Exploring the role of PPAR-gamma agonist in the management of major ocular complications as Diabetic Retinopathy and Dry Eye Disease: *in-vitro*, *ex-vivo* and *in-vivo* evidences.

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Abstract:

Diabetic retinopathy is one of the worst complications of diabetes and it is treated by invasive method. We prepared a surface modified poly (D, L-lactide-co-glycolide) i.e. PLGA nanoparticles for delivery of pioglitazone-a peroxisome proliferator-activated receptor-gamma agonist to posterior segment of the eye by topical administration. The present study investigated two grades of PLGA viz. 75:25 and 50:50. Surface modification was performed using polysorbate 80. Nanoparticles were prepared by single emulsion solvent evaporation method and optimized by using 3-factor 3-level Box-Behnken statistical design. Mean particle size, PDI and entrapment efficiency for optimized batch of PLGA 75:25 was found to be 163.23 nm, 0.286 and 91%, whereas; for PLGA 50:50 it was 171.7 nm, 0.280 and 93% respectively. DSC confirms the molecular dispersion of drug in polymer. In vitro release study showed biphasic drug release pattern with 58.48 ± 1.38% and 74.17 ± 1.38% cumulative drug release by PLGA 75:25 and 50:50 nanoparticles at the end of 10h. *In vivo* study on rat showed dose dependent reduction in vascular endothelial growth factor concentration in vitreous fluid. The study reveals significance of peroxisome proliferator-activated receptor-gamma in management of diabetic retinopathy.

In later stage optimized PLGA 50:50 NPs were then loaded in temperature sensitive *in situ* gel for treatment of dry eye disease. Effectiveness of optimized formulation in comparison to existing marketed formulation was estimated by Schirmer's test on mice. Prepared formulation showed induced tear production and further stabilized tear film for long period in comparison to marketed formulation.

1. Introduction:

Diabetes mellitus is recognized as a global epidemic which has already affected 382 millions of people [1]. Patients with diabetes are at high risk of development of the most devastating microvascular complication known as diabetic retinopathy (DR) [2, 3, 4]. Nearly all patients with type 1 diabetes and more than 60% patients with type 2 diabetes are at risk of development of diabetic retinopathy after 10 years of incidence of diabetes [5, 6]. Presently DR is treated by laser treatment, vitrectomy and intravetrous injections which is associated with pain and further fear of loss of vision [7-10]. Therefore; there is surge to develop alternative method for the treatment of diabetic retinopathy. Recently, several clinical studies proved the beneficial role of peroxisome proliferator-activated receptor-γ in management of diabetic retinopathy. Min K Song *et al.*(2012) had reported the presence of peroxisome proliferator-activated receptor-γ (PPAR-γ) in the mammalian eye [4]. Increased level of vascular endothelial growth factor (VEGF) can be acts as marker of progress of DR as reported by Saravia M. *et al.* [11]. By considering the involvement of PPAR-γ receptors in diabetic retinopathy, current research involves preparation of topical formulation of PPAR-γ agonist with aim to regulate VEGF. We have prepared the surface modified nano-particles of pioglitazone which is a PPAR-γ agonist by using two different grades of biodegradable polymer PLGA [poly (D,L-lactide-co- glycolide)] viz. PLGA 75:25 and PLGA 50:50 [12]. Further these nanoparticles were incorporated in temperature triggered *in situ* gel for treatment of dry eye disease (DED).

2.0 Materials and Methods:

2.1. *Materials*

Pioglitazone was obtained as a gift sample from Glenmark Pvt. Ltd. Nashik, India. PLGA 75:25 and 50:50 was gifted by Evonik Pvt. Ltd. Mumbai, India. All other solvents and materials used were of analytical grade.

2.2. Analytical method development

To quantitate the content of pioglitazone from samples, the UV-Visible Spectrophotometry **(**UV-Vis Spectrophotometer 1800 Shimadzu Co., Japan) method was used and validated as per ICH guidelines Q2(R1) in phosphate buffer pH 7.4. The absorbance was measured at 238 nm.

2.3 Fabrication of nanoparticles

PLGA NPs with grade 75:25 were prepared by using single emulsion solvent evaporation method. The method involves preparation of an organic and aqueous phase separately. Organic phase was consists of PLGA dissolved in dichloromethane (DCM) and Pioglitazone in methanol while aqueous phase containing PVA and Polysorbate 80 dissolved in water. The organic phase was added drop by drop into the aqueous phase during high speed homogenization (T25 IKA Digital Ultra Turrax) at 15000 rpm. It results in formation of o/w emulsion which further broken down in nano-droplets. Further to achieve smaller size prepared emulsion was subjected to High pressure homogenizer (NS 1001L Panda Gia Niro Soavi, Italy) at 600 bar pressure. Particle size and PDI of each batch was determined by using Zeta-sizer (ZS 90, Ver. 7.01 Malvern Instruments). All batches were subjected to solvent evaporation by heating the suspension on magnetic stirrer (1 MLH Remi equipments, India). This suspension was then passed through 0.45 um membrane filters (Millipore, Bedford, MA, USA). All filtrate were centrifuged at 15,000 rpm at 4°C for 20 min by using cooling centrifuge. The clear supernatant was collected and evaluated for entrapment efficiency [13]. One batch was also prepared by using same procedure but without addition of polysorbate 80 in order to check impact of surface modifier on nano formulation.

2.4 Formulation of PLGA nanoparticles by applying 3^3 Box-Behnken statistical Design (Experimental Design)

A 3-factor 3-level Box-Behnken statistical design was employed to investigate the variables and experimental trials performed at all 15 possible combinations. Polymer concentration, surface modifier concentration and number of cycles were selected as an independent variables while particle size, polydispersity index and entrapment efficiency were selected as dependent variables. The resultant data was fitted into Design Expert software 11.0.5.0. and analyzed statistically using analysis of variance (ANOVA). The statistical significance of the data was performed in terms of regression coefficients [14, 15].

Table 2 Translation of the coded levels in actual units

Independent variables	Coded levels		
	-1	**0**	**+1**
A= Polymer concentration (mg)	100	200	300
B= Surface modifier concentration(mg)	300	450	600
C= Number of cycles	6	7	8

The three-dimensional (3D) response surface plots were used to show graphically the relationship and interaction between the coded variables and the response. A 3^3 Box-Behnken statistical design layout is shown in Table 3

Optimized batch was subjected to spray drying (Labultima LU222, India) after 2-3 times washing with distilled water [16]. With same procedure one batch of PLGA 50:50 was also prepared at optimized condition of PLGA 75:25.

