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OPTIMIZATION OF 3^2 FULL FACTORIAL DESIGN BASED NORFLOXACIN NANOPARTICLES EMBEDDED THERMO SENSITIVE DROPPABLE GEL FOR OCULAR DRUG DELIVERY.

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ABSTRACT

The objective of the present study was to fabricate and optimize nanoparticle loaded thermo sensitive droppable gel contains norfloxacin(NFLX) for ophthalmic delivery. Norfloxacin nanoparticle were prepared by nanoprecipitation method and evaluated for particle size analysis, zeta potential, percentage entrapment efficiency and In-vitro drug release studies. Based on evaluation nanoparticle formulation (D1) was dispersed into thermo sensitive droppable gel base (NP-TDGL). A preliminary trail of 9 Experiments (runs) were performed according to a 3^2 full factorial design (FFD) to evaluate the effects of two factors such as Pluronic-F-127 and HPMC K-100-M. Polynomial equation was generated by Design Expert 11.0 for the model which assists in determining the effect of independent variables. 3D Response surface plots (RSM) were also generated by the software for investigate effect of the independent variables on the response. Linear model was found to be the best for responses (Y1) and response (Y4) and quadratic model for response (Y2) and response(Y3). The optimized formulation (NF1) evaluated for drug content of the formulations was 100.14%. pH of the formulations was in the range 7.4, gelation temperature was in the range 34.2^{o}C and cumulative % drug release was in the range of 67.33% at the end of 6 hr and simultaneously the release of drug based on the concentration of polymers used. Finally it was conclude that the developed NP-TDGL imparts prolonged corneal residence time and

controlled drug release could be considered a viable alternative to the conventional eye drops in achieving enhanced bioavailability.

KEYWORDS: Norfloxacin, Pluronic-F-127, design expert 11, quadratic model, Gelation temperature.

INTRODUCTION

Norfloxacin(NFLX) is a fluoroquinolone derivatives of broad spectrum antibacterial drugs for the treatment of bacterial conjunctivitis in the eye. Commercially it is available as eye drops in the concentration 0.3% w/v. However the dose range is one or two drops meant for instilled in the lower conjuctival-sac for every two hours up to eight times for first two days. It can inhibit deoxyribonucleic acid gyrase enzymes, leads to bacterial death and prevent deepening of microbial infection.[1] According to biopharmaceutical classification, norfloxacin has poor solubility and poor permeability drugs (BCS-IV).[2] A Traditional norfloxacin (NFLX) eye drops possess major demerits such as frequency dose administration, absence of bioadhesive properties and prolonged release effect, which cause quick prior-corneal elimination and low ocular absorption.[3] Thereby, researchers have tried to find ways to overcome the barriers and defense mechanisms of the eye in order to enhance the bioavailability of ocular drugs into target sites by using colloidal carrier systems like micelles, nanosuspensions, nanoemulsions, liposomes, polymeric and lipid nanoparticles, in-situ nanogel.[4] Among these new delivery systems, in situ nano gel appear to be the most promising carrier for ocular drug delivery. Nowadays, a kind of droppable gel called as ‘‘in situ nanogel’’ consisting of freeze dried nanoparticle blended in to thermo sensitive in-situ polymers base.[5] It is liquid at low temperature 25.0°C and becomes gel when it contacts with precorneal temperature 34.5°C.[6] The ideal critical temperature for this system is ambient and physiologic temperature so that phase transition does not require any external source of heat other than the body heat.[7] One of well known polymer types possessing thermo responsive behavior is Pluronic® so called Poloxamer. They are amphilic behavior due to hydrophilic poly-ethylene oxide (PEO) and hydrophobic poly-propylene oxide (PPO) domains. The gelation mechanism of pluronics could be explained by the changes in micellar structure as a function of concentration and temperature.[8] However, a major disadvantage of Pluronic is their low bioadhesive activity, therefore, some Pluronic based formulation have been improved by adding polymer providing bioadhesive property such as hydroxyl propyl methyl cellulose (HPMC-K-100-M).[9]

In the present study, dual carriers were employed for preparation thermo sensitive characters of droppable gel. Intially norfloxacin (NFLX) loaded polymeric nanoparticle was prepared using nanoprecipitation technique. The prepared nanoparticle was characterized and optimized based upon particle size, zeta potential, drug entrapment efficiency. Further the best formulations were blended in thermo sensitive droppable gel base comprising of Pluronic -F-127 and viscosity enhancer HPMC K-100-M. Which will provides benefit of prolonged release and higher transcorneal permeation owing to dual carriers like polymeric nanoparticle and droppable gel thus is likely to correct the high need for frequent dosing as in the case of conventional formulation. The nanoparticle loaded thermo sensitive droppable gel were designed using 3^2 full factorial design as per design expert software 11.1 (Trial version 30 days). Based on the principle of design of experiments (DOE), the methodology encompasses the use of various types of experimental designs, generation of polynomial equations and mapping of the response over the experimental domain to determine the optimum formulation. The technique requires minimum experimentation and time, thus proving to be far more effective and cost-effective than the conventional methods of formulating dosage forms.[10]

MATERIALS AND METHODS

Norfloxacin(NFLX) and Eudragit-RLPO was donated as a gift sample from Dr.Reddy's laboratories Ltd., (Hyderabad, India). Dichloro methane(DCM), methanol were purchased from thermofisherscientific Pvt. Ltd,(Haryana, India). Pluronic-F-127 and HPMC-K100-M wasprocured from yarrow chem. products, Mumbai, India and Poly vinyl alcohol purchased from loba chemie Pvt. Ltd, mumbai, India. Benzalkonium chloride(BAC) was obtained as gif sample from tablets India, chennai. Muller hinten agar medium, soya bean casein digested medium(SCDM), fluid thioglycollate medium(FTM) were purchased from Himedia laboratories. Deionized water was used throughout experimentation. All chemicals used for preparing artificial tear fluid (ATF) were analytical grade and purchased from S.D Fine chem. Ltd, Mumbai, India.

Preparation of norfloxacin (NFLX) nanoparticle by nanopreciptation technique

In Brief accurately weighed amount (30 mg) of norfloxacin drug and various amount of eudragit-RLPO polymer were dissolved in a mixture of water miscible solvents such as dichloromethane and methanol until to form a homogeneous solution**.** These organic solution were injected drop by drop into the aqueous solution consist of stabilizer polyvinyl alcohol

(PVA) respectively (Table 1). The mixture was agitated using a magnetic stirring at 900 rpm for 3 hr until to produce blue colour opalescence. Further it was sonicated and the organic solvent was evaporated by magnetic stirring for the 12hr at room temperature. All batches of Nanosuspension were lyophilized (Osterode Am Harz, Germany) by incorporating 1.5% mannitol as cryoprotectant to reduce agglomeration in the formulation. In our earlier journal publication norfloxacin loaded polymeric nanoparticle were developed and characterized.[11]

Evaluation of NFLX -loaded eudragit-RLPO nanoparticle

All the formulation was analyzed for mean particle size, zeta potential (surface charge) and poly dispersity index (PDI) using malvern-zetasizer instrument. Percentage drug entrapment efficiency of nanoparticle was determined by centrifugation method (Indirect method) respectively. The morphological characters of nanoparticle were determined using transmission electron microscopic technique (Philips EM-CM). The best formulation (D1) were selected based on particle size, high drug content and zeta potential value as presented in table 5.

3^2 Full factorial design (FFD) experiments

A 3-level 2-factor factorial design (3^2)was used for design and optimizing norfloxacin nanoparticulate based thermo sensitive droppable gels utilizing design expert software 11. (Stat-Ease).[12] The concentration of Pluronic-F-127 and HPMC K-100-M were selected as independent variable at three different levels low (-1), medium (0)and high (+1) totally nine formulation were coded and Gelation temperature (Y1)**,** Bio-adhesive force (Y2), Viscosity at physiological condition (Y3) and cumulative % drug release at the end of 6 hr (%) were chosen as dependent variables. (Table 2).

Optimization of formulation

Response surface methodology (RSM) is a widely practiced approach in the development and optimization of drug delivery devices. Based on the principle of design of experiments (DOE), the methodology encompasses the use of various types of experimental designs, generation of polynomial equations and mapping of the response over the experimental domain to determine the optimum formulation. The various computations for the current optimization study were performed using Design Expert software (Design expert 11. state ease inc., Minneapolis, USA). Various RSM computations for the current optimization study were performed employing Design Expert software. A 3^2 full factorial design(FFD) was constructed where the ratio of Pluronic-F-127 (X_1) and the amount of viscosity enhancer

HPMC-K-100-M(X_2) were selected as the independent variables i.e. factors The level of this factors is selected on the basis of initial studies and observations. Polynomial models including interaction and quadratic terms were generated for all the response variables using multiple linear regression analysis (MLRA) approach. The general form of the polynomial model is represented as equation below.

