition of the F5 with respect to F2 formulations seems to influence the release mechanism and consequently the release rate: the replacement of ALG with CAR and the increase in xanthan gum amount.

Sant et al. [34] have reported on the drug release retarding capacity of sodium CMC being higher than sodium ALG; findings that are in agreement with our data. In fact F3 formulation

showed a slow release, which could be related to the higher quantity of CMC. Chougule et al. [35] have developed different thin films containing an increasing amount of PVA, displaying a slower drug release with the gradual increase of PVA percentage in the formulation, which was attributed to the more viscous gel layer created upon hydration of polymer particles.
Considering mechanical, physicochemical properties, CyA *in vitro* release, and the intended therapeutic application, F3 and F5 were considered the most promising formulations. When combined with consideration of SEM analysis and folding endurance results, F3 formulation emerged as the potential prototype for Nano-CyA delivery and further *in vitro* and *in vivo* studies since F5 showed a rough surface which suggested potential heterogeneous and uneven nanomicelles distribution within the polymeric matrix.

Selected insert (F3) performance evaluation

In the beginning, the F3 insert was dissolved in 10 ml of water, a volume chosen to maintain the surfactant concentration higher than its critical micellar concentration (0.045%wt), which was verified by the determination of particle size distribution of the aqueous dispersion using the dynamic light scattering. Compared to the nanomicelles before their incorporation into the polymeric dispersion to obtain the final ocular insert, two populations were observed, the first with a mean particle size of 9.96 ± 0.14 nm relates to Nano-CyA nanomicelles, and the second (129.8±14.81 nm) is presumably ascribable to the polymeric mixture forming aggregates of distinct size. The size of the nanomicelles corresponded well with that determined before their addition to the polymeric dispersion (10.80 ± 0.30 nm,) while the sizes of the polymeric entities were consistent with those reported by Burgalassi et al. [36]. These authors evaluated the molecular size of a series of polymers, including XG, PVA, ALG, and CMC, reporting a hydrodynamic diameter value of around 100 nm for ALG, PVA, and CMC and about 200 nm for XG. This result appeared to confirm that the Nano-CyA nanomicelles were restructured, assembled, and formed once the insert was put in contact with water to give F3-Nanomicellar-CyA aqueous dispersion.

The following step was to prepare the fluorescent nanomicelles containing coumarin-6 (C6) as a fluorescent probe insoluble in water to verify both the ability of the nanomicelles to encapsulate, C6 facilitating its solubilization in an aqueous medium as well as promoting the miscibility of the nanomicellar dispersion with the polymer dispersion F3. The aqueous solution of C6 (***C6*-sol**, 10 μg/ml), colloidal dispersion of nanomicelles containing C6 ***(Nano-***

C6), F3 dispersion of C6 (***F3-C6$_{disp}$***), and the final F3-Nano-C6 dispersion were compared in terms of appearance, particle size analysis, and fluorescence distribution. Nano-C6 showed a particle size of 10.56 nm ± 0.78 (SD) with a PDI of 0.110, comparable with that of Nano-CyA. Furthermore, as shown in **Figure 6**, both nanomicellar (Nano-C6) and polymeric (F3-Nano-C6) dispersions showed uniformly distributed color under daylight, demonstrating successful incorporation of the fluorescence probe into nanomicelles and good compatibility of Nano-C6 with the polymeric dispersion of formulation F3.

To verify the nanomicelle's distribution inside the insert, a confocal laser scanning microscopy (CLSM) in fluorescent mode imaging experiment was performed on F3/Nano-C6. A representative image captured from CLSM of the fluorescent insert is shown in **Figure 7.** The fluorescent nanomicelles in the insert are colored in green. A homogeneous fluorescence at the C6 excitation wavelength (512 nm) suggests the successful incorporation and distribution of the laden nanomicelles along with the whole insert, further confirming the SEM results for F3. CLSM in florescence mode is an imaging technique that allows in-depth discrimination compared to classical fluorescent microscopy, and it is recognized as a suitable method to evaluate the quality of dispersion in composite films [37,38]. In fact, a strongly focused laser beam scans the specimen without disturbance from focus light, resulting in sharper images. Moreover, CLSM provides different information in terms of the analyzed area of the sample compared to other microscopy techniques used for nanometer-scale investigations (e.g., SEM, TEM, AFM, etc.).