Table 3 3^3 Box-Behnken statistical design layout, experimental runs and their combinations

Runs	**Batch no.**	**Factor 1 A**	**Factor 2 B**	**Factor 3 C**
1	B_1	0	0	0
2	B_2	-1	0	+1
3	B_3	0	+1	+1
4	B_4	-1	0	-1
5	B_5	+1	-1	0
6	B_6	+1	+1	0
7	B_7	0	0	0
8	B_8	+1	0	-1
9	B_9	0	-1	-1
10	B_{10}	0	+1	-1
11	B_{11}	0	0	0
12	B_{12}	0	-1	+1
13	B_{13}	-1	+1	0
14	B_{14}	+1	0	+1
15	B_{15}	-1	-1	0

2.5 Characterization of nanoparticles:

2.5.1. Particle Size and Polydispersity Index (PDI)

Average particle size and size distribution are important parameters because they influence the physicochemical properties and fate of NPs after *in-vivo* administration. The particle size and polydispersity index of nanoparticles were measured using dynamic light scattering (Zetasizer Nano ZS 90, Malvern Ltd., UK) [15].

2.5.2. Entrapment Efficiency

The total percentage of drug entrapped (% EE) was determined by spectrophotometric analysis. Before spray drying, the nanoparticles suspension was centrifuged at 15,000 rpm at 4°C for 20 min. using cooling centrifuge. The clear supernatant was collected and the concentration of Pioglitazone in the supernatant was determined spectrophotometrically at 238 nm [17].

$$E.E.(\%) = \frac{Total\ amount\ of\ the\ drug - \ Amount\ of\ the\ free\ drug}{Total\ amount\ of\ the\ drug} \times 100$$

2.5.3. Zeta Potential measurement

The zeta potential of the optimized batch of PLGA 75:25 (with and without surface modification) and PLGA 50:50 was determined by dynamic light scattering [14, 15].

2.5.4. Drug Loading Efficiency

Drug loading efficiency was calculated by using following equation after weighing the spray dried nanoparticles (both grade).

$$DL\ (\%)\ = \frac{Total\ amount\ of\ the\ drug - \ Amount\ of\ the\ free\ drug}{\text{Weight of spray dried nanoparticles}} \times 100$$

2.5.5. Product Yield

Product yield were calculated as the weight of the final product after spray drying with respect to the initial total amount of the polymer and drug used for preparation [15].

$$\%\ Yield = \frac{Total\ weight\ of\ spary\ dried\ nanoparticles}{Drug\ +\ polymer\ weight +\ dispersing\ agent} \times 100$$

2.5.6. Total drug content

Spray dried PLGA NPs equivalent to 10 mg of pioglitazone were weighed accurately. It was dissolved in 100ml of phosphate buffer (pH 7.4) to give concentration of 100 ug/ml. From solution, 1ml solution was withdrawn and further serially diluted to make 10ug/ml. Absorbance was measured at 238nm by UV-visible spectrophotometer.

2.5.7. *In-vitro* drug release

The *in vitro* release study of surface modified PLGA nanoparticles of pioglitazone (PLGA 75:25 and 50:50) was performed in triplicate as per procedure given by R. Kesarla et al. (2016) and Salama, Mahmoud and Kamel (2016) [18, 19]. Phosphate buffer (pH 7.4) was used as dissolution medium. A dialysis membrane (mol. Weight cut off 14000, restricts diffusion of particles above 150 nm) previously soaked overnight in the diffusion medium was tied from one end and 1ml of formulation was accurately pipetted into dialysis sac and it's another end was closed tightly. The dialysis membrane sac was suspended in a beaker containing 100 ml of diffusion medium at 37± 0.5 °C. This assembly was kept on magnetic stirrer at 50 rpm. The 5ml of aliquots was withdrawn at specified time interval during 8h and analyzed by spectrophotometrically at 238 nm. Fresh medium was added to replenish withdrawn samples. Cumulative percentage drug released was calculated. Drug release data of optimized batch was subjected to describe drug release kinetics.

2.5.8. Shape and Surface Morphology

Morphological analysis of optimized batch of pioglitazone loaded surface modified PLGA nanoparticles (PLGA 75:25 and 50:50) were examined by scanning electron microscopy (JEOL model JSM-6390LV) [20].

2.5.9. Thermal analysis

Thermal behaviour of pioglitazone, polyemer and spray dried drug loaded nano particles (both grade) was conducted using differential scanning calorimeter (PerkinElmer 4000) at rate of heating 10^0C/min.

2.5.10. *In vivo* evaluation

In vivo evaluation of formulation was carried out on Wistar rats (200-250 gm). The animals were acclimatized and maintained on a normal food and water *ad libitum* for a week. The experimental protocol was approved by Animal Ethical Committee. Nanoparticles dispersion was placed in UV chamber with 100 μJ/cm^2 dose (1.5 h) for sterilization prior to instillation. After a week the rats were weighed and tail-snip baseline blood glucose was measured with biochemical method using ready mix kit (Erba diagnostic kits, India). The animals were divided in 6 groups (n=6). One group as control, second group as diabetes control, third group as with diabetes and treated with Pioglitazone PLGA 75:25 nano-suspension (4 mg/ml), fourth group as with diabetes and treated with Pioglitazone PLGA 50:50 nano-suspension (4 mg/ml), fifth group as with diabetes and treated with Pioglitazone PLGA 50:50 nano-suspension (2 mg/ml) and sixth group as with diabetes and treated with Pioglitazone PLGA 50:50 nano-suspension (6 mg/ml). Animals were treated with single intraperitoneal injection of 65 mg/kg streptozotocin (STZ) or control vehicle buffer pH 4.5. After 3 days tail snip blood glucose was again verified to ensure hyperglycaemia in STZ treated rats (glucose level more than 250 mg/dl). After a week of diabetes, treatment was initiated when STZ-treated diabetic rats demonstrate significantly elevated VEGF expression. The non-diabetic control rats treated with distilled water while STZ treated animals were treated with one drop of sterile nano formulation prepared in distilled water as mentioned above. After 4 weeks of continuous treatment, VEGF protein concentrations in the retina (vitreous fluid) from each group was determined [21, 22].

Analysis of VEGF level: Rats were sacrificed using CO_2 anaesthesia. The vitreous fluid from both eyes was rapidly isolated and kept in deep freezer. VEGF concentration in vitreous fluid was then determine by using Rat ELISA Kit (R&D Systems, Inc Minneapolis) and analysed on ELISA reader (PowerWave XS, Biotek, India).

2.5.11 Accelerated stability testing

PLGA NPs 50:50 were subjected for accelerated stability testing as per condition prescribed by ICH Q1A (R2). Product designed for storage in refrigerator need to be tested at 25^0C ± 2^0C /60% RH ± 5% RH for three months. NPs were filled into 3 glass vial (amber colour) and then sample was analysed for particle size at 1, 2 and 3 months.