Linear model

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X$ **…….**

Quadratic model

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^{\ 2} + \beta_5 X_2^{\ 2}$ **……**

where, β_0 is the intercept representing the arithmetic average of all quantitative outcomes of 9 runs; β_1 to β_5 are the coefficients computed from the observed experimental values of Y(response) and X_1 and X_2 are the coded levels of the independent variables. The terms X_1X_2 shows how the response values change when two factors are simultaneously changed. The polynomial terms are included to investigate the nonlinearity. The polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries (i.e., positive or negative). In the equation represents that by making a minor change in the setting of that factor one may obtain a significant change in the dependent variables. Statistical validity of polynomials was established on the basis of analysis of variance (ANOVA) provision in the design expert software level of significance was considered significant at P>0.005. The best-fitting mathematical model was selected based on the comparison of several statistical parameters including the coefficient of variation. The multiple correlation coefficient(R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicted residual sum of squares (PRESS) provided by the software. Which indicates how well the model fits the data and for the chosen model it should be small relative to the other model under consideration.

Validation of experimental model

According to desirability value and composition of variables, formulation was prepared and evaluated for response. The predicted and observed response was compared and percentage error was calculated to confirm the validity of design for optimization. The percentage prediction error was calculated by using following formula,

$$\text{Percentage prediction error} = \frac{\text{Observed value (OV)} - \text{Predicted value (PV)}}{\text{Observed value (OV)}} \times 100$$

Redispersability study of lyophilized NFLX nanoparticle

Accurately weigh 20 mg of optimized lyophilized nanoparticle (D1) was dispersed in deionized water after manual hand shaking of the container. The purpose of the study to determine any agglomeration or redisperbility in a selected aqueous vehicle and characterized the stabilility of nanoparticulate in thermo sensitive droppable gel formulation.[13]

Development of nanoparticle laden thermo reversible droppable gel

Norfloxacin nanoparticle loaded thermo sensitive droppable gel(NP-TDGL) was prepared by slight modification in cold technique as shown in figure 1. In brief, accurately weighed quantity of Pluronic-F-127 and HPMC-K-100M were dissolved separately in the little portion of cold deionized water and stirred on magnetic stirrer for a period of 1 hr at 200-300 rpm. The low speed was used to avoid the foam formation in the solution and partially dissolved polymeric solution were closed with aluminum foil and refrigerated overnight soaking at 4-5^{o}C. Both the solutions were then blended by constant stirring for 1 hr at 200-300 rpm. Accurately weighed optimized freeze dried nanoparticle(D1) formulation (equivalent to 30 mg /10 ml) was dispersed in previously prepared thermo sensitive gel base with continuous stirring at 200-300 rpm in magnetic stirrer for 1 hr. Then add required quantity of preservative benzalkonium chloride and tonicity modifier sodium chloride were dispersed in deionized water, added to the polymer solution and further stirred until uniformly mixed (Table 4). All the sample solutions were adjusted to pH 7.4 by 0.1 N sodium hydroxide solution and then sterilized by membrane filtration sterilizer passed through 0.22 µm polycarbonate (PC) membrane unit. Finally formulation was terminally sterilized screw cap glass container.[14,15]

Appearance and pH measurement of NP-TDGL

Before performing characteristics studies all developed formulations was determined by visual examination under black and white background. The pH of each formulation was measured by using pocket pH meter which was calibrated using buffers of pH 4 and pH 7 before the measurements. Each recording was made in triplicates when they are in solution characters.[16]

Surface characters of nanoparticle loaded -TDGL

The morphological examination of nanoparticles was performed by transmission electron microscopy (TEM) CM12; Philips, USA). The selected nanoparticle dispersion was dropped onto Formvar-coated copper grids and after complete drying the samples were stained using

2% w/v aqueous solution of uranyl acetate. The outer appearance formulation (NF1) was compared against reference image of D1 nanoparticles.[17] Morphology characters were analyzed using scanning electron microscopy (SEM) (Philips, XL30). One drop of freshly prepared NP-TDGL with or without polymer was deposited on sample stub covered with carbon tab. The samples were then air-dried and covered with gold. To avoid deformation measurements were performed at low voltages (10 kV).

Particle size and Zeta potential measurement of NP-TDGL

Nanoparticle size distribution and zeta potential were determined using photon correlation spectroscopy (Zetasizer, HAS 3000; Malvern Instruments, Malvern, UK). The size distribution analysis was performed at a scattering angle of 90 degrees and at a temperature of 25°C using samples appropriately diluted with filtered water, whereas zeta potential was measured using a disposable zeta cuvette.[18]

Determination of gelation temperature of NP-TDGL

For measurement of gelation temperature, 10 mL of the sample solution and a magnetic bead were put into a beaker that was placed in a low-temperature water bath. A thermometer was immersed in the sample solution. The temperature was increased gradually with continuous stirring at 100–200 rpm. The temperature was determined as gelation, at which the magnetic bead stopped moving due to gelation. Each sample was measured at least in triplicate.[19]

Measurement of gelation temperature in ATF

The solution to gel phase transition temperature was determined as described above after dilution with ATF pH 7.4. The temperature was increased gradually. The measurements were taken at 15–37°C, the temperature in the conjunctival sac of the eye. To mimic the properties in the eye, if all applied polymer solution (40 µL) was immediately mixed with the available tear fluid (7 µL), which would be the critical condition of the polymer solution was mixed with artificial tear fluid in a ratio of 40:7.

Measurement of Solution to gel phase transition time of NP-TDGL

Gelling capacity of the representative formulations was determined by placing a drop of the sample (about 20µl) into a test tube containing 2ml of pH 7.4 artificial tear fluid (ATF) equilibrated at 35±1°C. Artificial tear fluid were composed of sodium chloride (0.67 g), sodium bicarbonate (0.2 g), calcium chloride·$2H_2O$ (0.008 g) and purified water added to

(100 g).[20] The visual assessment of gel formation and dissolution with time record was performed in triplicate.

Drug content

Drug content was determined by dissolving nanoparticulate droppable gel (equivalent to 30mg drug) with 10 ml of methanol in a 100ml volumetric flask and volume was made up to 100 ml with ATF pH 7.4. After suitable dilution absorbance was measured at 277 nm using UV spectrophotometer to calculate the percentage drug content.[21]

Bio-adhesive force measurement

A modified physical balance method was used to determine the bio-adhesive performance of different formulations by measuring the force required to detach the gel from a mucosal surface (Figure 3 b). Fresh sheep goat corneal mucosa was obtained from a local slaughterhouse and used within 2 hours. The mucosal membrane was separated by removing underlying fat and loose tissues then membrane was washed with distilled water and then with proper medium at 37°C. A piece of mucosa was tied to the glass vial, which was filled with phosphate buffer. NP-TDGL was spread on an area of 1 cm^2 on another piece of mucosa, which was adhered to another glass vial put on a moving platform. The two sides of the balance were made equal before the study, by keeping a 5-g weight on the right-hand pan. A weight of 5 g was removed from the right-hand pan, which lowered the pan along with the NP-TDGL over the mucosa. The balance was kept in this position for 5 minutes contact time. The water (equivalent to weight) was added slowly with an infusion set (100 drops/min) to the right-hand pan until the vial detached from the mucosal surface. This detachment force gives the bio-adhesive strength of the NP-TDGL in grams. The weight of water required to detach bio-adhesive formulation from corneal mucosa was noted as bio-adhesive strength in grams. From the bio-adhesive strength following parameter was calculated as follows,

$$\text{Bio-adhesive strength dynes/ cm}^2 = \frac{M \times g}{A}$$

$$\text{Force of adhesion (N)} = \frac{\text{Bioadhesive strength (gm)} \times 9.8}{1000}$$

Where, m is the weight required for detachment in grams, g is the acceleration due to gravity (980 cm/s^2) and A is the surface area of mucosa exposed to the formulation (cm^2).