Ilcikova et al. [37] used this technique to visualize nanocomposites inside a polymeric matrix, while Aw et al. [38] confirmed a good distribution of Vit E-TPGS - based nanomicelles inside porous and nanotubular implants. Our observations demonstrate that C6 remained anchored to the nanocarrier hydrophobic core due to its highly lipophilic nature (log P = 5.43 [39])

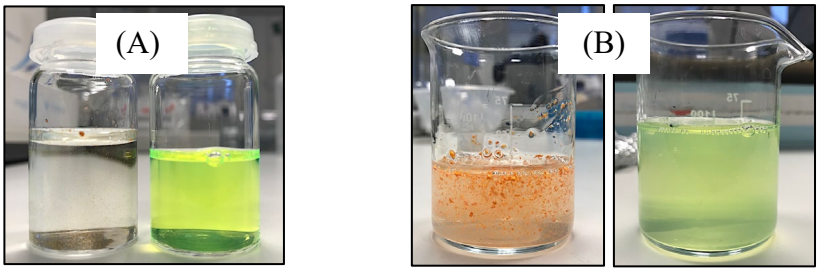


Figure 6. Coumarin-6 (C6) A) in water (left) as such and loaded into nanomicelles (fluorescent) and B) C6 in F3 polymeric dispersion as such and in F3 dispersion as Nano-C6.

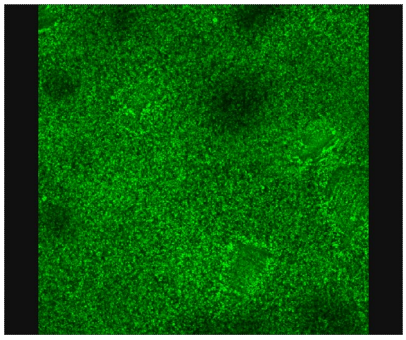


Figure 7. CLSM image of the selected formulation (F3) loaded with Coumarin-6. Magnification: 40x; Wavelength of excitation: 512nm.

HETCAM assay / conjunctival irritation

To investigate the conjunctival irritation potential of the hybrid ocular formulation of cyclosporine A nanomicelle-laden polymeric inserts, the HETCAM assay was adopted. This was necessary to establish whether the selected formulation (F3) could be safely studied in an appropriate animal model (New Zealand Albino rabbit eye on this occasion) without any concerns around potential undesirable conjunctival or ocular surface irritation.

Figure 8 (Fig. 8 - A) demonstrates the characteristic inflammatory responses (hyperemia, hemorrhage, clotting, and coagulation) observed at 300 seconds (T300) when a strong irritant (0.1 M NaOH) solution was brought into contact with the CAM. To further validate the responsiveness of the HETCAM model, a moderate irritant (propane 1,2 diol, so-called propylene glycol) was tested where responses mainly of hyperemia were observed at T300 (**Fig. 8 - B**). On the other hand, our tested formulation (F3) did not induce any vascular responses when brought in contact with the CAM, as such deemed free of any conjunctival irritation effect, hence suitable to study in an appropriate animal model (**Fig. 8 - C**).