2.6 Preparation of nanoparticles loaded in situ gel

Nano particle laden temperature triggered *in situ* gel was formulated by using Poloxamer 407 and HPMC K4M. Accurately weighed HPMC K4M was transfer into half of desired volume of distilled water and placed in refrigerator to obtain clear solution. Desired quantity of Poloxamer 407 was added in HPMC K4M solution with continuous agitation. Mixture was stored at 4ºC to obtain clear solution. Sodium chloride (tonicity modifier) and Polyquaternium-1 (preservative) were dissolved in small possible quantity of distilled water and transferred to the polymeric solution. Final volume was adjusted to 100 ml with purified water. At last prepared formulations were subjected for terminal sterilization by autoclaving at 121ºC, 15 p.s.i. for 20 minutes. Prepared freeze dried nanoparticles were placed in UV chamber for 1 hr. Nanoparticle (equivalent to 100 mg of drug) aseptically transferred into sterile *in situ gel* [23-25]. Composition of nanoparticle loaded in situ gel is shown in table 4.

Table 4 Composition of nanoparticle loaded in situ gel

Name of ingredients	Composition (%w/v)								
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
Pioglitazone (equivalent weight)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Poloxamer 407	14	14	14	16	16	16	18	18	18
HPMC K4M	0.5	0.75	1.0	0.5	0.75	1.0	0.5	0.75	1.0
Sodium chloride	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85
Polyquaternium-1*	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Water	100	100	100	100	100	100	100	100	100

*used in v/v

2.7 Experimental design for nanoparticle loaded in situ gel

For optimization of pioglitazone nanoparticle loaded *in situ* gel, 3^2 randomized full factorial design was selected. The amount (%) of temperature sensitive polymer, Poloxamer 407 (X1) and the amount (%) of viscosity modifier, HPMC K4M (X2) were selected as independent variables. These two factors were evaluated at 3 levels as higher, middle and lower levels. The

dependent or response variables included viscosity at 37ºC and 20rpm (Y1), cumulative % drug release at 10h (Y2) and gelation temperature (Y3).

2.8 Characterization of nanoparticle laden in situ gel:

2.8.1. Clarity and pH

Prepared formulations were evaluated for clarity and pH by visual inspection (against black and white background) and by using digital pH meter respectively [24].

2.8.2 Gelling capacity

Gelling capacity was determined by adding 100µl of sample in 2ml Simulated tear fluid (STF) present in a vial. Temperature was maintained at 37 ± 0.5ºC and gel formation assessment was done visually. Time required for sol-to-gel as well as for gel-to-sol transition was also noted [26].

2.8.3 Viscosity

Viscosity of formulation was measured by using Brookfield viscometer. Viscosity was measured at 20 rpm and two temperature viz. 25ºC (non-physiological) and 37ºC (physiological) [27].

2.8.4. *in-vitro* drug release

It was performed as per procedure discussed in 2.5.7. Drug release from optimized batch was subjected to describe drug release kinetics.

2.8.5. Gelation temperature

To determine gelation temperature, 2 ml prepared *in situ* formulation was transferred in test tube and immersed in water bath. The temperature of water bath was increased gradually to the temperature at which gel formed [24].

2.8.6 Sterility testing

Sterility testing of prepared formulation was carried out as per Indian Pharmacopoeia (2010) by direct inoculation method [28].

2.8.7. Preservative efficacy testing

Preservative efficacy study of optimized *in situ* gel was performed by challenging the formulation with *Pseudomonas aerugenosa and Staphylococcus aureus* as per Indian Pharmacopoeia (2010) [28].

2.8.9 *In vivo* study

Animal: Swiss albino mice weighing about 18-22 gm were used for this experiment. Mice were provided with standard feed and water. Experimental protocol was approved by CPCSEA, New Delhi (MET-IOP-IAEC/2019-20/07).

Experimental Procedure

Thirty albino mice were divided randomly into five groups each containing six as Group 1- Control, Group 2- Untreated, Group 3- Nanoparticles treated, Group 4- Formulation treated, Group 5- Standard formulation treated

Topical application of 0.2 mg / ml of benzalkonium chloride solution caused DED in all animals twice daily for seven days (except in control group). After 7 days, Schirmer Test tested mice for DED. Therapeutic and normal group mice were treated by drop of formulation (1 mg/ml) and marketed formulation (5 mg / ml) twice daily at a time interval of 3 h. The development of the tear was measured in all groups by the Schirmer test. A Whatman filter paper sheet of 0.5 x 3.0 mm was positioned under the lower lid within each eye close to the medial canthus and, after 2 minutes, the length of the wetting strip was quantified at 10x magnification by a micrometre on the dissecting microscope. During Schirmer test, the animal was slightly sedated with pentobarbitone sodium (45mg/kg) [29, 30, 31].

3.0 Results and Discussion:

3.1 Experimental design

From the results of preliminary batches, 3^3 Box-Behnken Statistical Design was selected for optimization of surface modified PLGA nanoparticles. The amount of drug in the formulation was fixed to 50 mg and speed and duration of high speed homogenizer was fixed to 15000 rpm for 35 minutes. Prepared formulation was then immediately passed through the high pressure homogenizer at 600 bar pressure with different number of cycles as per optimization design. Total 15 runs with the three center points were prepared and shown in table 5.

Table 5 Experimental runs

Runs	Batch No.	Factor 1 A: PLGA (mg)	Factor 2 B: P80 (mg)	Factor 3 C: cycles (numbers)	Response1 Particle size(nm)	Response 2 PDI	Response 3 E.E. (%)
1	B_1	200	450	7	182	0.323	85
2	B_2	100	450	8	163	0.286	91
3	B_3	200	600	8	179	0.359	86
4	B_4	100	450	6	172	0.310	82
5	B_5	300	300	7	189	0.388	91
6	B_6	300	600	7	186	0.364	94
7	B_7	200	450	7	182	0.323	85
8	B_8	300	450	6	192	0.387	90
9	B_9	200	300	6	185	0.289	87
10	B_{10}	200	600	6	186	0.321	88
11	B_{11}	200	450	7	185	0.368	86
12	B_{12}	200	300	8	173	0.283	88
13	B_{13}	100	600	7	169	0.267	82
14	B_{14}	300	450	8	186	0.354	93
15	B_{15}	100	300	7	166	0.288	81

The regression analysis for responses R1, R2, R3 and ANOVA of each model has shown in table 6 and 7 respectively.