Measurement viscosity of formulation

Viscosity of instilled formulation is an important factor in determining residence time of drug in the eye. From the literature, it is evident that the formulations in the solution form need to have viscosity ranging 5–1500 cps and gels with viscosity ranging about 50–50,000 cps. Viscosity of the solutions was measured at 25°C (room temperature) and of the gels (after addition of artificial tear fluid (ATF) at 37°C ± 0.5°C (body temperature) with the thermostatic water bath connected to the small sample adapter. The viscosity of gels was measured with 1:3 ratio of formulation and ATF, respectively.[22]

In-vitro drug release of nanoparticulate droppable gel

In vitro drug release studies were carried out in franz diffusion cell using dialysis membrane previously soaked overnight in the ATF-pH 7.4(M.wt cutoff-12,000–14,000 Dalton) respectively. NP-TDGL and ATF was placed (1:3) in the donor compartment, which was in contact with receptor medium. The receptor medium (ATF) was stirred continuously at 50 rpm to simulate blinking action of eyelids. The whole assembly was adjusted on magnetic stirrer and maintained at 37°C ± 0.5°C to mimic physiological condition at which gelation occurs. Aliquots of medium (1ml) were withdrawn at 1 hr time interval for extended period of 12 hr. Equal volumes of fresh media were added to replace the withdrawn samples. Finally, the withdrawn samples were diluted and estimated by UV spectrophotometer at 277 nm using the ATF as blank.[23]

Kinetic modeling of drug release mechanism

To investigate the mechanism of drug release, the in-vitro release data were analyzed mathematically according to the following models zero-order kinetics (cumulative % drug released vs. time), first-order kinetics (log % drug retained vs. time), Higuchi model (cumulative % drug released vs. square root of time) and Korsmeyer–Peppas equation (log amount of drug released vs. log time). The correlation coefficient (r) values were calculated for the linear curve obtained by regression of the above plots.[24]

Antibacterial Studies of NP-TDGL

The zone of inhibition studies were conducted by using “cup plate technique”. Sterile solutions of marketed norfloxacin eye drop (Norflox-0.3%-Control) and the optimized formulations (NE1) were diluted at different concentration (10 µg/ml) these solutions were poured in to cups bored into sterile nutrient agar previously seeded with test organisms (*Pseudomonas aeruginosa and Staphylococcus aureus*) after allowing diffusion of the

solutions for 2 hr, the agar plates were incubated at 37^{o}C for 24hr. The zone of inhibition (ZI) measured around each cup and was compared with that of control. The entire operation except the incubation was carried out in a laminar airflow unit.[25]

Sterility characteristics of NP-TDGL

The test of sterility was carried out as per the reported method in Indian pharmacopeia 1996 by the direct inoculation method. Measure two ml of the developed formulation was withdrawn with a sterile syringe thereafter, aseptically transferred to thioglycolate medium (FTM) and soyabean- casein digest medium (SCDM) separately. The inoculated media incubated 14 days at 30-35^{O}C in case of fluid thioglycolate medium and 20- 25^{O}C in case of soyabean casein-digest medium.[26]

Hen's egg test on chorioallantoic membrane (HET-CAM) for **NP-TDGL**

The ocular tolerability of the developed formulation was analyzed by using a modified HET-CAM test. The potential irritancy of compounds may be detected by observing adverse changes that occur in the chorio-allantoic membrane of the egg after exposure to test chemicals. Briefly, fertilized hen's eggs were obtained from a poultry farm. Eggs weighing between 50 and 60 g were selected and divided into three groups each containing three eggs. These eggs were incubated in a humidified incubator at a temperature of 37±0.5◦C for 3 days. The trays containing eggs were rotated manually in a gentle manner after every 12 hr. On the third day, 3 ml of egg albumin was removed by using sterile techniques from the pointed end of the egg. The hole was immediately sealed by 70% alcohol-sterilized Para film with the help of a heated spatula. The eggs were kept in the equatorial position for the development of chorioallantoic mem-brane (CAM) away from the shell. The eggs were candled on the fifth day of incubation, and every day thereafter nonviable embryos were removed. On the tenth day, a window (2 cm × 2 cm) was made on the equator of the eggs through which formulations (0.5 ml) were instilled directly onto the CAM surface and left in contact for5 min. The membrane was examined for vascular damage as the sign of haemorrhage, hyperemia and coagulation. Normal saline (0.9% NaCl solution) was used as a control as it is reported to be practically nonirritant. The scores were recorded according to the scoring as follows No visible haemorrhage-0 (Non irritant), Just visible membrane discoloration-1(Mild irritant), haemorrhage-2(Moderately irritant), Structure are covered totally due to membrane discoloration or haemorrhage-3(Severe irritant).[27]

Isotonicity study

Three different concentration of NaCl solution were prepared to obtain hypertonic (3% w/v), hypotonic (0.2% w/v) and isotonic (0.9% w/v) concentrations. Isotonicity has to be maintained to check tissue damage. Four clean slides were taken. They were labeled as hypertonic, hypotonic, isotonic and test. A small drop of blood was applied to the center of each slide along with a drop of heparin solution (1% w/v) to prevent coagulation of blood. A drop of each test solution was placed on the respective slides. A drop of optimized formulation was placed on the slide labeled as test (T). Using the edge of the cover slip, the contents were mixed and observed under microscope to observe the morphology of red blood corpuscles.[28]

In vivo eye irritation test (Modified driaze test)

The in vivo eye irritation test of the NFLX-NP-TDGL was conducted in five New Zealand White rabbits. All tests were carried out in the identical laboratory with constant artificial lighting. After 60 minutes of acclimatization in restrainer boxes, 50 µL of the NFLX-NP-TDGL formulation was instilled into the conjunctival sac of the rabbit's right eye, the left eye was kept without manipulation (control). The test eye was observed at 0, 0.08, 0.17, 0.5, 1, 6, 12, 24, 48 and 72 hr to compare changes in the cornea, iris, conjunctiva, secretion and chemosis with the control. Eye irritation levels were scored using the modified draize test.[29] The experimental protocol was approved by institutional ethical committee (IAEC) of QIS co llege of pharmacy, AP, India vide Reg.No:1921/PO/Re/S/16/CPCSEA.

RESULT AND DISCUSSION

Norfloxacin(NFLX) loaded eudragit-RLPO nanoparticle were prepared by nanoprecipitation technique. This comprised of water miscible organic phase and aqueous phase containing PVA as stabilizer. The formulation were evaluated for zeta potential, particle size, percentage entrapment efficiency to choose best formulation (D1) for preparation of thermo sensitive droppable gel by cold method. The prepared nanoparticle incorporated thermo sensitive droppable gel were designed and optimized by using design expert software 11.0 (Trialversion 30 days).

A 3^2 full factorial design formulation were characterized for pourability, appearance, gelation temperature at room temperature and physiological temperature, gelation time, bioadhesive force, viscosity measurement at physiological condition and in vitro diffusion study. In the present investigation, combination of Pluronic -F-127 (X_1) and HPMC-K100-M (X_2) were

studied using 3^2 full factorial design(FFD). The 3^2 is an effective approach for investigating variables at low level(-1), medium level (0), high level (+) with a total number of nine experimental runs are shown in table 3. Mathematical models developed for all the dependent variables such as gelation temperature (Y_1), bioadhesive force(Y_2), viscosity in physiological condition(Y_3) and cumulative % drug release (Y_4) shows linear and quadratic polynomial model equations. According to the software linear model fitted for response Y1, Y4 and quadratic model fitted for response Y2,Y3. The polynomial equation to investigate the Pluronic F-127 and HPMC K-100 -M on the gelation temperature as follows,

$Y_1 = + 33.49 + 0.0233\ X_1 + 1.90X_2 - 0.1675\ X_1X_2 - 3.11X_1^2 + 1.39X_2^2$

The positive sign of coefficient indicate a synergistic effect while negative term indicates an antagonistic effect or inverse effect up on the response. From the ANOVA results of response Y1 was found that this model was insignificant effect(Table 9) on gelation temperature. The observed experimental value of gelation temperature in the range of 28.4±0.78 to 35.18 ± 0.48. 3 D response surface curve (RSM) and 2 D contour plot for the response Y_1 are represented in figure 5a and figure 7a**.** The gelation temperature measured for all range of developed formulation was found to be in the concentration dependence higher is the concentration of Pluronic F-127 and HPMC K-100-M. The gelation mechanism of Pluronic F-127 aqueous solution may occur due to different ratio of PPO-PEO in Pluronic F-127 and ideal gelation temperature can be modulated by mixing specific amount of Pluronic F-127 and HPMC K-100-M in aqueous solution.[30] Poloxamers-407(Pluronic F-127) were previously proven to undergo thermal gelation or sol-gel transition at a temperature of about 25 to 35°C. Below the transition temperature, poloxamer solutions allow a comfortable and precise delivery by the patient to the cul-de-sac, where thermogelation occurs. If the gelation temperature of thermo sensitive formulation is lower than 25°C, a gel might be formed at room temperature and if the gelation temperature is higher than 34°C, a liquid dosage form still exists at corneal surface temperature, resulting in the drainage of the formula from the eyes.