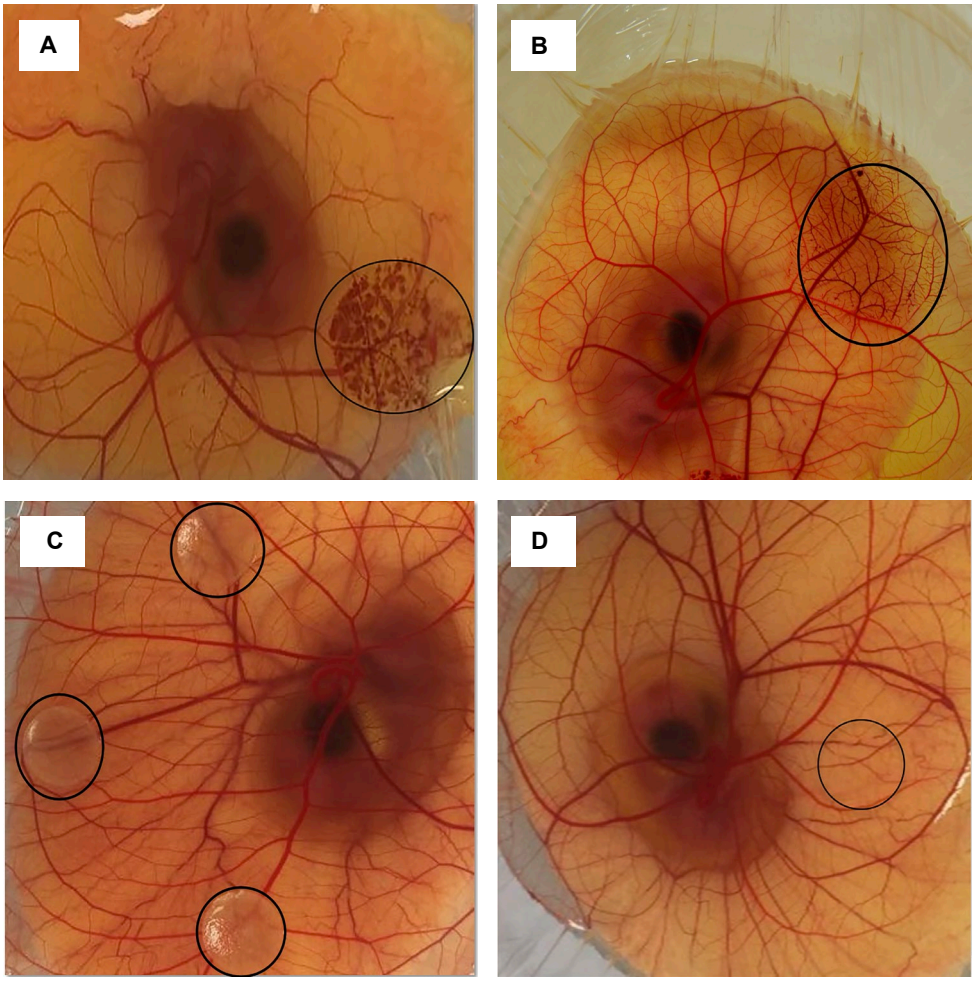


Figure 8. Representative 10-day old CAM exposed to positive controls (A) 0.1 M NaOH (strong irritant) and (B) propane 1,2 diol (moderate irritant), (C) F3 ocular film formulation and (D) negative control. NaOH induced clotting, hemorrhage, and hyperemia the only hyperemia was induced with propane 1,2 diol, and no irritation response was caused by F3, similar to the negative control. Pictures were taken 300 seconds post-application of test substances.

Biological studies

Tear fluid pharmacokinetics was evaluated after the insertion of F3 formulation into the lower conjunctival sac of the rabbit's eye. Nano-CyA dispersion and commercial product Ikervis® were used as references (controls). The CyA concentration in the tear fluid (mean ± SE) versus time profiles of all formulations under study are shown in **Figure 9,** while the relevant pharmacokinetic data are summarized in **Table 5**. F3 insert demonstrated a remarkably higher bioavailability (AUC=2942±155.3 μg/mL min^{-1}) compared to both the nanomicellar dispersion (AUC=1426 ± 92.99 μg/mL min^{-1}) and the commercial emulsion (AUC=1813±354.1 μg/mL min^{-1}) with statistically significant differences ($p<0.05$).

