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DEVELOPMENT AND CHARACTERIZATION OF CYCLOSPORINE LOADED BIOADHESIVE NANOPARTICLES FOR THE TREATMENT OF DRY EYE

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ABSTRACT

Dry eye is a common disease of the tear film caused by decreased tear production or increased evaporation. Dry eye is characterized by chronic dryness of the cornea and conjunctiva which is caused by unstable tear film associated with abnormality of the lipid, protein, and mucin profiles. Changes in tear composition resulting from lacrimal dysfunction, increased evaporation, and poor clearance have pro inflammatory effects on the ocular surface. According to the Dry Eye Workshop (DEWS, 2007) report, prevalence of dry eye ranges from 5–30% in people aged 50 years and older. Prevalence of DES is estimated that about 3.2 million women and 1.7 million men, for a total of 4.9 million patients 50 years and older, have dry eye. Topical cyclosporine is currently the only and safe pharmacologic treatment of dry eye. The objective of this study was to evaluate the potential effectiveness of cyclosporine nanoparticles (NPs) for the treatment of dry eye. Cyclosporine belongs to BCS (Biopharmaceutical Classification System) Class II and a group of immunosuppressive compounds having an anti-inflammatory activity. Arginine is having positive charge and a potent vasodilator. Hyaluronic acid is having a negative charge and act as a natural lubricating agent. Therefore, when these are mixed with cyclosporine by ionic-gelation method, it leads to formation of nanoparticles, consequently improve retention time and provide controlled release. So, it will retard the evaporation of water and also improve water holding capacity of eyes. The aim of the proposed study was to develop a controlled release formulation of cyclosporine using natural bioadhesive carriers for the management of dry eye treatment. So, objectives of the proposed study was to formulate cyclosporine bioadhesive nanoparticles for dry eye treatment to achieve control release of cyclosporine from the formulation at the target site and to improve therapeutic outcome.

KEYWORDS: Dry Eye, Chronic Dryness, Mucin Profile, Lacrimal Dysfunction, Pro Inflammatory Effects, Prevalence, Immunosuppressive Compound, Cyclosporine, Arginine, Potent Vasodilator, Hyaluronic Acid.

1. INTRODUCTION

Keratoconjunctivitis Sicca (KCS) or Dry Eye is a common disease characterized by unstable tear film associated with abnormality of the lipid, protein, and mucin profiles. Changes in tear composition also promote inflammation on the ocular surface. For untreated patients, the risk of ocular infection increases at considerable level and clinical course of the disease may proceed up to infection, corneal ulcer and blindness.[1-3] Cyclosporine, which has a selective immunosuppressive effect, is used for several autoimmune and contingent eye diseases because it specifically inhibits the T-cell-dependent immune reactions at high rates.[4-6]

In other words, Dry eye disease can also be known as Keratoconjunctivitis Sicca, either due to insufficient tear production or excessive tear evaporation, both resulting in tears hyperosmolarity that leads to symptoms of discomfort and ocular damage.[7] Dry eye disease is a prevalent disease that affects visual acuity, activities of daily living, and quality of life. Various environmental factors like contact lenses, pollution, working at video display terminals can affect the tear film and proceed up to infection, corneal ulcer, and blindness. It prevents T-cells from releasing cytokines that incite the inflammatory component of dry eye. Topical cyclosporine is an immunomodulator and anti-inflammatory agent. It is a fungal peptide which inhibits expression of various immune mediators such as Interleukin (IL) 2, IL-4 and Interferon (IFN) gamma, and through interaction with T cells inhibits lymphocyte proliferation.[8-10]

Although the reported prevalence of DES varies among populations, DES affects millions of individuals worldwide. In American men, Schaumberg et al. found prevalence rates ranging from 3.9% in men aged 50–54 years to 7.7% in those 80 years or older.[7] In American women, the prevalence also increased with age, from 5.7% among women younger than 50 years to 9.8% among women aged 75 years or older.[8] Other studies have found DES in 14% of individuals aged 65–85 years.6 Prevalence rates in Asian populations appear to be even higher. However, at least some of the variation between studies relates to differences in the definition of disease used.[9] The overall incidence of DED has significant geographic variation, with published data ranging from 7.8% to 14.6% in the USA, 5.5% to 16.6% in Australia, and 27% to 33% in Asian studies.[10] In addition to the natural occurrence of DED,

iatrogenic causes induce a similar form of OSD in a much larger population subjected to anterior segment ocular surgery or using long-term topical medications. Specifically, cataract surgery is known to disturb the tear film and induce surface inflammation up to 2 months following surgery.[11-13]

Nanoparticles systems accelerate the drug penetration, increase corneal uptake, and avoid systemic absorption. It is able to deliver more intact drug at site of action as compared to free drug.[24] After administration, colloidal drug carriers can remain at the application site (cul-de-sac) and the prolonged release of the active ingredient starts by particle degradation or erosion, drug diffusion, or a combination of both, depending on the biodegradable or inert nature of the polymer.[14] Based on these considerations, various strategies have been employed to modify NPs properties, including the use of cationic or mucoadhesive polymers. We have evaluated the particle formation process of the NPs prepared from HA polymers with the benefit of positively charged in different ratios, which could interact with the anionic mucins present in the mucus layer at the surface of the eye.[23] The current study aimed at developing and optimizing a bioadhesive nanoparticles of Cyclosporine for ocular delivery to improve corneal uptake efficiency to treat dry eye.[11]

Prior studies with cyclosporine were only directed at patients with moderate to severe disease; we wished to include a cohort of patients with mild disease. The purpose of this study was to evaluate the effect of cyclosporine on treating mild, moderate, and severe dry eye disease that was unresponsive to artificial tears therapy.[12-15]

2. MATERIALS AND METHODS

2.1 Materials

Cyclosporine (Sigma Aldrich Pvt. Ltd.), Hyaluronic acid (Himedia Laboratories), Arginine (Himedia Laboratories), Cetrimide (Himedia Laboratories) etc.