Based on 3^2 factorial design, the factor combinations of Pluronic F-127 and HPMC K-100 -M resulted in difference response variable for bioadhesive force. The equation derived by best fit mathematical model(quadratic) to relate the response as follows,

$Y2 = + 130.80 + 34.12X_1 + 23.85\ X_2 - 5.00X_1X_2 - 22.79X_1^2 + 6.24X_2^2$

ANOVA result of the equation suggested the model p value indicating that the model is significant. 3D response plot and 2D contour plot of bioadhesive force is portrayed in figure 5b and figure 7b. Good bioadhesive properties of ocular formulations are important to improve their retention time in the eye. The results, listed in table 10**,** reveal that, as the amount of Pluronic F-127 and HPMC K-100-M increased, gel strength increased as well; this must be due to the additional effect of the concentration of both polymers. As HPMC K-100-M concentration increased, the bioadhesive force also increased. However, bioadhesive force was less affected by the increase of Pluronic-127 concentration than that of HPMC K-100-M. This may be due to the interaction of HPMC K-100-M with the mucin of the cul-de-sac. Increased viscosity due to formation of a denser polymeric network between the long p-polyoxypropylene- polyoxyethylene chains of pluronicF-127 with the HPMC-K-100 -M resulted in increased gel strength of the formulation.[31]

The transition of the formulation from solution to gel at 34^{o}C was also verified by viscosity measurements. For instance if viscosity is too high it will lead to difficult instillation if viscosities too low it will give rise to increase drainage. The independent variables such as combinations of Pluronic F-127 and HPMC K-100–M resulted in difference response variable for viscosity in physiological condition. The best fit mathematical quadratic model relate the response as follows,

$Y3=+\ 2143 +392.00X_1+436.17X_2-164.00X_1X_2-181.33X_1^2+187.17X_2^2$

ANOVA of the equation suggested the model p value indicating that the model (table 11) is significant. Three dimensional response plot and 2D contour plot of viscosity in physiological medium is portrayed in figure 6a and figure 8b. All the prepared formulations exhibited shear thinning flow behavior at 34± 0.1°C, at which condition the viscosity of the tested formulations decreased by increasing the shear rate. It was observed that decreasing the concentration of PF-127 from 21% in to 16% w/v is showed a decrease in viscosity, despite the presence of HPMC in the latter. Table 3shows indicates that the gel acquires greater entanglement and rigidity at higher PF-127 concentrations. El-Kamel stated that, when the polymer concentration increases, the number and size of micelles within the gel structure increase, the inter-micellar distance becomes shorter, which result in greater number of cross-links between neighboring micelles leading to higher viscosity. However, it is reported that in situ gels with high viscosities, may cause irritation to eye. Since the ocular shear rate is very high, ranging from 0.03S⁻1 during inter-blinking periods to 4500–28500S^{-1} during blinking, a

pseudo plastic fluid is optimal for ocular application as it will interfere as little as possible with the pseudo plastic character of the pre-corneal film.[32] Furthermore, the high viscosity of gel at low shear rate will aid in maintaining contact between the corneal surface and the delivery system, whereas the shear thinning nature of the gel in response to blinking will allow a good distribution of the formulation over the surface of the eye.[33] Hence the viscoelastic fluids having high viscosity under low shear rates and low viscosity under high shear rates called as pseudo plastic fluid are often preferred. The response of in vitro drug release obtained at various levels of three independent variables were subjected to multiple regression to give linear polynomial equation as follows,

$Y4= +17.13 -6.25X_1-6.49X_2$

The experimental value for in-vitro drug release study of NP-TDGL released drug varied from 57.67% to 85.61% at the end of 6 hr respectively (Figure 4). For the response-Y4, the interaction between factors X_1 and X_2 can be elucidated by using response surface plot, 2 D contour plot as illustrated in figure 6b and 8b. Moreover that incorporation of nanoparticle into thermo sensitive droppable gel resulted in a significant slower release of drug. This is probably attributed to the diffusion restriction of the free fraction of drug imposed by the polymeric network of the gel. Although, it was revealed that as the concentration of PF- 127 increases, the viscosity increases and the rate of drug release decreases. This result may be due to the presence of the HPMC K-100- M in the latter, which may have played a significant role in retarding the release of the drug, probably by slowing down polymer erosion.

To optimize all the responses(Y1-Y4) with different targets, a multi criteria decision approach a numerical optimization techniques by the desirability function and a graphical optimization technique by the overlay plot was used as shown in figure 9a. The optimized nanoparticle loaded thermo sensitive droppable gel formulation was obtained by applying constraints(table 2) on responses. These constraints are common for all the formulation was calculated by the DOE from above plots has highest desirability near to 1.0 as shown in figure 9 b. The comparison of predicted and experimental shows very close agreement, indicating the success of the design combined with desirability function for the evaluation and optimization of NP-TDGL formulation (Table 13).

All the prepared formulations (NF1-NF9) were homogenously dispersion of pourable liquids at room temperature and translucent characters owing to dispersion of polymeric nanoparticle. The pH of NP-TDGL formulation was found to be in appropriate range of 6.8–

7.4 which is similar to the lachrymal fluid pH as shown in table 6. The pH is within acceptable range and hence do not cause any irritation of the ocular tissue. Bacterial contamination of ophthalmic products must be avoided and of the different sterilization techniques available, autoclaving is the method of choice. Hence necessary to examine the effect of autoclaving on the mechanical properties of the polymer solution. The viscosity profiles of the NP-TDGL before and after autoclaving were studied and results showed that autoclaving did not affect the viscoelastic properties of the NP-TDGL and the pH remains unchanged (Table 7). Thus, to meet the criteria of sterility for the ocular formulation, an assembling procedure maintaining a strategic distance from amazingly high temperature, for example, utilizing aseptic system with sterilization by filtration at room temperature ought to be considered as a more proper strategy for fabricate of NP-TDGL than the procedure with terminal sterilization via autoclaving.

Table 5 shows comparison results of nanoparticle formulation(D1) and optimized NP-TDGL formulation(NF1). The average particle size range of optimized NP-TDGL is 185nm, poly dispersity index value 0.256 and high zeta potential around 29.3 mV, which clearly indicates all nanoparticle s were monodispersed without any aggregation. The zeta potential value showed high positive charge which cans electrostatic interaction with mucin a negative charged protein in the ocular tissue with tolerability in eye. TEM photograph (Figure 2a) of optimized formulation (NF1) revealed that dispersed nanoparticle possessed spherical shape with discrete droplets as observed from thermo sensitive droppable gel and SEM image (Figure 2b) are clearly exhibited that nanoparticle are retained their smooth spherical structure.

The gelling capacity grades and gelation time measured for all prepared formulation is presented in table 6. All the formulations showed instantaneous gelation when contacted with artificial tear fluid pH 7.4. Formulations NF7 showed immediate gelation in 212 sec and dissolved rapidly (+) whereas the formulations NF2 and NF8 revealed that immediate gelation occur 192, 164 sec withstand remained for few hours (++) and NF1, NF3, NF4, NF5, NF9 exhibited gelation immediate remains for extended period(+++)when maintained at the 37^{o}C. It is clear from the data shown that at constant pluronic F-127, the gelation time of different formulation decreased as the concentration of pluronic F-127 increased. All formulations were found to have drug content in the range of 95.33 ± 0.16 to 100.14 ± 0.25%, thus NF1 showing distribution of drug uniformly in the nanoparticle loaded thermo sensitive

droppable gel as reported in table 7. To investigate the drug release mechanism of drug from nanoparticle thermo sensitive droppable gel, the release data were fitted to the kinetics models such as zero order, Higuchi and korsmeyer peppas. Table 8 represents that the release mechanism fitted with zero order plots because the correlation coefficient (R^2) was ranged from 0.993 with n values were obtained in the range of 0.34, which indicate non-fickian diffusion. It has been shown that in the case of hydrophilic polymers, swelling and erosion of the polymer occur simultaneously.

The sterility test was performed on autoclaved packaging according to Indian pharmacopeia standards. Based on the study report (Figure 10) no microbial growth was observed in the medium when compared to control and it pass the sterility test (Table14). The optimized formulation (NF1) shows clear zone of inhibition (ZO-I) against *staphylococcus auerus* (21.32mm) compare to commercial formulation(19.56 mm). Results revealed that optimized nanoparticulate droppable gel having more antibacterial efficacy than commercial formulation(Norflox 0.3%w/v) were given in figure 11.