Table 6 Summary of results of regression analysis for responses

Response	Model	Sequential p-value	Adjusted R^2	Predicted R^2
Particle size (R1)	Quadratic	0.0001	0.8880	0.8468
Polydispersity index (R2)	Linear	0.0053	0.8807	0.8685
Entrapment efficiency (R3)	Linear	0.0001	0.8819	0.8305

Table 7 Analysis of variance of calculated model for responses

Result of ANOVA	Particle size (nm)	Polydispersity index (PDI)	Entrapment efficiency (%)
Sum of squares	1094.08	0.0152	202.25
Degree of freedom (df)	9	3	3
Mean squares	121.56	0.0051	67.42
F value	39.86	7.46	35.85
P value	0.0004	0.0053	0.0001

3.2.1 Particle Size:

Observed particle size for prepared batches is shown in table 5. From results of the optimization studies it was observed that, as concentration of polymer and surface modifier increases, the particle size also increases. On the other hand as number of cycles of HPH increases there is decrease in particle size [15, 32]. The quadratic polynomial equation generated for particle size is given as follows:

$$R_1=183+10.37*A+0.8750*B-4.25*C-1.50*AB+0.7500*AC+1.25*BC-4*A^2-1.50*B^2-0.7500*C^2 \quad (1)$$

The model F value of 39.86 implies the model is significant and values of probability less than 0.05 indicates that model terms are significant.

3.2.2 Polydispersity index:

Polydispersity Index (PDI) is used to describe the degree of non-uniformity of a size distribution of particles. Values of 0.3 and below are most commonly deemed acceptable in practice for polymer-based nanoparticle materials. PDI for prepared batches is shown in table 5.

The linear polynomial equation generated for Polydispersity index is given as follows:

$$R_2= 0.327 + 0.0427*A + 0.0079*B - 0.0031*C \quad (2)$$

Above equation (2) indicates positive impact of A and B on PDI. While; the impact of C was found to be negative on PDI. However; impact of A slightly more significant than B [14]. The model F value of 7.46 implies the model is significant and values of probability less than 0.05 indicates that model terms are significant.

3.2.3. Entrapment Efficiency:

Results of entrapment efficiency for prepared batch is shown in table 5. From results of the optimization studies it was observed that, as concentration of polymer increases there is increase in EE; as concentration of surface modifier increases there is increase in EE and as number of cycles of HPH increases there is increase in EE [23]. The linear polynomial equation generated for entrapment efficiency is given as follows:

$$R_3 = 86.73 + 5*A + 0.3750*B + 0.3750*C \quad (3)$$

The model F value of 35.85 implies the model is significant and values of probability less than 0.05 indicates that model terms are significant. In this case, A, B, C are significant model terms. **Development of the optimized batch**: Based on the statistical evaluations the software gave further 31 possible solutions for the optimization of the batches. Formulation B2 was selected based on the criteria. A new optimized formulation was prepared according to the predicted model and evaluated for the responses as shown in table 8.

Table 8 Results of predicted batch and experimental batch with residual error

Response	Predicted value	Experimental value	Residual error (%)
Particle size (nm)	162	163.23	0.759
PDI	0.284	0.286	0.70
% Entrapment Efficiency	90	91	1.11

3.3. Characterization of Optimized Batch

3.3.1. Particle size and PDI:

The average particle size and polydispersity index of nano particles were determined and results are summarized in table 9.

Table 9 Particle size and PDI of nano-particles

Batch	Particle Size (nm)	PDI
Surface modified nano-particles of PLGA 75:25	163.23	0.286
Surface unmodified nano-particles of PLGA 75:25	174.36	0.287
Surface modified nano-particles of PLGA 50:50	171.7	0.280

3.3.2. Zeta potential measurement

Zeta potential of unmodified PLGA 75:25 nano particles was found to be -6.22 mV because of the terminal carboxyl acid end groups of PLGA molecules located on the surface of nanoparticles. The zeta potential of the surface modified PLGA 75:25 NPs was found to be -10.8 mV. This change in zeta potential confirms the surface modification of nano particle by Polysorbate 80. Zeta potential of surface modified PLGA 50:50 nano-particles was found to be -7.49 mV [33].

3.3.3. Entrapment Efficiency, Drug Loading Efficiency, Product yield and Total drug content

NPs were evaluated for various parameters and results are shown table 10.

Table 10 NPs evaluation

Nano Particles	Entrapment efficiency (%)	Drug loading efficiency (%)	Product Yield	Total drug content
Surface modified PLGA 75:25	91	7.89	67%	93%
Surface modified PLGA 50:50	93	8.12	66%	90%

3.3.4. *In-vitro* drug release

Comparative *in vitro* drug release study between surface modified PLGA 75:25 and PLGA 50:50 nanoparticles was carried out for 10 h. Surface modified PLGA 75:25 nanoparticles showed 58.481 ± 1.383 % drug release at the end of 10 h. On the other hand surface modified PLGA 50:50 polymer showed 74.178 ± 1.384% drug release at the end of 10 h (Figure 1). In both case drug release pattern was found to be biphasic which might be due to initial burst release [34,35].

Data obtained from *in vitro* drug release study was further subjected to mathematical treatment to determine drug release kinetic profile. The drug release from both grades of PLGA was best explained by Higuchi kinetic with highest R^2 value and by Quasi fickian mechanism (table 11 and 12).

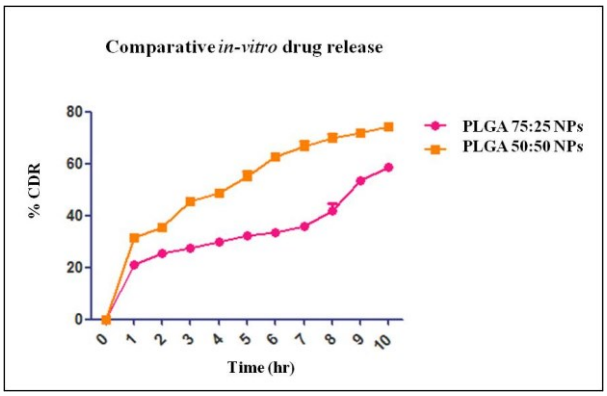

Fig. 1 Percentage drug release from PLGA 75:25 and PLGA 50:50 NPs (n= 3, mean ± SD).

Table 11 Model fitting for release profile

Nano particles	Coefficient of determination (R^2)				Best fit model
	First order	Zero order	Higuchi	Hixon-Crowel cube root	
Surface modified PLGA 75:25	0.0275	0.9369	0.9654	0.9583	Higuchi
Surface modified PLGA 50:50	0.9578	0.9262	0.9979	0.9762	Higuchi

Table 12 Korsmeyer-Peppas drug release kinetics

Nano particles	R^2	N value	Mechanism
Surface modified PLGA 75:25	0.9895	0.5150	Quasi fickian
Surface modified PLGA 50:50	0.9895	0.5103	Quasi fickian

3.3.5. Shape and Surface Morphology

The morphology of the surface modified PLGA 75:25 and PLGA 50:50 nano particles was studied by scanning electron microscopy. Results for both grades of PLGA are shown in figure 2. SEM analysis of particles reveals that all particles were spherical and possessed smooth surface without any fracture.