2.2 Preparation of Cyclosporine loaded Arginine-Hyaluronic acid nanoparticles

Hyaluronic acid-Arginine (HA-Arginine) nanoparticles was prepared by ionic-gelation method as reported by Oyarzun Ampuero *et al*. (2011). Nanoparticles were obtained using 0.1 molar of HA and 0.2 molar of Arginine in order to provide a molar equivalency. An arginine solution was dropped into a HA solution using a pipette under magnetic stirring (1,000 rpm). After this step drug which is dissolved in methanol, mixed drop by drop to the above solution. Final colloidal solution was then subjected for homogenization at 10000 rpm.

2.2.1 Optimization of ratio of Arginine and Hyaluronic acid

Different conc. Of arginine and hyaluronic solution were prepared in molar concentration such as 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M and nanoparticles are prepared by ionic-gelation method to get the desired size range of nanoparticles at different speed and time by homogenization.

2.2.2 Optimization in the concentration of cetrimide

Selected ratio of Hyaluronic acid and Arginine was kept constant and the concentration of Cetrimide was changed and the concentration with least particle size was selected.

2.2.3 Optimization in the concentration of water

Ratio of Hyaluronic acid and Arginine and concentration of Cetrimide with least particle size was kept constant and the percentage of stabilizers was changed to achieve the desired size of nanoparticles.

2.2.4 Optimization of process parameters

Homogenization speed (RPM), Time are the important processing parameters, which are used in the optimization of nanoparticles formulations. These are described in the following section.

A. Speed (rpm): The nanoparticles were prepared on different RPM to get the desired size.

B. Time: Time is also play a vital role in preparation of nanoparticles at different speed of homogenizer.

2.3 Physicochemical characterization

2.3.1 Particle Size and Polydispersity index (PDI)

Particle size and size distribution of prepared Hyaluronic acid and Arginine Nanoparticles were determined in the low volume quartz cuvette by using Delsa-Nano Zeta-Sizer. A minimum of three measurements per sample were made and the mean values was reported.

2.3.2 Scanning electron microscopy

The surface morphology and size of prepared Cyclosporine loaded Nanoparticles were analyzed using a Scanning Electron Microscope (SU8020, Hitachi). Scanning Electron Microscope uses a focused beam of high-energy over the nanoparticles, producing various signals that help to obtain information about the surface morphology and composition.

2.3.3 X-Ray diffraction

X-Ray Diffraction screening was performed to detect the physical state of Cyclosporine loaded Nanoparticles. The sample was exposed to monochromatic nickel-filtered copper radiation (45kV, 40mA) in a wide angle X-ray diffractometer (D8 Advance, BRUKER, Germany) with 2Ɵ scan ranges from 4-450 with a step size of 0.1º.

2.3.4 Differential scanning calorimetry

DSC is a thermo-analytical technique, which is used to study thermal behavior and the decomposition of the prepared Cyclosporine loaded Nanoparticles. The phase transitions of Cyclosporine NPs was analyzed by Differential Scanning Calorimetry (SIIO 6300, SIIO, Japan) in a perforated aluminium pans at a heating rate of 50C/min from 30 to 4000ºC using nitrogen as blanket gas (50ml/sec).

2.3.5 IR spectroscopy

IR study was performed for identification and structural analysis of sample using FT-IR spectrometry. A small quantity of the sample was triturated with KBR preferably in the ratio of 1:100. This mixture was then compressed into a pallets which was then placed into the FT-IR assembly and the IR-spectrum was obtained out at temperature from 400 - 4000.

2.3.6 Solubility

It is the amount of the solute which can be dissolved in a given amount of solvent under standard conditions of temperature, pressure and pH to form homogeneous solution. Solubility of drug was investigated in water, ethanol, chloroform and methanol.

2.3.7 pH

The pH values of optimized formulations were determined using digital pH meter (ME 963-P). The pH was repeated for formulations after 1day, 3days, 7days, 14days and 28 days.

2.3.8 In-vitro release study

The in-vitro release study was performed using Franz diffusion cell to evaluate the drug release profile of the optimized formulations. The pre-treated dialysis membrane was used and mounted on the Franz diffusion cells. The receptor compartment contained PBS (100 ml) of pH 7.4. The temperature of diffusion media was thermostatically controlled at 37±0.5ºc by surrounding water in the outer jacket and the medium was stirred by magnetic stirrer at 100 rpm. Drug loaded nanoparticles were placed on the dialysis membrane, which was fixed in

between donor and receptor compartment. The donor compartment was then capped to prevent evaporation. Samples of 1 ml were taken after 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,12, 13, 14, 15,and 16 hours and replaced by an equal volumes of fresh PBS to maintain the sink conditions. After suitable dilutions with PBS, samples were analysed by UV Spectrophotometer at 210 nm against the blank and atomic absorption spectrophotometer for nanoparticles. The release experiments of each sample were performed in triplicate and average value are reported.