On the basis of the pharmacokinetic profiles, it is possible to note a typical pulse entry release of CyA from both liquid formulations (NanoCyA nanomicellar dispersion and Ikervis® emulsion). Upon instillation of both liquid formulations, the concentration of drug in the tear fluid was typically high, immediately after instillation (C_{1min}: 769.16±43.50 and 458.18 ± 115.87 μg/mL, for Ikervis and Nano-CyA, respectively) followed by a drastic decrease in concentration to arrive at undetectable levels in less than 30 min, with differences related to the type of formulation used, as reported by Terreni et al. [10]. Consequently, each liquid eye drops delivery are expected to produce a short duration of action for CyA. When compared with the liquid vehicles, topical administration of ophthalmic inserts containing CyA in nanomicellar form prolonged the duration of action where appreciable drug concentrations were found up to 180 min. The AUC values after application of the cyclosporine insert (F3) were about 2-fold greater than those obtained with the commercial emulsion and nanomicellar dispersion. As such, our ocular insert may overcome the disadvantage of the rapid precorneal clearance of the CyA instilled as eye drops, maintaining constant therapeutic levels for a relatively long period of time. In fact, there was a significant reduction of the rate of drug elimination from the tear fluid (K_e = 0.0309± 0.0041 min^{-1}; $t_{1/2}$ = 24.57±3.33 min) of about 7-fold with respect to that obtained by Ikervis® (p=0.0003) and about 4-fold compared to Nano-CyA dispersion (p=0.0187), confirming that the formulation delays the elimination of the drug from the precorneal area which is particularly desirable when treating ocular surface inflammation characteristic of dry eye. Moreover, F3 showed a shift of the time to reach the CyA concentration peak (C_{max} = 51.94 ± 7.49 μg/mL) from 1 to 30 min. These results seemed to confirm the sustained drug delivery, already observed *in vitro* experiments, wherein the formulation F3 produced linear release kinetics of CyA following a hydration/swelling process when the insert came in contact with the biological medium; the tear fluid penetrated the matrix, swelling occurred, and, after polymer chain relaxation, drug diffusion took place. It is known that drug release and dissolution in tear fluid is a crucial issue in reaching the desired steady-state concentrations in the site of action because only the released or dissolved drug can be absorbed into the eye.

The underlying mechanism behind the increase in CyA bioavailability when loaded in the F3 insert, compared to the reference formulations, could be attributed to two unique characteristics, they are:

- The fact that F3 is a solid formulation which renders it less susceptible to the processes

of dilution by the tear fluid and the subsequent physiological elimination via nasolacrimal drainage [40,41]; furthermore,

- F3 consisted of a mixture of biocompatible polymers (PVA, XG, ALG, and CMC) known to have remarkable viscosity enhancing and mucoadhesive properties [36,42,43].

Terreni et al. [10] have already demonstrated the positive effects of mucoadhesion on the bioavailability of CyA loaded into the same nano-micellar system used in the current research (Nano-CyA), albeit with an additional mucoadhesive polymer, hyaluronic acid (NanoHA-CyA). The presence of HA improved the bioavailability of CyA from a value of AUC of 1426 μg/mL min^{-1} to 2142 μg/mL min^{-1}.

The current study demonstrated the added benefit of combining a solid polymeric insert formulation with the nanostructured vehicle of CyA as demonstrated by a further increase in CyA's bioavailability (AUC = 3092 μg/mL min^{-1}) in tear fluid.

It is noteworthy that F3 insert did not cause any apparent ocular adverse effects when applied in the lower conjunctival sac of the rabbit eye probably due to its favorable characteristics, including flexibility and ability to hydrate that appeared to reduce the foreign body sensation, ocular discomfort, excessive tearing, hyperemia, swollen lids characteristic of the application of solid ocular inserts, including commercially available ones [44, 45].

It has been previously reported that the use of Ikervis® by dry eye patients is associated with undesirable adverse effects (instillation pain experienced by 29.2–54.5% of users) and subsequent discontinuation of treatment (9.9–10.4%) [46,47]. Our hybrid formulation offers a viable alternative to overcome some of these adverse effects, hence improve patient adherence.