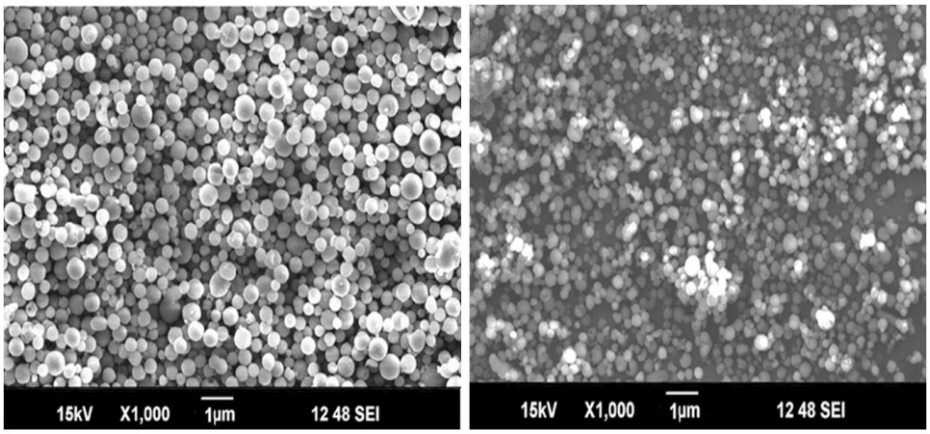

Fig. 2 SEM image of surface modified Pioglitazone loaded nano particles of a) PLGA 75:25 and b) PLGA 50:50

3.3.6 Thermal analysis:

The DSC thermogram of Pioglitazone showed sharp characteristic endothermic peak at 198.20°C which is also reported by Faruksha & Vetrichelvan, 2013 [36]. Characteristic peak of drug was disappeared in thermogram of surface modified PLGA 75:25 as well as PLGA 50:50 nano particles given in Figure 3

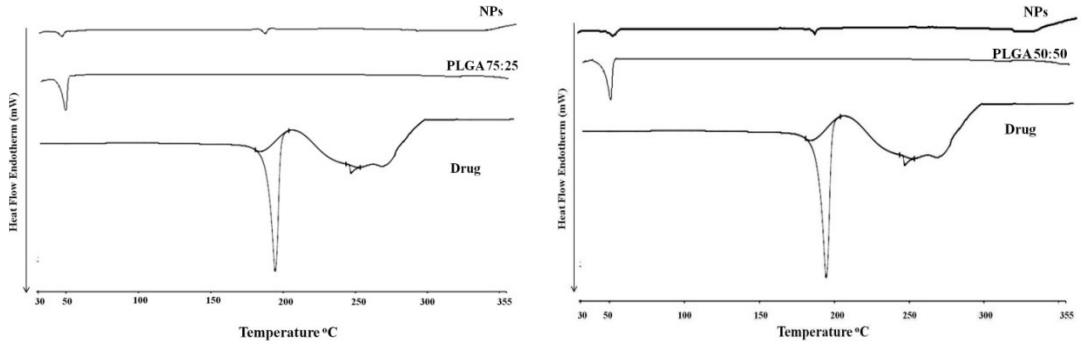

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Fig. 3 I. Comparative DSC thermogram

3.3.7 *In vivo* evaluation:

The effect of prolong treatment of surface modified Pioglitazone nano particles of PLGA 75:25 and 50:50 on VEGF protein in vitreous fluid of STZ-induced diabetic rats was determined using ELISA. Animals were grouped as non-diabetic, diabetic without treatment, diabetic with administration PLGA 75:25 Nano suspension (4 mg/ml), three groups as diabetic with treatment

of PLGA 50:50 nanosuspension with different concentration viz. 2 mg/ml, 4 mg/ml and 6 mg/ml. After 4 weeks of study the VEGF level in vitreous was found to be less in entire treatment rats as compared to untreated rat. Moreover; the VEGF level was significantly reduced in PLGA 50:50 nano suspension treated animal than in PLGA 75:25 nano suspensions (Figure 4).

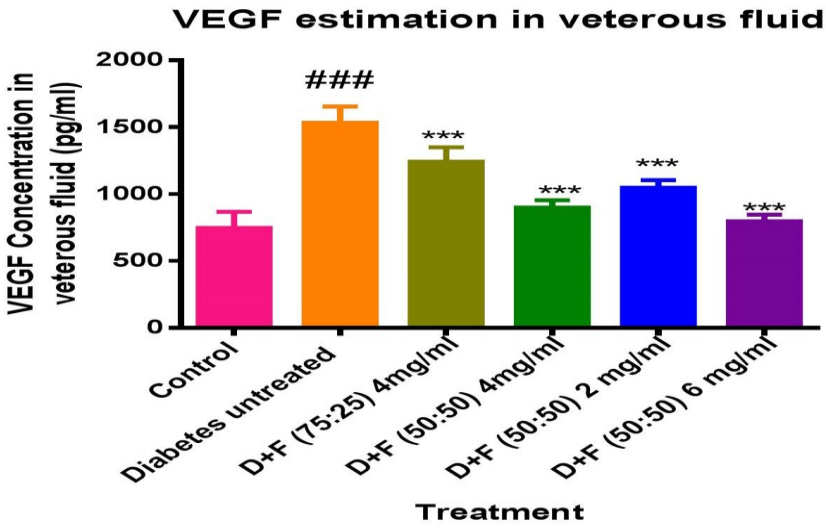

Fig. 4 Effect of formulation on VEGF in rats

3.3.8 Accelerated stability study

Fabricated PLGA NPs were subjected to accelerated stability testing of three months and results are shown in table 13. Therefore; prepared formulation was found to be stable.

Table 13 Accelerated stability study

Batch	One month		Two month		Three month	
	Particle Size (nm)	PDI	Particle Size (nm)	PDI	Particle size (nm)	PDI
Surface modified nano-particles of PLGA 50:50	174.30	0.287	182.63	0.323	185 nm	0.289

3.4 Preparation of nanoparticle loaded in-situ gel

Nanoparticles loaded temperature sensitive in situ gel was prepared by cold method. Lyophilised nanoparticles were sterilised under UV radiation and aseptically transferred in autoclaved polymeric system.