2.3.9 Sterility test

The sterility test of nanoparticles was performed by comparing turbidity of the test inoculation with test of Mc-farland standards. The culture media was prepared by using nutrient broth and it was autoclaved to sterilize the culture media. The nanoparticles were transferred into the culture media and incubated for 48 hrs. After the stipulate time period the samples were taken and analysed under UV-Spectroscopy. Solution consisting of 1% $Bacl_2$ and 1% H_2SO_4 was used to produce a series of Mc-farland standards with respect to their composition and equivalence. The absorbance of prepared dispersion was observed at 210 nm using UV-Spectrophotometer.

2.3.10 Isotonicity

Isotonicity of the developed formulation was observed for swelling, bursting and shrinking of RBC, the RBC were taken and diluted with the Hayem's solution, which was observed under the Motic microscope and the another part of test RBC was incubated with formulation. After incubation the diameter of red blood cells was measured. The mean diameter of RBC was determined by manual image analysis of approximately 200 to 500 RBC. Change in diameter of RBC was compared with control group to interpret toxicity.

3. Ex-vivo permeability study[26]

Permeability study were done on freshly excised cornea of goat obtained from local slaughter house. The cornea was carefully removed from other ocular tissue and was washed several times before use for removal of protienous matter. After its removal the excised cornea was immediately fixed between donor and receptor compartments of an all-glass modified Franz diffusion cell in such a way that its endothelial surface faced the receptor compartment and epithelial towards the donor. The receptor compartment was filled with 10 mL freshly prepared bicarbonate ringer solution (pH 7.2)/normal saline, and before start the experiment all air bubbles were expelled from the compartment. An aliquot (1 mL) of optimized

formulations and marketed formulation was placed on the cornea and the receptor fluid was kept at 37 ± 1°C with constant stirring using a Teflon-coated magnetic stir bead. Permeation study was continued for 240 min and samples were withdrawn from receptor compartment at regular time intervals and analyzed for drug (Cyclosporine) content by UV spectrophotometer (Pharmaspec 1700, Shimadzu, Japan). Results were expressed as amount permeated and percentage permeation. The permeation (%) was calculated as follows:

% Permeation = (Amount of drug permeated in receptor/Initial amount of drug in donor) × 100

4. In-Vivo studies

Rabbits (2-2.5kg) were used in in-vivo study of formulations (Hyaluronic acid and Arginine nanoparticles, Cyclosporine loaded nanoparticles and Cyporin (Marketed formulation) ophthalmic emulsion 0.05% w/w Cyclosporine. Animals were procured from the registered breeders and were housed under standard laboratory conditions with free access to food and water. The protocol was approved by Institutional Animal Ethical Committee (IAEC) at ISF College of Pharmacy, Moga, India. The experiments were conducted as per CPCSEA (Committee of prevention, Control and Supervision of Experimental Animals ISFCP/IAEC/CPCSEA/Meeting No.24/2019/ Protocol No. 403) guidelines. Animals were divided into 3 groups of 3 animals per group as shown in table. The first group was treated with Cyporin (Marketed formulation). The animals of second group received Arginine-HA nanoparticles. The third group received final formulation.

S. No.	Groups	Animal Species	No. of animals
01.	Cyporin (Marketed formulation)	Rabbit	03
02.	Arginine-HA nanoparticles		03
03.	Final formulation		03

4.1 Measurement of tear production[25]

Nine rabbits will be used for the disease induction. One eye of each rabbit will be chosen randomly for twice-daily topical administration of 0.1% Benzalkonium chloride (1-2 drops) for 14 days. The other untreated eyes of 9 rabbits that will be exposed to no solution are used as a control group. In both, the Benzalkonium chloride treated and the control groups, Schirmer test will be perform before and after Benzalkonium chloride treatment on days 3, 5, 7, and 14.

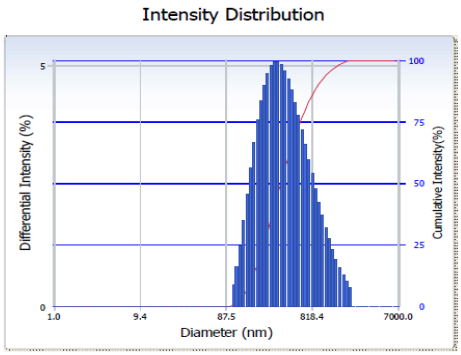
Tear production will be measure using Whatman 41 filter paper strip on days 0, 3, 5, 7, and 14. Intramuscular injection of a mixture of 40 mg ketamine and 25 mg chlorpromazine will be administer to keep the rabbits immobile. After topical application of formulation, paper strip will be insert into the conjunctival sac location around the junction of the middle and outer thirds of the lower lid. The wetted length (mm) of the paper strip will be read after 5 minutes.

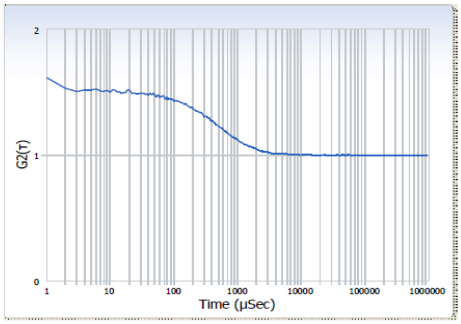
4.2 Irritancy test[26]

Ocular irritation testing (Tolerance studies) will be perform in order to evaluate the tolerance of different formulations. The animals will be divided into three groups (each having three animals) and 10 µL of the formulation are placed in the cul-de-sac of both eyes of the animal of each group. Group 1 receives Cyporin (Marketed formulation), group 2 receives Arginine-HA NPs without drug and group 3 receives final formulation, when instilled in both eyes of the rabbits and then their blinking rate/hr, discomfort (redness and irritation), discharge rate and swelling of cornea and conjunctiva will be examined for 1 day at regular intervals of 1, 3, 6, 8, 12, and 24 h.