The eye irritancy test was carried out to identify the irritation potential of the optimized NF1 sodium chloride solution (Isotonic) was taken as a control as it is practically nonirritant. CAM of hen's egg is similar to the vascularized mucosal tissues of the human eye, which can provide information on the effects that may occur in the conjunctiva following exposure to a test substance. From the scores obtained in the HET-CAM test, it was observed that a mean score of 0 for saline solution and optimized formulation (NF1) throughout the duration of study were reported in table 15. Isotonic nature of the formulation is important to maintain the normal functioning of the cells or tissues at the site of instillation. Hypotonic and hypertonic preparations tend to irritate sensitive ocular tissue when instilled to eye. The degree of irritation is related to level of divergence from isotonicity. If a preparation is isotonic character, the tone of the cell will not be disturbed by either ingress of water from the instilled solution (hypotonic) or egress of water of the cell (hypertonic) The optimized formulation NF1 did not show swelling or shrinkage of blood cells are depicted in figure 13. Consequently optimized formulation (NF1) were tested for their eye irritation in albino rabbit and results showed that the formulation was practically non-irritating reported in table 16. Therefore, it concluded that developed formulation was considered safe and non-irritant for ocular tissues.

Table 1: Composition of norfloxacin loaded eudragit-RLPO nanoparticle formulation D1.

S.No	Formulation code	Norfloxacin : Eudragit -RLPO	Organic : Aqueous phase	PVA (%w/v)
1.	D1	1:4	1:2	0.8

Table 2: Variables and their levels for 3^2 full factorial design.

INDEPENDENT VARIABLES			DEPENDENT VARIABLES	
Coded level	Factors (Actual level)		Response	Criteria
	Pluronic-F127, X1 (% w/v)	HPMC -K-100-M X2(% w/v)	Gelation temperature in ATF (Y1)	28-35^oC
Low(-1)	16	0.1	Bioadhesive force (Y2)	Maximum
Medium(0)	18.5	0.3	Viscosity in physiological medium (Y3)	Maximum
High(+)	21	0.5	Cumulative % Drug release at 6 hr (Y4)	70%

Table 3: 3^2 Full Factorial experimental Design (FFD).

	Factor 1	Factor 2	Response 1	Response 2	Response 3	Response 4
Run	A:Pluronic-F-127 (%w/v)	B:HPMC-K-100-M (%w/v)	Gelation temperature in ATF(^oC)	Bioadhesive force (dynes/cm^2)	Viscosity in physiological medium (cps)	Cumulative % drug release
	Coded level	Coded Level				
1	+1	0	34.23±0.82	139.21	2589	67.33
2	-1	0	28.4±0.78	88.26	1467	82.31
3	+1	+1	33.64±0.54	164.32	2652	65.02
4	+1	-1	29.15±0.65	135.33	2197	69.06
5	0	0	34.64±0.92	119.34	2013	64.27
6	-1	+1	35.32±0.08	97.43	2365	70.99
7	-1	-1	30.16±0.93	48.43	1254	85.61
8	0	-1	33.45±0.33	110.21	1871	77.94
9	0	+1	35.18±0.48	175.32	2922	57.67

Table 4: Composition of different batches of norfloxacin nanoparticle loaded droppable gel.

Run	Norfloxacin nanoparticle equivalent to (%w/v)	Pluronic-F-127 (%w/v)	HPMC K-100 -M (%w/v)	Nacl (% w/v)	BAC (% w/v)	Deionized water (ml)
NF1	0.3	21	0.3	0.9	0.01	100
NF2	0.3	16	0.3	0.9	0.01	100
NF3	0.3	21	0.5	0.9	0.01	100
NF4	0.3	21	0.1	0.9	0.01	100
NF5	0.3	18.5	0.3	0.9	0.01	100
NF6	0.3	16	0.5	0.9	0.01	100
NF7	0.3	16	0.1	0.9	0.01	100
NF8	0.3	18.5	0.1	0.9	0.01	100
NF9	0.3	18.5	0.5	0.9	0.01	100

Table 5: Characterization of particle size and zeta potential of NP-TDGL.

Formulation	Average diameter ± SD* (nm)	Zeta potential ± SD*(mV)	Poly dispersity Index(PDI)	Drug content (%±SD)
NP-TDGL(NF1)	185.3 ±1.3	24.3 ± 1.5	0.256±0.58	100.14±0.25
NP(D1)	100.7±4.2	21.3±1.9	0.234± 0.22	94.46±0.12

*Mean ±SD, *n*=3.

Table 6: Evaluation test of nanoparticle loaded thermo sensitive droppable gel.

Batch code	Appearance	Pourability at room condition	Initial pH (Before autoclave)	Gelling capacity grades	Solution to gel transition Time (sec)
NF1	Translucent	Free flowing liquid	7.2	+++	41
NF2	Translucent	Free flowing liquid	6.8	++	198
NF3	Translucent	Free flowing liquid	7.4	+++	35
NF4	Translucent	Free flowing liquid	7.3	+++	45
NF5	Translucent	Free flowing liquid	7.2	+++	88
NF6	Translucent	Free flowing liquid	7.4	++	164
NF7	Translucent	Free flowing liquid	7.4	+	212
NF8	Translucent	Free flowing liquid	6.8	++	160
NF9	Translucent	Free flowing liquid	7.1	+++	74

+ Gelation after few minutes, dissolves rapidly, ++ gelation immediate, remains for few hours, +++ gelation immediate remains for extended period.

Table 7: Evaluation test of nanoparticle loaded thermo sensitive droppable gel.

Batch Code	pH (After autoclave)	Drug content (%)	Viscosity at non physiological condition
NF1	7.3	100.14±0.25	600
NF2	6.8	97.42±0.42	134
NF3	6.9	98.86±0.19	134
NF4	7.3	98.33±0.36	242
NF5	7.2	99.75±1.34	210
NF6	7.4	97.34±0.42	315
NF7	7.3	95.53±0.16	124
NF8	6.8	99.15±0.65	155
NF9	7.1	100.41±0.21	832

Table 8: In -vitro drug release kinetics data for optimized NP-TDGL.

Batch code	Zero order R^2	First order R^2	Higuchi's R^2	Korsemayer and poppas pattern		Drug release mechanism
				R^2	n	
NF1	0.9482	-0.9871	0.9908	0.9934	0.34	Non fickian diffusion

Table 9: ANOVA for Linear model gelation temperature in ATF (Response Y1).

Source	Sum of square	df	Mean square	F Value	P Value	Significance model
Model	21.59	2	10.79	1.84	0.2386	Non significant
A-Pluronic-F-127	0.0033	1	0.0033	0.0006	0.9820	
B-HPMC-K-100-M	21.58	1	21.58	3.67	0.1037	
Residual	35.25	6	5.88			
Cor Total	56.84	8				

F Value – Fischer's value, P value – Significance value.

Table 10: ANOVA for Quadratic model for bioadhesive force (Response Y2).

Source	Sum of square	df	Mean square	F Value	P Value	Significance model
Model	11616.31	5	2323.26	9.33	0.0477	Significant
A-Pluronic-F-127	6986.41	1	6986.41	28.05	0.0131	
B-HPMC-K-100-M	3412.93	1	3412.93	13.70	0.0342	
AB	100.10	1	100.10	0.4019	0.5711	
A^2	1039.07	1	1039.07	4.17	0.1337	
B^2	77.79	1	77.79	0.3124	0.6152	
Residual	747.15	3	249.05			
Cor Total	12363.46	8				

Table 11: Quadratic model for viscosity in physiological condition (Response Y3).

Source	Sum of square	df	Mean square	F Value	P Value	Significance model
Model	2.3070+06	5	4.6140+05	9.34	0.0476	Significant
A-Pluronic-F-127	9.2200+05	1	9.2200+05	18.67	0.0228	
B-HPMC-K-100-M	1.1410+06	1	1.1410+06	23.11	0.0171	
AB	1.0760+05	1	1.0760+05	2.18	0.2365	
A^2	65763.56	1	65763.56	1.33	0.3321	
B^2	70062.72	1	70062.72	1.42	0.3193	
Residual	1.4820+05	3	49390.37			
Cor Total	2.4550+06	8				

Table 12: ANOVA for linear model for cumulative % drug release (Response 4).

Source	Sum of square	df	Mean square	F Value	P Value	Significance model
Model	486.97	2	243.48	8.19	0.0193	Significant
A-Pluronic-F-127	234.37	1	234.37	7.88	0.0308	
B-HPMC-K-100-M	252.59	1	252.59	8.50	0.0268	
Residual	178.38	6	29.73			
Cor Total	665.35	8				

Table 13: Percentage prediction error or Bias.

Response	Predicted value	Experimental or observed value	Prediction error (%)	Optimized formulation
Gelation temperature (Y1)	35.31	31.23	-0.418	NF1
Bio-adhesion force (Y2)	143.50	139.21	-3.081	
Viscosity in physiological condition (Y3)	2466.48	2589	4.732	
Cumulative % drug release (Y4)	67.87	67.33	-0.80	

Table 14: Sterility test of nanoparticle loaded temperature sensitive droppable gel.