3.5 Data analysis, statistical optimization and validation

Two independent variables as concentration of Poloxamer 407 (X1) and HPMC K4M (X2) with their lower, middle and upper design points in their actual and coded form is shown in table 14.

Table 14 Design parameters, formulation composition, experimental conditions and characterization of nanoparticles.

Run	FC	Coded levels of variables		Viscosity 20 rpm and 37°C	%CDR at the end of 10 h	Gelation temperature	Gelling capacity
		% Concentration of Poloxamer 407	% Concentration of HPMC K4M				
1	F1	14	0.5	7220 ± 137	58.37 ± 0.25	38.43 ± 0.20	++
2	F2	14	0.75	8189± 168	54.89 ± 0.38	37.50 ± 0.10	++
3	F3	14	1.0	8927± 150	50.21 ± 0.16	36.43 ± 0.32	+++
4	F4	16	0.5	11221 ± 154	49.97 ± 0.41	35.10 ± 0.26	+++
5	F5	16	0.75	12132 ± 122	46.37 ± 0.31	33.70 ± 0.40	+++
6	F6	16	1.0	13029 ± 117	42.31 ± 0.39	31.35 ± 0.70	+++
7	F7	18	0.5	14141± 104	40.71 ± 0.24	27.36 ± 0.85	+++
8	F8	18	0.75	14689± 168	37.77 ± 0.32	26.76 ± 0.75	+++
9	F9	18	1.0	15441 ± 121	35.07 ± 0.21	25.10 ± 0.10	+++

Responses Viscosity (Y1), Percent cumulative drug release (CDR) (Y2), Gelation temperature (Y3) were found to be in the range of 7220 ± 137 to 15441 ± 121, 35.07 ± 0.21 to 58.37 ± 0.25, and 25.10 ± 0.10 to 38.43 ± 0.20 respectively. Detail results are depicted in table 5. Software suggested best fitted model as Quadratic for Y1, 2FI for Y2 and linear for Y3. As value of probability is less than 0.05 in each model, results of ANOVA and regression analysis confirmed that suggested models were significant for operational parameters (table 15).

Table 15 Summary of results of regression analysis and ANOVA for measured responses.

Responses	Model	R^2	Adjusted R^2	Predicted R^2	SS	DF	MS	F value	P value	Model Significance
Y1	Quadratic	0.9993	0.9983	0.9920	9.601	2	4.801	30.86	< 0.005	Significant
Y2	2FI	0.9990	0.9984	0.9954	1.59	1	1.59	16.48	0.0097	Significant
Y3	Liner	0.9726	0.9635	0.9416	193.74	2	96.87	106.51	<0.0001	Significant

Y1: Viscosity; Y2: % Cumulative drug release; Y3: Gelation temperature; SS: Sum of Square; DF: Degree of freedom; MS: mean square.

Software suggested following polynomial equations for dependent variable;

$$Y1 = +12131.89 + 3322.50\ X1 + 802.50\ X2 - 101.75\ X1\ X2 - 692.83\ (X1)^2 - 6.83\ (X2)^2 \quad (4)$$

$$Y2 = +46.19 - 8.32\ X1 - 3.58\ X2 + 0.6300\ X1\ X2 \quad (5)$$

$$Y3 = +32.41 - 5.32\ X1 - 1.33\ X2 \quad (6)$$

Equation 4 indicates that concentration of both polymers had positive impact on viscosity which means that as the concentration of Poloxamer (X1) and HPMC (X2) increases the viscosity increases. However; effect of X1 was more intense than X2.

X1 and X2 showed negative impact of %CDR (equation 5). Therefore; as concentration of polymer increases, drug release decreases. It also proved sustained release property of polymeric system. X1 as well as X2 showed negative impact on gelation temperature (equation 6).

Process was optimized for responses Y1, Y2 and Y3. Optimized nanoparticle loaded in-situ gel was selected by desirability search approach. Formulation having gelation at physiological temperature, maximum drug release and optimum viscosity at 37ºC were the selection parameters. Additionally; gelling capacity was also taken into consideration while selecting optimized batch. Batch F3 prepared by using 14% w/v Poloxamer 407 and 0.75% w/v HPMC

K4M with optimum viscosity (8927± 150), physiological gelation temperature (36.43 ± 0.32) with 50.21± 0.16 % cumulative drug release and good gelation capacity (+++) was selected as optimum batch.

3.6 Characterization of nanoparticles loaded in situ gel:

3.6.1. Clarity and pH

All prepared formulation showed satisfactory clarity. The pH of all formulation was in the range of 6.4 to 6.9 [24].

3.6.2. Gelling capacity

Gelling capacity of all prepared batches was determined in simulated tear fluid at 37ºC results are shown in table 14.

3.6.3. Viscosity

Viscosity of all prepared batches was found in range of 7220 ± 137 to 15441 ± 121 (cP) when measured at 37ºC and 20 rpm.

3.6.4. Drug release

Drug release study of all prepared batches was carried by using dialysis sac. The percent cumulative drug release for prepared batches at the end of 10h was found to be in the range of 35.07 ± 0.21% to 58.37 ± 0.25%. Drug release of optimised batch was further compared with simple nanoparticles. Optimized nanoparticle loaded in situ gels showed 50.21 ± 0.16% drug release in comparison to simple nanoparticles which showed 74.17±1.38%. This is due entrapment of nanoparticles into gel matrix formed by Poloxamer and HPMC at physiological temperature [37]. Comparative drug release profile is shown in figure 5.

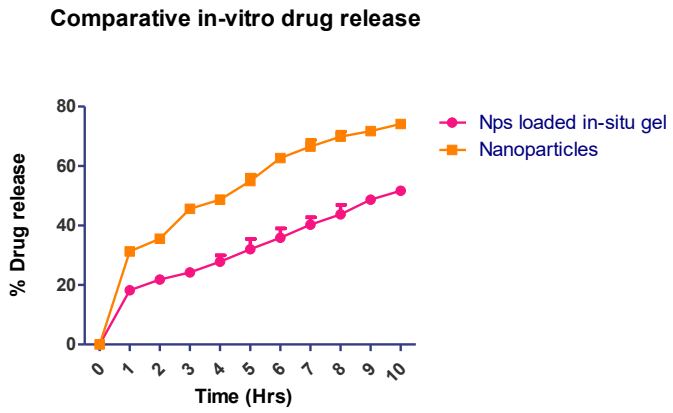

Fig 5. Percentage drug release from nanoparticle loaded *in situ* gel and simple nanoparticles (n= 3, mean ± SD).

Data obtained from *in vitro* drug release study was further subjected to mathematical treatment to determine drug release kinetic profile. The drug release from NPs loaded in-situ gel was best explained by Higuchi kinetic with highest R^2 value and by Fickian mechanism table 16 and 17).