5. RESULT AND DISCUSSION

5.1 Particle Size and PDI of nanoparticles without drug





Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	499.5	373.3
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	499.5	373.3
Residual :	1.514e-002	(O.K)

Cumulants Results

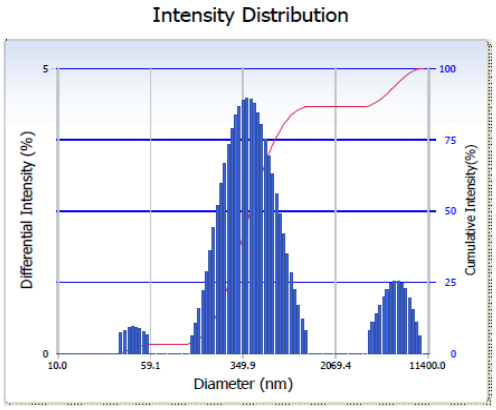
Diameter	(d)	: 387.5	(nm)
Polydispersity Index	(P.I.)	: 0.249	
Diffusion Const.	(D)	: 1.273e-008	(cm²/sec)

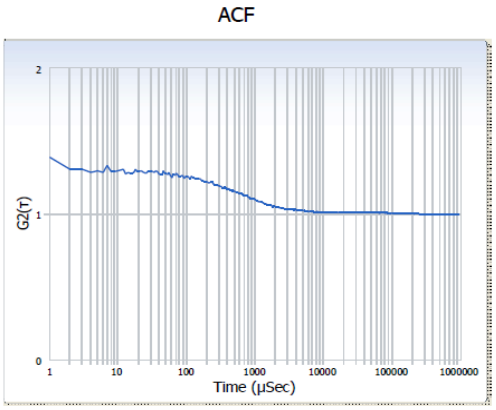
Measurement Condition

Temperature	: 25.1	(°C)
Diluent Name	: WATER	
Refractive Index	: 1.3328	
Viscosity	: 0.8858	(cP)
Scattering Intensity	: 10185	(cps)

Polydispersity index (PDI): 0.219
Diameter: 387.5 nm

5.2 Particle Size and PDI of Drug loaded nanoparticles





Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	43.3	6.7
2	433.7	211.2
3	6,702.6	1,692.7
4	0.0	0.0
5	0.0	0.0
Average	1,260.8	2,238.1
Residual :	1.790e-002	(O.K)

Cumulants Results

Diameter	(d)	: 620.0	(nm)
Polydispersity Index	(P.I.)	: 0.261	
Diffusion Const.	(D)	: 7.934e-009	(cm^2/sec)

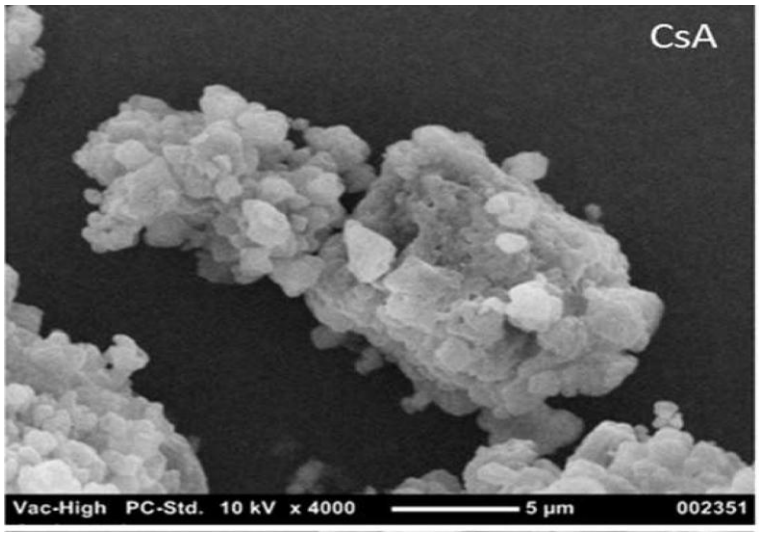
Measurement Condition

Temperature	: 25.0	(°C)
Diluent Name	: WATER	
Refractive Index	: 1.3328	
Viscosity	: 0.8878	(cP)
Scattering Intensity	: 8518	(cps)