Batch		Day of sterility testing at 32°C ±2.5°C in FTM						Day of sterility testing at (22.5°C ±2.5°C in SCDM						
Control	Positive	+	+	+	+	+	+	+	+	+	+	+	+	+
	Negative	-	-	-	-	-	-	-	-	-	-	-	-	-
Sterilized & optimized formulation (NF1)		-	-	-	-	-	-	-	-	-	-	-	-	-

(-)Absence of microbial growth (Bacterial, fungal), (+)Presence of microbial growth (Bacterial, fungal).

Table 15: HET-CAM test of optimized formulation (NF1).

FORMULATION	SCORES IN HET-EGG						Cumulative score for 5 minutes A+ B+ C
	A-Haemorrhage		B-Hyperaemia		C-Coagulation		
	Time(min)		Time (min)		Time (min)		
	0.5	5	0.5	5	0.5	5	
Optimized formulation (NF1)	0	0	0	0	0	0	0
Normal saline	0	0	0	0	0	0	

Table 16: In vivo eye irritation study of nanoparticle loaded temperature sensitive droppable gel.

Parameters	Score for each lesion	Score obtained from assessment	Safety rating
Conjuctival edema (Chemosis)			Non irritant
No swelling	0	0	
Any swelling	1		
Prominent swelling along with partial lid eversion	2		
Swelling with half closed lids	3		
Swelling with totally closed lids	4		
Redness in conjunctiva			
Absent	0	0	Non irritant
Abnormal conjuctival injection	1		
More diffuse and deeper hyperemia	2		
Diffuse and dense hyperemia	3		
C-Secretion			
Absent	0	0	Non irritant
Any abnormal secretion	1		

Secretion leading to wet eye lashes closer to lids	2		
Secretion leading to wet lids and whole periorbital area	3		
D-Corneal capacity		0	Non irritant
Absent	0		
Scattered or diffused areas detail of the iris discernible	1		
Detail of the iris slightly darkened	2		
Opalescent areas, size of the pupil barely discernible	3		
E-Iris involvement			
Absent	0	0	Non irritant
Pronounced deep folds, congestion, deep swelling, circumcorneal injection	1		
No response, hemorrhage	2		
	Total score	0	

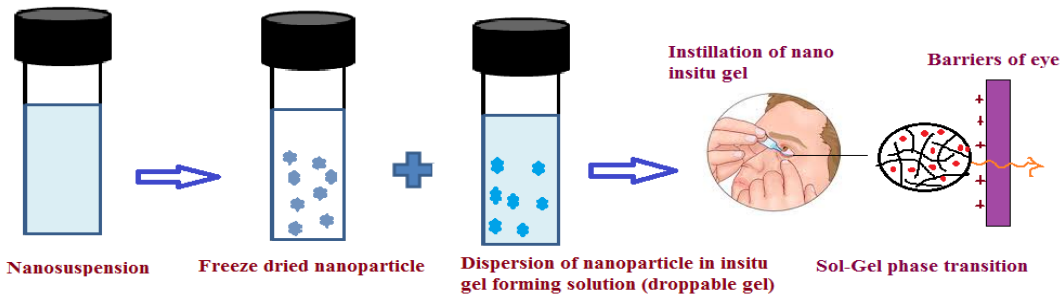

Figure 1: Diagrammatic representation of nanoparticle loaded thermo sensitive droppable gel formulation.

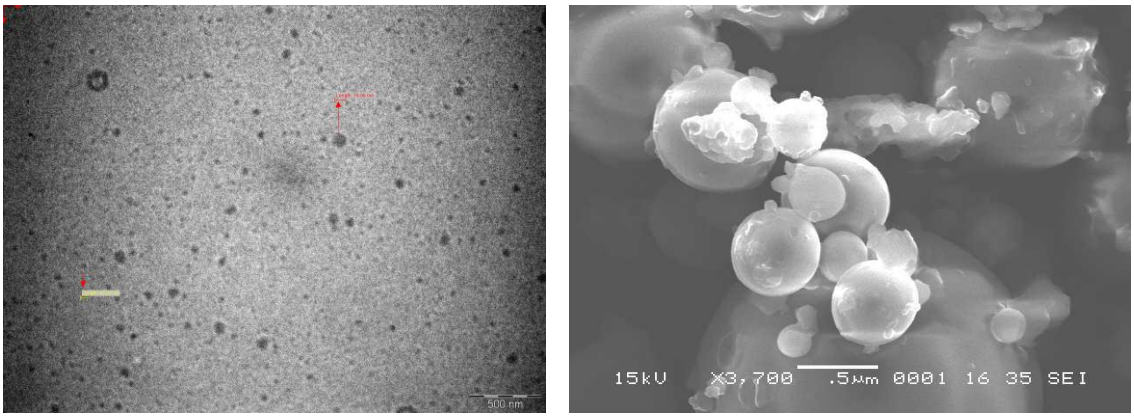

Figure 2: a) TEM and b) SEM image of optimized NP-TDGL formulation (NF1).

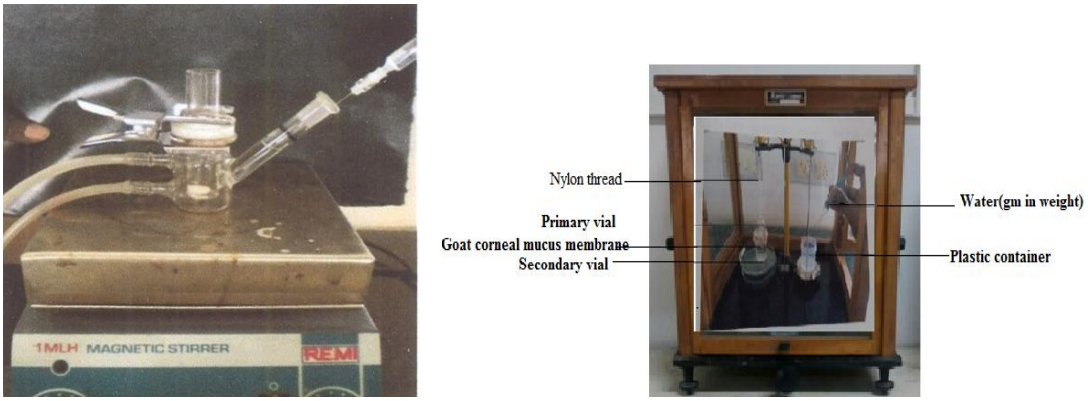

Figure 3: a) Franz diffusion cell apparatus for in-vitro drug release study b) Modified physical balance for bio-adhesive force measurement.

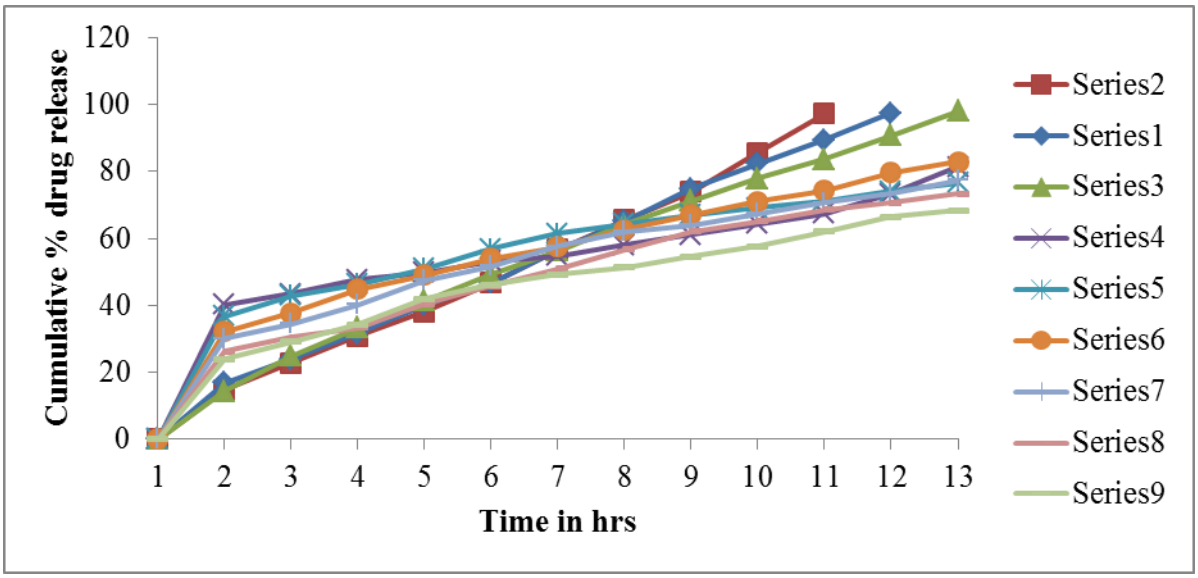

Figure 4: Drug release profile of nanoparticle based thermo sensitive droppable gel.