Table 16 Model fitting for release profile

Formulation	Coefficient of determination (R^2)				Best fit model
	First order	Zero order	Higuchi	Hixon-Crowel cube root	
NPs loaded in-situ gel	0.9578	0.9155	0.9879	0.9374	Higuchi

Table 17 Korsmeyer-Peppas drug release kinetics

Formulation	R^2	N value	Mechanism
NPs loaded in-situ gel	0.9895	0.455	Fickian

3.6.5. Gelation temperature

Prepared all batches showed gelation at temperature in the range 25.10 ± 0.10 °C to 38.43 ± 0.20°C. Batch F3 was selected as optimized batch which showed gelation at temperature 36.43 ± 0.32 °C.

3.6.6. Sterility testing

Optimized formulation was subjected for 14 days sterility testing as per Indian Pharmacopoeia. No turbidity was observed after incubation for 14 days at specified condition in comparison to positive control. Thus selected method of sterilization was found to be effective.

3.6.7. Preservative efficacy study

Effectiveness of added preservative was evaluated by 'Preservative Efficacy Testing' as per specified by Indian Pharmacopoeia. No microbial growth was observed at the end of 28 days of incubation which confirmed the added concentration of Polyquaternium-1 was significant.

3.7 *In vivo* evaluation

Healthy mice were randomly defined in 5 groups as controlled, untreated, treated nanoparticles, treated in situ formulation and treated as standard. Dry eye was induced by systemic application of benzalkonium chloride (BAC) for 7 days throughout all the groups except the control group. After seven days, dry eye illness was verified by quantification of the value of tear fluid with the assistance of a filter strip of paper that was positioned under the lower eyelid for 2 minutes (Schiemers test). Control group showed 2.6 ± 0.5 mm wetting of filter paper. While the animals of other groups showed less waiting compared to the control group (Table 18) which stated the DED in BAC-treated mice. Mice from the nanoparticle group, in situ formulation and standard treatment received 1 drop of formulation. Upon 15 minutes, the animals have been tested again by Schirmer. Treatment of mice with Nanoparticle showed less increase in the secretion of tears. Whereas; in situ gel-loaded nanoparticles showed significant increase in the secretion of tears. On the other hand, the standard treated mice showed significantly increase in tear secretion compared to an in situ formulation of the treated groups. Schirmer 's test was then performed 3 hours before the formulation was administered and 15 minutes after the formulation was administered. Results are shown in Table 18 below. Mice received nanoparticles loaded with in situ gel retained tear secretion near the control group prior to the second dose. The study shows the efficacy of the prepared formulation.

Table 18 Tear secretion measurement by Schirmer's test

Group	Filter paper wetting (mm) at the 7th day of BAC treatment	After 15 minutes of formulation administration	Before second dose administration	After 15 minutes of dose administration
1	2.6 ± 0.5	2.7 ± 0.3	2.6 ± 0.4	2.6 ± 0.5
2	1.9 ± 0.3	1.8 ± 0.4	1.9 ± 0.5	2.0 ± 0.6
3	2.1 ± 0.2	2.4 ± 0.5	2.3 ± 0.3	2.5 ± 0.4
4	1.9 ± 0.2	2.6 ± 0.2	2.6 ± 0.6	2.7 ± 0.4
5	2.2 ± 0.5	2.4 ± 0.4	2.2 ± 0.2	2.5 ± 0.3

(n= 6, mean ± SD).

4.0 Conclusion:

Present research showed the involvement of PPAR-□ receptors in management of these two major ocular complications i.e. Diabetic retinopathy and Dry eye disease.

Developed nanoparticles showed remarkable reduction in vitreous VEGF levels of rats. Moreover; we investigated that the VEGF level was significantly less in PLGA 50:50 nano suspension than PLGA 75:25 nano suspension which might be due to rapid biodegradation of PLGA 50:50. Furthermore; we found dose dependent reduction in VEGF. This down regulation of VEGF in comparison to untreated group proved the involvement of PPAR-gamma in diabetic retinopathy management as well as movement of nanoparticles towards the posterior segment.

Further, proposed study also showed the increased tear fluid secretion and tear film stabilization in mice when treated with PLGA 50:50 nanoparticles loaded in situ gel prepared by Poloxamer 407 and HPMC K4M. Moreover; drug loaded nanoparticle also showed promising results which supports the involvement of PPAR-□ in dry eye disease. Therefore; based on our evidence of preclinical studies developed formulation can be consider as notable substitute for present treatment of dry eye disease.

5.0 References:

[1] Das A, Stroud S, Mehta A, Rangasamy S, New treatments for diabetic retinopathy, Diabetes, Obesity and Metabolism 17 (2015) 219-230.

[2] Gologorsky D, Thanos A, Vavvas D, Therapeutic Interventions against Inflammatory and Angiogenic Mediators in Proliferative Diabetic Retinopathy, Mediators of Inflammation (2012) 1-10.

[3] Kowluru RA, Zhong Q, Santos JM, Thanddampallayam M, Putt D, Gierhart DL, Beneficial effects of the nutritional supplements on the development of diabetic retinopathy, Nutrition and Metabolism 11 (2014) 1-10.

[4] Song MK, Roufogalis BD, Huang THW, Modulation of diabetic retinopathy pathophysiology by natural medicines through PPAR-gamma-related pharmacology, British Journal of Pharmacology 165 (2012) 4-19.

[5] Abdulrahman A, Alghadyan MD, Diabetic Retinopathy: An update, Saudi Journal of Ophthalmology 25 (2011) 99-111.

[6] Tarr JM, Kaul K, Chopra M, Kohner EM, Chibber R, Pathophysiology of diabetic retinopathy, ISRN Ophthalmology (2013) 1-13.

[7] Ciudin A, Hernandez C, Simo R, Molecular Implication of the PPARs in Diabetic Eye, PPAR Research (2013) 1-11.

[8] Boyd K. Retinopathy Treatment. Published by American Academy of ophthalmology; 2019. https://www.aao.org/eye-health/diseases/diabetic-retinopathy-treatment (accessed on 03 Jan 2020)

[9] The electronic Medicines Compendium (eMC): https://www.medicines.org.uk/emc/product/307/smpc (accessed on 15 April 2019).

[10] European Medicines Agency: https://www.ema.europa.eu/en/medicines/human/EPAR/eylea (accessed on 15 April 2019).

[11] Saravia M, Zeman L, Ingolotti M, Schlaen A, The VEGF paradox: Dose diabetic retinopathy protect from age related macular degeneration?, Medical Hypotheses 109 (2017) 156-161.