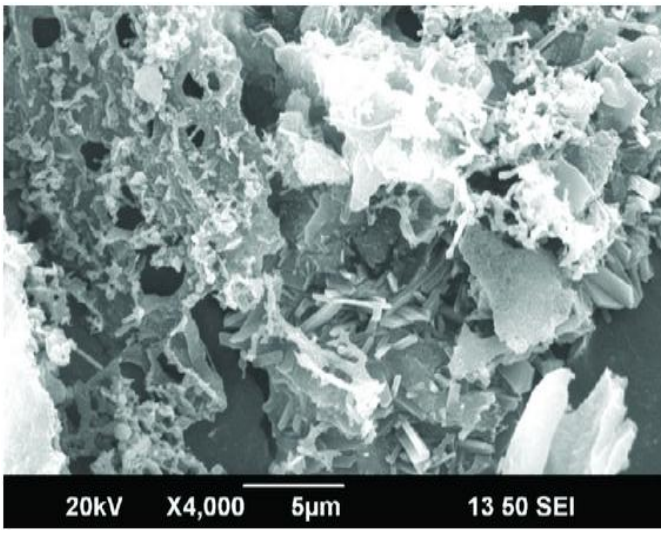
Polydispersity index (PDI): 0.261

Diameter: 620 nm

5.3 SEM of Cyclosporine and Formulation

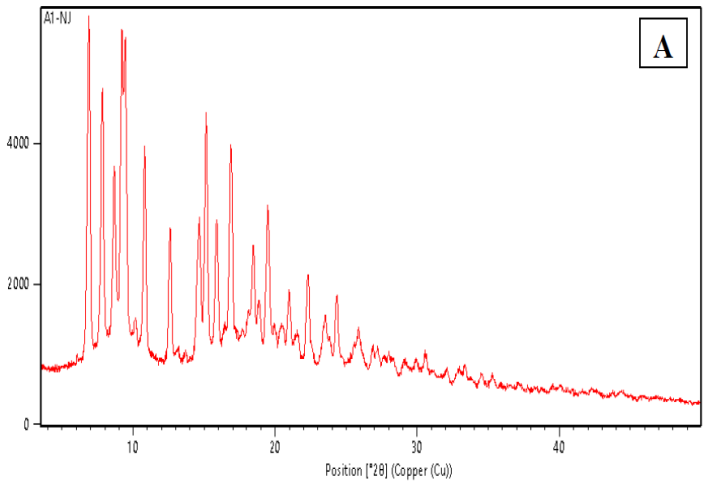


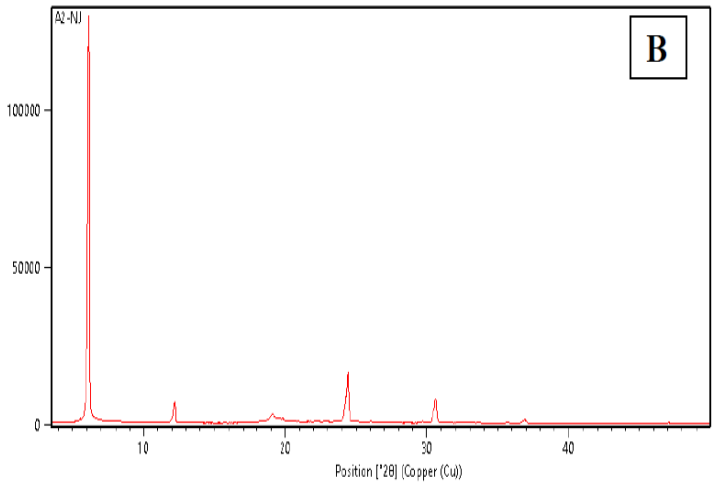
(SEM of Cyclosporine)



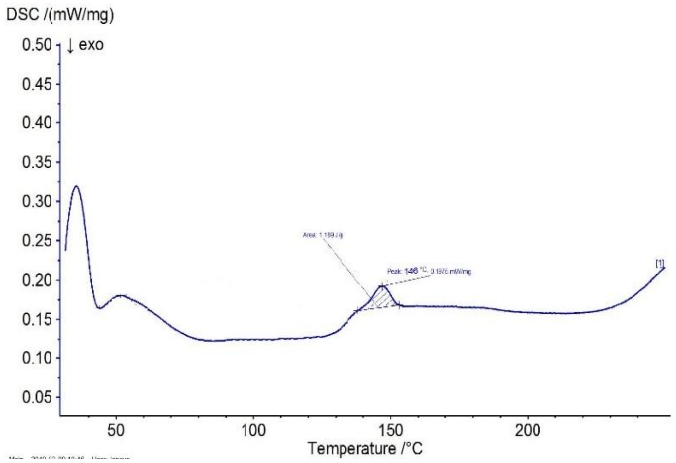
(SEM of Formulation)

5.4 X-ray diffraction of Plain Drug (A) & Formulation (B)

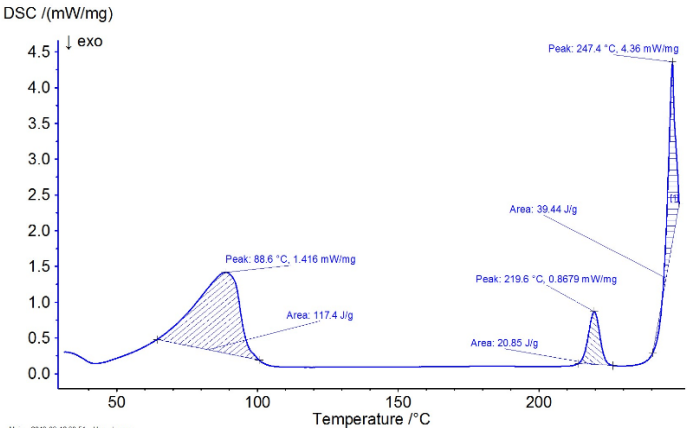




5.5 DSC of Cyclosporine and Physical Mixture

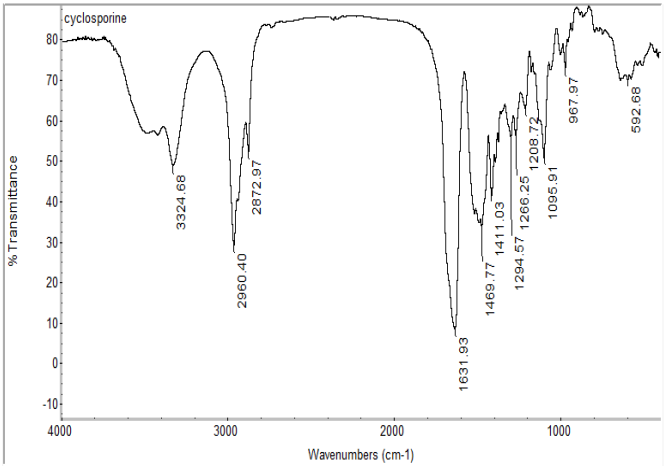


(DSC Graph of Cyclosporine)