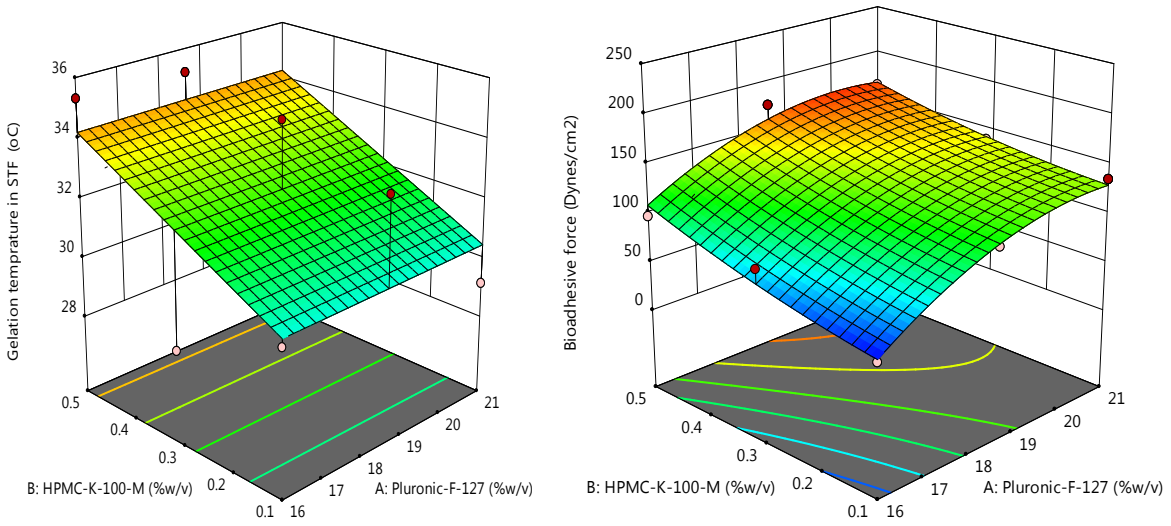

Figure 5: Response surface plot showing the effect Pluronic F-127 and HPMC K-100-M on a) Gelation temperature b) Bio-adhesive force.

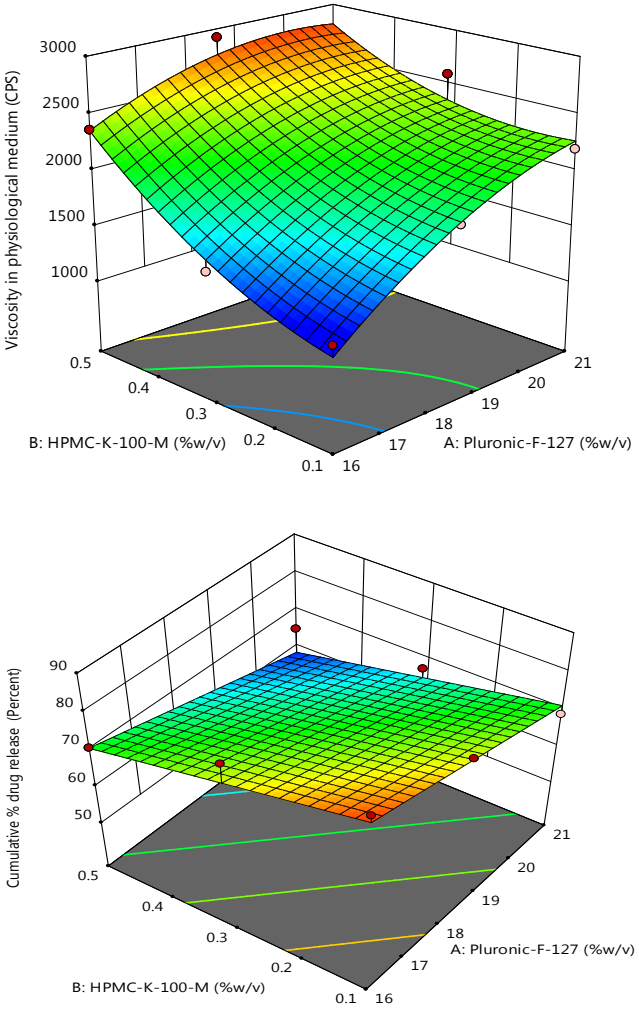

Figure 6: Response surface plot (3D) showing the effect Pluronic-127 and HPMC K-100 M on a) Viscosity in physiological medium b) Cumulative % drug release.

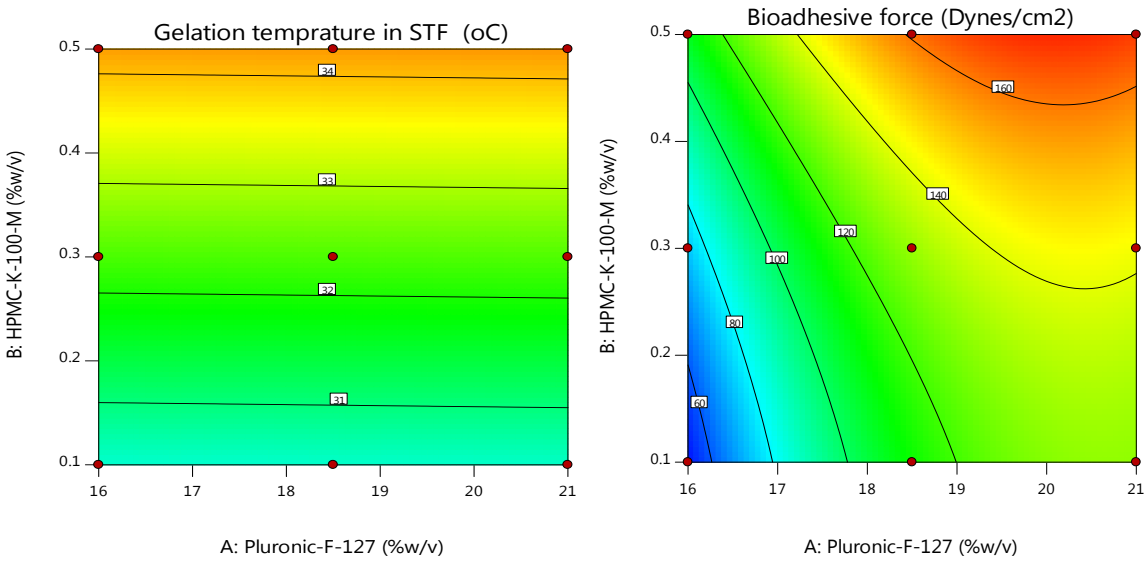

Figure 7: Contour plot (2D) showing the effect Pluronic-127 and HPMC K-100 M on a) Gelation temperature b) Bio-adhesive force.

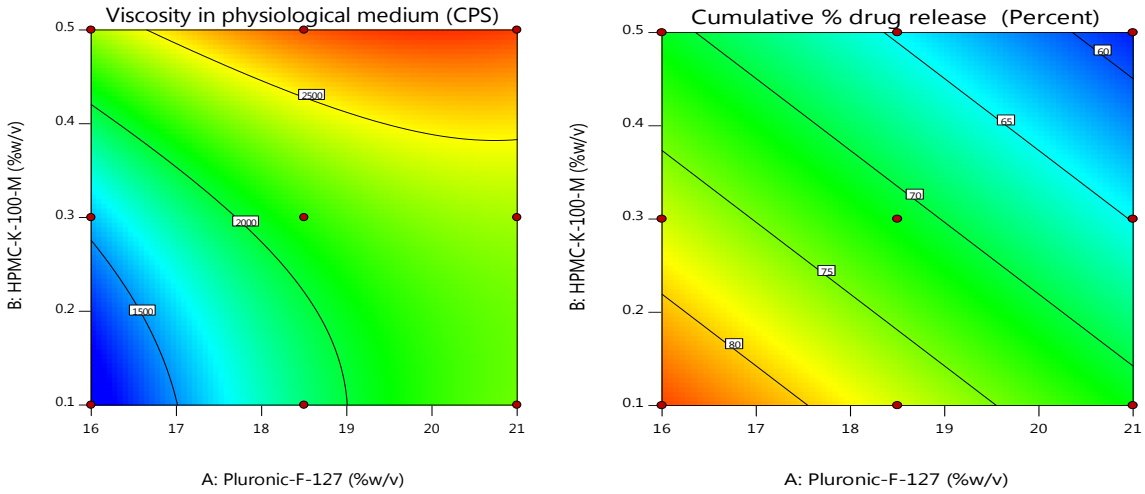

Figure 8: Contour plot (2D) showing the effect Pluronic-127 and HPMC K-100-M on a) Viscosity in physiological medium b) Cumulative % drug release.