Table 5. In vivo pharmacokinetic parameters in rabbit tear fluid (mean ± SE, n=6).

Formulation	**AUC (μg/mL . min^{-1})**	**C_{max} (μg/mL)**	**t_{max} (min)**	**K_e (10^2 min^{-1})**	**$t_{1/2}$ (min)**
F3*	2942± 155.3	51.94 ± 7.49	30	3.09±0.41	24.57±3.33
Nano-CyA	1426 ± 92.99	458.18 ± 115.87	1	11.9±3.8	7.12±1.42
Ikervis	1813±354.1	769.16±43.50	1	20.5±3.3	3.83±0.74

*All pharmacokinetic parameters obtained by administration of F3 insert are significantly different from the reference formulations (Nano-CyA and Ikervis®)

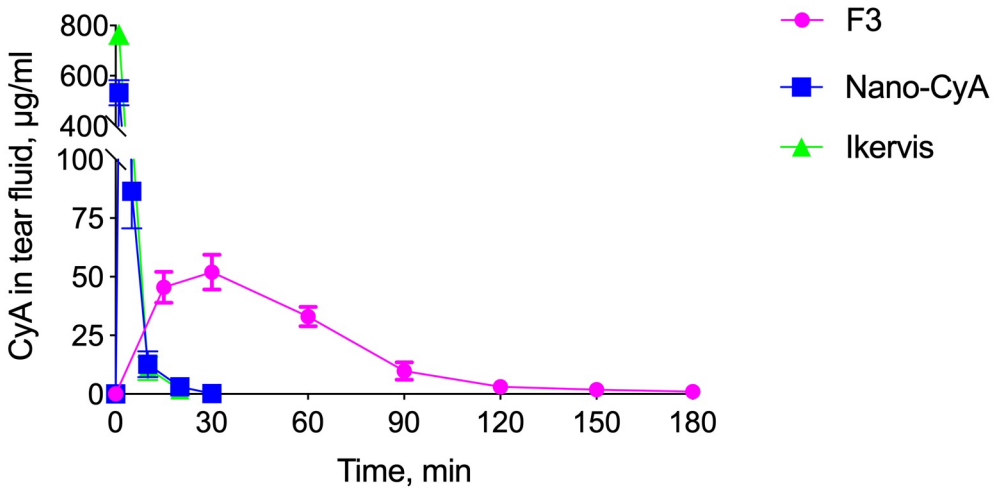


Figure 9. CyA concentration in the tear fluid of rabbits vs. time profile, following topical administration of F3 insert, nanomicellar dispersion (Nano-CyA), and commercial product (Ikervis®). Results are expressed as mean ± standard error (S.E.), n=6.

CONCLUSIONS

CyA is available as a cationic emulsion formulation (Ikervis®) for once daily use for the treatment of severe keratitis in adult patients with dry eye syndrome. The use of Ikervis® is associated with undesirable side effects leading to poor patient adherence. An ocular insert comprising a hybrid system of CyA nanomicelles embedded in a mucoadhesive polymeric film has been developed and shown to provide a superior effect and improved ocular tolerability profile. The developed formulation (F3) demonstrated prolonged precorneal residence relative to Ikervis®. Furthermore, it was devoid of ocular adverse effects when tested in the rabbit eye, which could be ascribed to the desirable physicochemical and mechanical properties of this formulation. Testing this insert in human volunteers would be an attractive proposition for patients, clinicians, and pharmaceutical companies.

Authors' contributions

Conceptualization, E.T., P.C., R.G.A., A.A.A-K and D.M.; methodology, E.T., E.C and S.T.; validation, E.T.; investigation, E.T., S.T., E.C and S.B.; writing—original draft preparation, P.C., R.G.A., A.A.A-K and D.M.; writing—review and editing, E.T. D.M, R.G.A. and A.A.A-K; supervision, P.C, D.M., R.G.A. and A.A.A-K.; funding acquisition, P.C. and S.B. All authors have read and agreed to the published version of the manuscript.

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