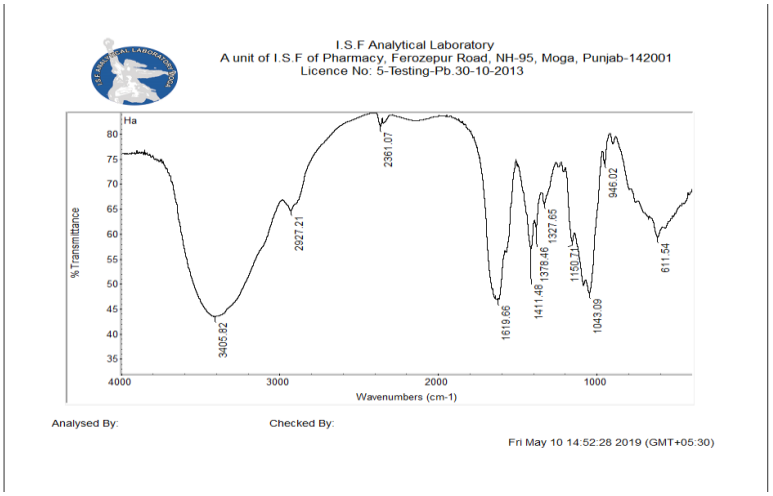


(DSC Graph of Physical Mixture: Cyclosporine + HA + Arginine)

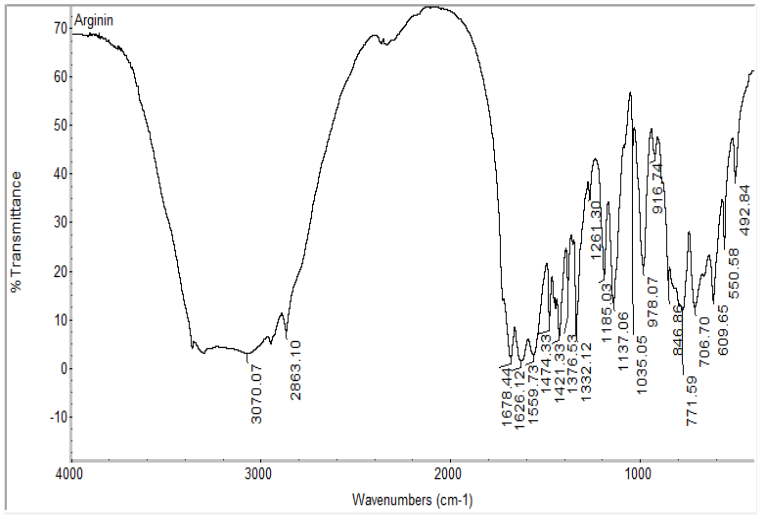
5.6 Infra-red (IR) Spectroscopy



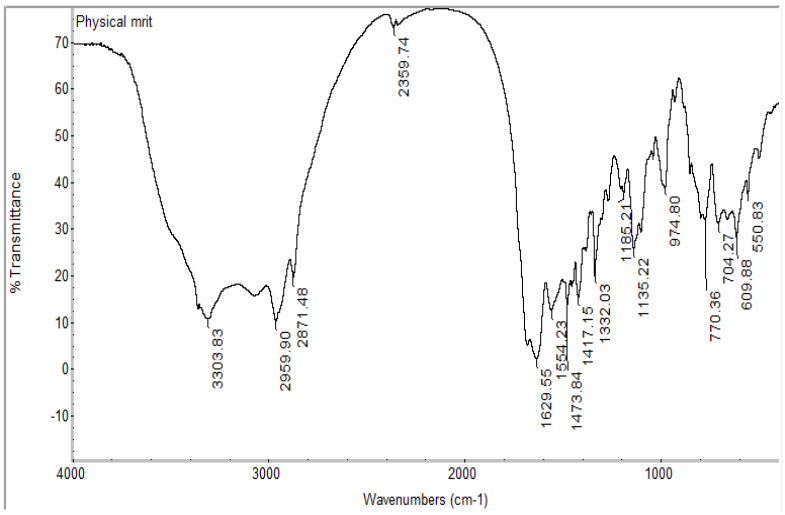
(IR Spectra of Cyclosporine)



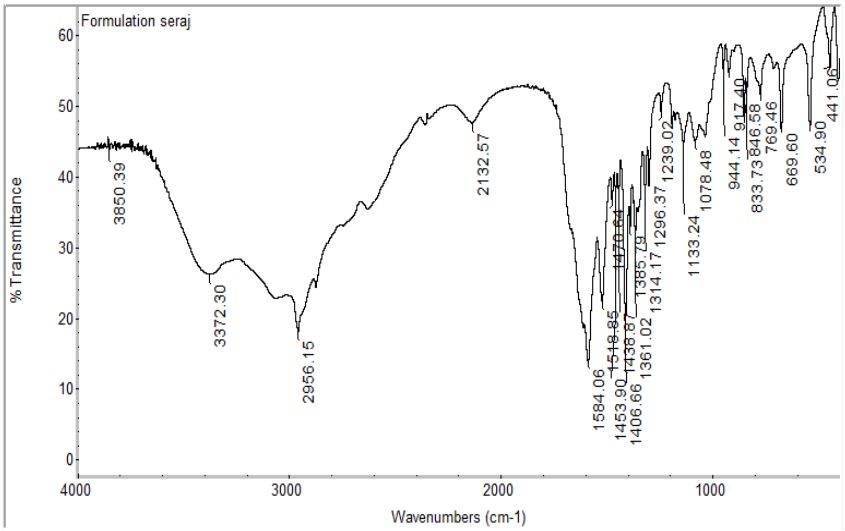
(IR Spectra of HA-Hyaluronic Acid)