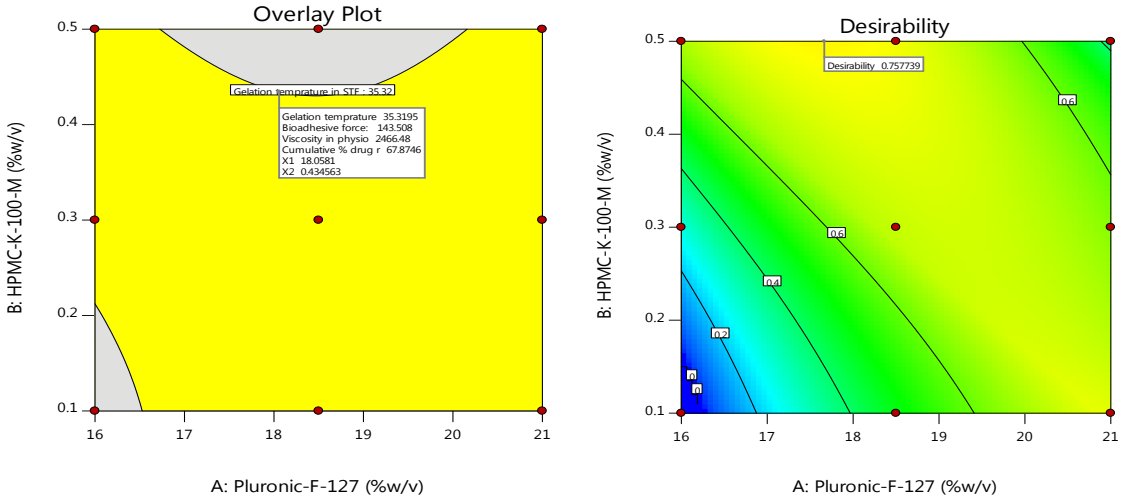

Figure 9: a) Overlay plot of optimized formulation b) Desirability value of all formulation.

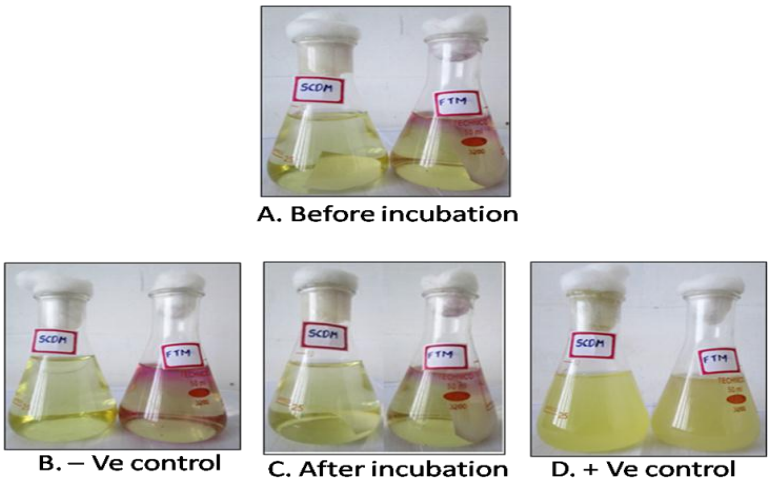

Figure 10: Sterility testing of optimized NP-TDGL formulation (NF1).

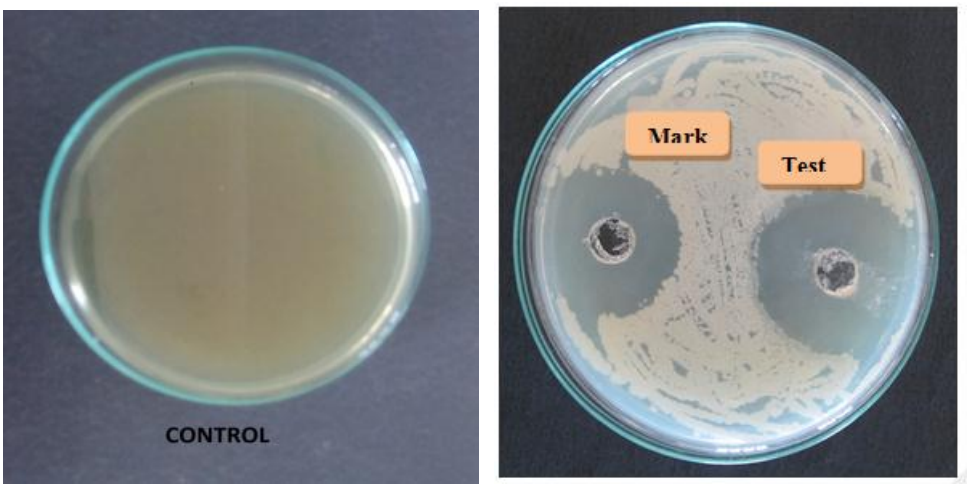

Figure 11: Antimicrobial testing of optimized NP-TDGL formulation against *staphylo coccus areus.*

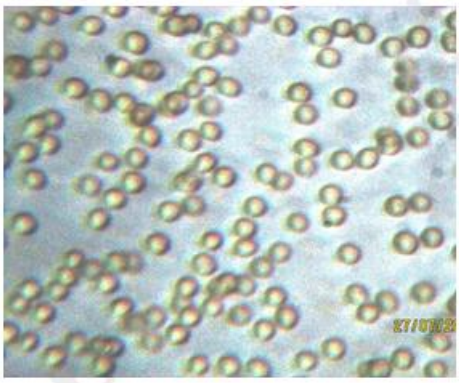

Figure 12: Isotonicity test of optimized NP-TDGL formulation (NF1) with RBC.

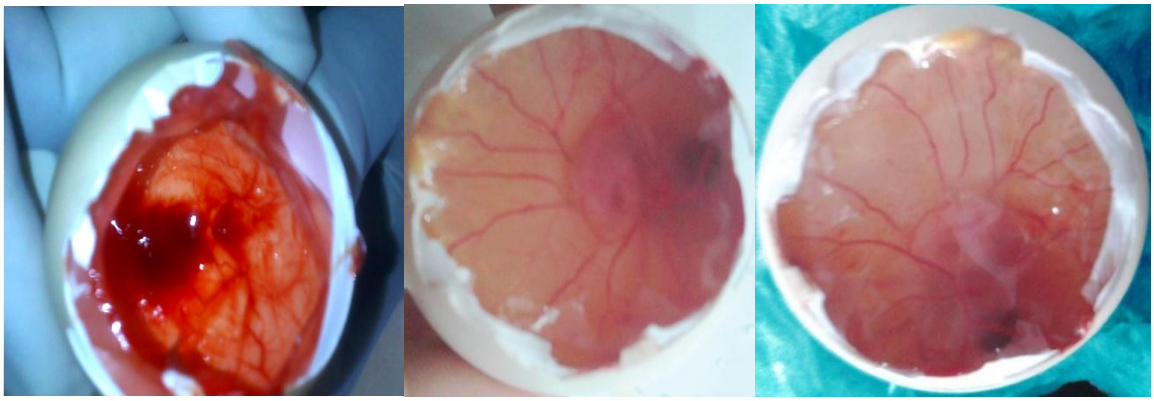

Figure 13: HET-CAM test for a) Positive Control b) Negative control) Optimized NP-TDGL (NF1).

CONCLUSION

In present study concludes that, nanoparticle based thermo sensitive droppable gel (NP-TDGL) was prepared and characterized for the treatment external bacterial infection in eye. A

3^2 full factorial design (FFD) employed for optimization of norfloxacin(NFLX) nanoparticle loaded droppable gel. It is free-flowing liquid at room temperature and could be converted into a semisolid gel after it was instilled into a pre corneal temperature in eye. The optimized NP-TGL formulation (NF1) showed prolonged drug release over a period of 12 hr. Ocular irritation tests and iso-tonicity study revealed that the developed formulation was non-irritant, having good ocular tolerance and significant antimicrobial study. The developed NP-TDGL formulation can be a viable alternative to conventional eye drops due to presence of nanoparticle ability may enhance bioavailability through its prolonged precorneal residence time. Hence, NP-TGL improves patient compliance due to decrease in frequency of administration with ease of administration.

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CONFLICT

There are no conflicts of interest.

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