[12] Tahara K, Karasawa K, Onodera R, Takeuchi H, Feasibility of drug delivery to the eye's posterior segment by topical instillation of PLGA nanoparticles, Asian Journal of Pharmaceutical Science 12 (2017) 394-399.

[13] Rafati H, Mirzajani F, Atyabi F, Fabrication of biodegradable Poly (d,llactide- co-glycolide) nanoparticles containing tamoxifen citrate, Iran Polym J 19 (2010) 437-446.

[14] Pandit J, Sultana Y, Aqil M, Chitosan coated PLGA nanoparticles of bevacizumab as novel drug delivery to target retina: optimization, characterization and in-vitro toxicity evaluation, Artificial cells, Nanomedicine and Biotechnology (2016) 1-11.

[15] Wagh V, Apar D, Cyclosporine a loaded PLGA nanoparticles for dry eye disease: In vitro characterization studies, Journal of Nanotechnology (2014) 1-10.

[16] Bhambere D, Shirivastava B, Sharma P, Gide P, Effect of polymer and formulation variables on properties of self-assembled polymeric micellar nanoparticles. Journal of Nanomedicine and Biotherapeutic Discovery 4 (2014) 1-11.
[17] Sah AK, Suresh PK, Verma VK, PLGA nanoparticles for ocular delivery of loteprednol etabonate: a corneal penetration study, Artificial cells, nanomedicine, and biotechnology (2016) 1-9.
[18] Salama A, Mahmoud A, Kamel R, A novel method for preparing surface- modified Fluocinolone Acetonide loaded PLGA nanoparticles for ocular use: In vitro and In vivo evaluations, AAPS PharmSciTech 17 (2015) 1159-1172.
[19] Kesarla R, Tank T, Vora PA, Shah T, Parmar S, Omri A, Preparation and evaluation of nanoparticles loaded ophthalmic in situ gel, Drug Delivery 23 (2016) 2363–2370.
[20] Lapez GP, Iglesias I, Beneda J, Lozano R, Teijan JM, Paclitaxel-loaded polyester nanoparticles prepared by spray-drying technology: in vitro bioactivity evaluation, J Microencapsul 28 (2011) 417-429.
[21] Kusari J, Sheila X, Edwin P, Kenneth G. Clarke, Daniel WG, Inhibition of Vitreoretinal VEGF Elevation and Blood–Retinal Barrier Breakdown in Streptozotocin-Induced Diabetic Rats by Brimonidine, Retina IOVS 51 (2010) 1044-1051.
[22] Angela KWL, Amy CYL, Animal Models of Diabetic Retinopathy: Summary and Comparison, Journal of Diabetes Research (2013) 1-29.
[23] Upadhay Shivam U, Chavan Siddhi K, Gajjar Devarshi U, Upadhya Umeshkumar M, Patel Jayvadan K, Nanoparticles laden in situ gel for sustained drug release after topical ocular administration, Journal of Drug Delivery Science and Technology, (2020).
[24] Barse R, Kokare C, Tagalpallewar A, Influence of hydroxypropylmethylcellulose and poloxamer composite on developed ophthalmic in situ gel: Ex vivo and in vivo characterization, Journal of Drug Delivery Science and Technology, (2016), 66-74.
[25] Tapia-Guerrero Y S, Del Prado-Audelo M L, Borbolla-Jiménez F V, Giraldo Gomez D M, García-Aguirre I, Colín-Castro CA, Morales-González JA, Leyva-Gómez G, Magaña J J, Effect of UV and Gamma Irradiation Sterilization Processes in the Properties of Different Polymeric Nanoparticles for Biomedical Applications, Materials, 13 (2020), 1-19.
[26] Bhandwalkar M, Avachat A, Thermoreversible nasal in situ gel of venlafaxine hydrochloride: formulation, characterization and pharmacodynamic evaluation, AAPS PhaciTech, 14 (2013) 101-110.
[27] Balasubramanium J, Kant S, Pandit J, In vitro and in vivo evaluation of the Gelrite_ gellan gum-based ocular delivery system for indomethacin, Acta Pharmaceutica, 53 (2003), 251–61.
[28] Indian Pharmacopoeia, Indian Pharmacopoeia Commission, Ghaziabad, (2010), PP. 27-28, 56-63,2224.
[29] Jain G K, Pathan S A, Akhter S, Microscopic and spectroscopic evaluation of novel PLGA-chitosan nanoplexes as an ocular delivery systems, Colloids and Surface B, 82 (2011) 397-403.

[30] Zhirong Lin, Xiaochen Liu, Tong Zhou, Yihui Wang, Li Bai, Hui He, Zuguo Liu, A mouse dry eye model induced by topical administration of benzalkonium chloride, Molecular Vision, 17 (2011), 257-264.

[31] Dilek D, Min W, Dagoberto M, De-Quan Li, Balakrishna L, Michael E S, Stephen C P, A Mouse Model of Keratoconjunctivitis Sicca, Investigative Ophthalmology and Visual Science, 43 (2002), 632-638.

[32] Jose S, Juna B, Cinu T, Jyoti H, Aleykutty N, Carboplatin loaded Surface modified PLGA nanoparticles: optimization, characterization and in vivo brain targeting studies, Colloids and Surfaces B: Biointerfaces 142 (2016) 307-314.

[33] Tahara K, Yamamoto H, Kawashima Y, Cellular uptake mechanisms and intracellular distributions of polysorbate 80-modified poly (D,L-Lactide-Co-Glycolide) nanospheres for gene delivery, Eur J Pharm Biopharm. 75 (2010) 218–224.

[34] Zhang D, Tan T, Gao L, Zhao W, Wang P, Preparation of azithromycin nanosuspensions by high pressure homogenization and its physicochemical characteristics studies, Drug Development and Industrial Pharmacy (2007) 569– 575.

[35] Kondo M, Niwa T, Okamoto H, Danjo K, Particle characterization of poorly water-soluble drugs using a spray freeze drying technique, Chemical and Pharmaceutical Bulletin 57 (2009) 657–662.

[36] Faruksha AU, Vectrichelvan, T, Formulation, characterization and optimization of pioglitazone hydrochloride nanoparticles by solvent displacement method using 3^2 factorial design, International Journal of PharmTech Research 5 (2013) 754-766.

[37] Nabil K A, Ameeduzzafar Z, Syed S I, Khalid S A, Nasser H A, Sultan A, Nabil A Al, Abdulaziz I A, Muhammad A. Stimulus Responsive Ocular Gentamycin-Ferrying Chitosan Nanoparticles Hydrogel: Formulation Optimization, Ocular Safety and Antibacterial Assessment, International Journal of Nanomedicine, 15 (2020) 4717–4737.

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