(IR Spectra of arginine)



(IR Spectra of physical mixture)



(IR Spectra of final formulation)

5.7 Solubility

The solubility trend of drug in different solvents has been given in the table. Results of solubility studies indicate that drug exhibited good solubility in methanol, acetone, ethanol and DMSO. It is slightly soluble in water.

Table: Solubility profile of cyclosporine

Solvent	Saturation solubility (mg/ml)	Parts of solvent required for one part of solute	Standard	Interpretation
Methanol	35	28	10-30 parts	Soluble
Acetone	36	27	10-30 parts	Soluble
Diethyl ether	32	26	10-30 parts	Soluble
Water	4	786	100-1000 parts	Slightly Soluble
Ethanol	32	25	10-30 parts	Soluble
DMSO	33	28	10-30 parts	Soluble

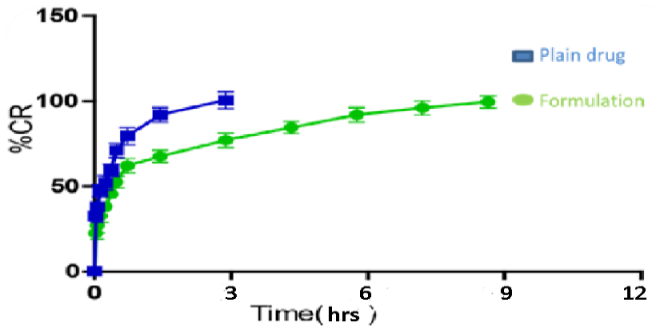
5.8 pH

pH of formulation at different time intervals

Days	pH
Days 1	7.4
Days 3	7.4
Days 7	7.3
Days 14	7.3
Days 28	7.3

5.9 In-Vitro drug release

The % cumulative drug releases from optimized formulation with time are shown in figure which is given following. The release pattern shows that there was a sustained release followed by burst release from the nanoparticles which may be due to surface adherence. Initial burst release of approximate 40% of applied to surface drug, following burst release, an extended drug release was observed which is attributed to diffusion mechanism. Similar findings were observed in earlier studies by our group, where sustained release behaviour of cyclosporine was observed from HA nanoparticles up to 12 hours. Extended drug release mechanism also a function of poor drug solubility and slow degradation of polymeric constituents. Further the comparative study indicated that HA significantly reduced the drug release compared to plain drug solution.



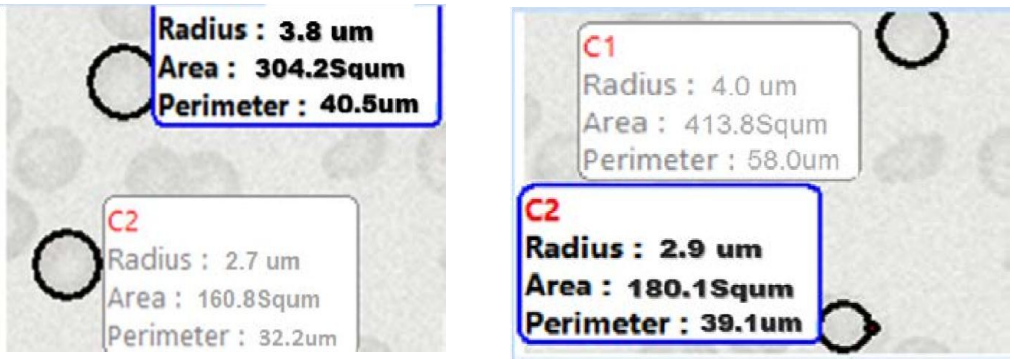
5.10 Sterility test

The sterility test of nanoparticles was performed by comparing the turbidity of the test formulation with that of Mc-farland standard. It was found that the absorbance of both the standard and the test samples was found to be in the almost same range, which shows that there was no microbial growth. The observed absorbance of standard and the test samples are in range from 0.78- 0.79 which shows that there is no sign of microbial contamination in drug loaded nanoparticles.

5.11 Isotonicity

Isotonicity of the formulation was examined using RBC dilution method. 500 RBC were counted and their mean were reported. Results indicated that there is no change in mean diameter was observed in the treated group compare to untreated group, confirmed the

isotonicity of the developed formulation. High molecular weight of hydrophobic polymer and insoluble nature of drug rendering fewer ionized particles to influence tonicity. Tonicity results also confirm that the developed product avoid discomfort and irritation, thus contribute useful platform for ophthalmic drug delivery. The mean value of 500 RBC were examined and reported in figure that is given below.

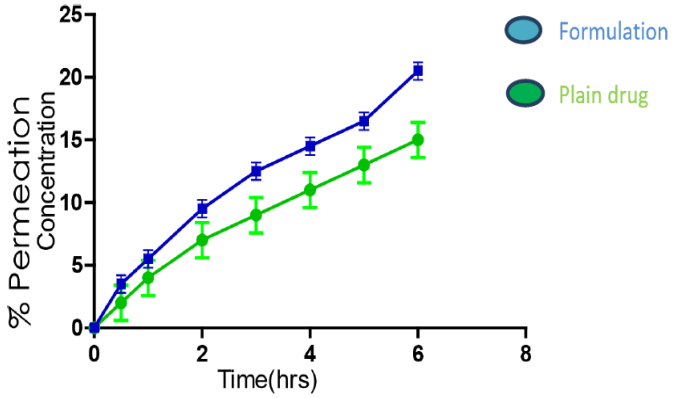


Standard (RBC with Hayems sol.) Test (RBC with Hayems sol.)

(Isotonicity of Standard and Test)

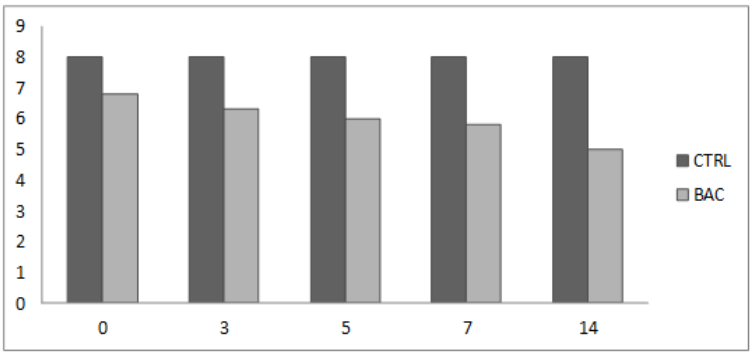
5.12 Ex-Vivo permeability study

Results of permeability study in fig.4.11 indicated a significantly higher drug permeation in nanoparticles group compared to the plain drug suspension. Higher drug permeation in nanoparticles attributed high surface drug, better wettability and nanonization of drug in nanoparticles.

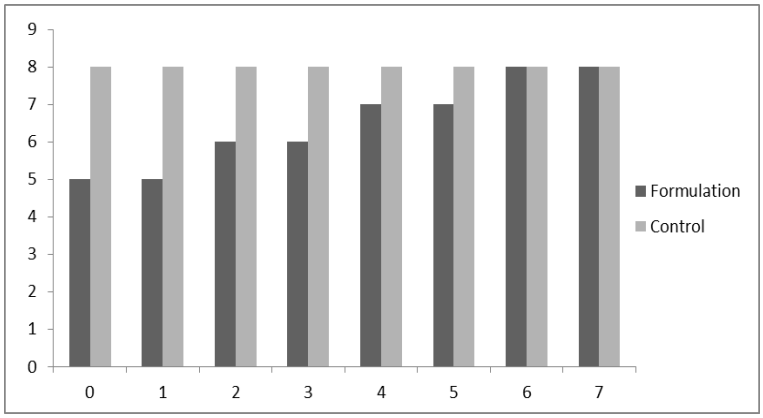


6. In-Vivo studies

6.1 Measurement of tear production



(Schirmer test showing statistically significant differences between the BAC-treated and the control (Ctrl) groups at days 5, 7, and 14.)



(Figure shows that after topical administration of formulation, tear production has increased from day 0 to 6)

6.2 Draize test

Results of eye irritation studies indicated that there is no visual sign of irritation was observed in all experimental groups shown in table which is given following. Outcomes of ocular irritation studies concluded that the prepared formulation has no ocular toxicity and safe for ocular use.

Draize test scoring:

Different Formulations	Time (Days)	Irritation	Blinking of Eye	Tear Flow	Inflammation Changes	Redness
Plain drug	2	2	1	1	0	1
	6	0	0	0	0	0
	10	1	0	0	0	0
Formulation	2	2	2	1	0	1

	6	1	1	0	0	1
	10	0	0	0	0	0
Marketed formulation	2	1	1	1	0	1
	6	1	0	0	0	1
	10	1	0	0	0	0

Tested Formulation	**Observation Period**		
	2nd Day	**6th Day**	**10th Day**
Plain Drug			
Nanoparticle Formulation			
Marketed Preparation			

7. CONCLUSION

The present study reports the preparation and physicochemical characterization of Cyclosporine loaded bioadhesive NPs which combine the negatively charged Hyaluronic acid with the positively charged properties of Arginine.

Cyclosporine loaded bioahesive nanoparticles successfully prepared using ionic-gelation method. Polymer concentration, applied homogenizer speed (RPM), homogenization time seems to be the key determinant that accounts for the morphology of nanoparticles. In vitro characterization demonstrated that the prepared nanoparticles show high entrapment efficiency, controlled drug release behavior, adequate mechanism, suitable for ophthalmic application. Ex-vivo permeability shows a relatively high drug permeation in prepared formulation compare to the plain drug and the marketed formulation. Above findings accounts for the suspected transition of drug in nanoparticles. In vivo observations clearly

indicates the therapeutic potential of nanoparticles with higher drug availability at the target site. Tear production and draize test shows no adverse outcomes of the prepared nanoparticles. All the findings support the application of preservative free bioahesive nanoparticles as a suitable carriers for drug delivery into the posterior eye segment.

In conclusion, we have demonstrated that NPs with different properties could modulate the drug release in the cul-de-sac or in the ocular tissues after uptake by epithelial cells. The system interacts with eye surface, ensuring optimal contact between the formulation and the mucosa. It provides sustained drug release in the Cul de-sac for an extended period of time. These results indicate that Cyclosporine loaded bioadhesive NPs have great potential as drug delivery systems and are promising formulations in the management of external inflammatory/autoimmune ocular diseases.

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