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Research paper

Formulation of nanoparticles loaded in situ gel for treatment of dry eye disease: In vitro, ex vivo and in vivo evidences

Umesh D. Laddha *, Sanjay J. Kshirsagar

MET's Institute of Pharmacy, Bhujbal Knowledge City, Affiliated to Savitribai Phule Pune University, Nashik, 422003, MS, India

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ABSTRACT

Dry eye disorder is the most common ophthalmic complication which has already affected millions of people worldwide. Due to protective response of eye, conventional drops get rapidly eliminated from eye site. Moreover; current treatment of dry eye syndrome demands frequent administration of formulation. Henceforth; there is need to develop novel ocular drug delivery system to meet the need. By considering the involvement of Peroxisome proliferator-activated receptor-γ *in dry eye, we prepared Pioglitazone loaded* Poly (D, L-lactide-co-glycolide) nanoparticles which were then suspended in temperature sensitive *in situ* gel prepared by combination of Poloxamer 407 and HPMC K4M those also contribute in treatment as lubricant. Nanoparticle system and *in situ* formulation was optimised by 3^2 factorial design. Optimized nanoparticle system was evaluated for particle size, PDI, % entrapment efficiency, XRD, DSC and then suspended in polymeric *in situ* gel. *In situ* gel was characterized by viscosity, %drug release, gelation temperature, gelling strength, sterility test, preservative efficacy test. Ocular irritation potential was confirmed by histology study on goat eye cornea. Formulation retained the normal structure of cornea and found non-irritant. Effectiveness of optimised formulation in comparison to existing marketed formulation was estimated by Schirmer's test on mice. Prepared formulation showed induced tear production and further stabilised tear film for long period in comparison to marketed formulation. It can be concluded that prepared formulation can be consider as alternative for existing treatment.

1. Introduction

Eye is convoluted organ amongst different part of body which is safeguarded by several mechanisms. It may suffer from several conditions like dry eye syndrome, glaucoma, allergic conjunctivitis, uveitis, cataract, age-related macular degeneration (AMD), diabetic retinopathy macular edema (DME), proliferative vitreoretinopathy and many more [1,2]. At present more than 258 million peoples are visually impaired and 39 million out of them are completely blind. There is an increased need to explore the research in contest with ocular site for improved therapeutic benefits [3].

Dry eye disorder (DED) is the most common ophthalmic manifestation which has already affected millions of people worldwide [4]. It is the chronic condition caused by lack of production of adequate tears [5]. Dry eye disease is a multifactorial issue of the ocular surface and more related to tear film stability [6]. It is alternately known as xerophthalmia, keratoconjunctivitis sicca (KCS), sicca syndrome and keratitis sicca [7]. Sjogrens syndrome, diseases or disorder of lachrymal glands, long term use of contact lenses are the major contributors of development of DED [8]. Insufficient tear production significantly damages the ocular surface and leads to symptoms those causes discomfort and visual disturbance [7]. Prevalence of DED increases with age. Peoples with age 50 and above are more prone for DED [9]. However; recent study showed that due to modern life style, middle age people are also at high risk of dry eyes [10]. Moreover; studies also showed that 54% of diabetic patients are more sensitive to DED [11].

Current treatment of DED involves topical administration of artificial tears, autologous serum eye drops, NSAIDs, corticosteroids, cyclosporine. However; each approach is associated with numerous limitations. Eye drops get rapidly eliminated from eye site due to defence mechanism of eye and leads to significant loss of drug. Although 'artificial tears' is the most common treatment of DED, it needs to be instilled frequently and sometimes even after every 30–50 min [8]. Moreover; drugs like corticosteroids reported adverse effects like ocular infection, glaucoma and cataracts on long term use. Cyclosporine is reported safe for long term use but may leads to some systemic complications [7]. Major problem of current treatment is high frequency of administration which leads to decrease in patient compliance. Therefore; there is need

* Corresponding author.
E-mail address: umeshladdha698@gmail.com (U.D. Laddha).

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to develop some novel treatment for DED which will reduce dosing frequency.

Recent study showed the involvement of Peroxisome proliferator-activated receptor γ in vision threatening eye diseases. It has been found effective in management of major blinding diseases [12]. Moreover; Chen et al. [13] showed the benefits of PPARγ activation in DED by increase in the stability of tear film and tear fluid secretion as well as it helps to clear the injury on the ocular surface. Additionally; study also revealed the inhibition of NO secretion by PPARγ activation, which otherwise affects lacrimal organ normal functioning. There is need to explore more the utilization of PPAR γ in management of DED.

By considering the connection of PPAR γ in DED, current research involves the preparation of topical formulation of PPAR γ agonist- Pioglitazone. Molecular formula and melting point of pioglitazone is $C_{19}H_{20}N_2O_3S$ and 183–184 °C respectively. Pioglitazone selectively stimulates the nuclear receptor (Peroxisome Proliferator Activated Receptor-γ and to lesser extent to PPAR-α). Drug has not reported any toxicity. Since; drug has poor solubility, we have prepared surface modified nano-particles (NPs) by using PLGA 50:50 [poly (D,L-lactide-*co*-glycolide)]. Furthermore; to enhance ocular retention time of prepared nano-particles, concept of 'nano-particle laden in situ gel' was used. Temperature triggered in-situ gel was prepared by using Poloxamer 407 and HPMC K4M. In prepared formulation Poloxamer as well as HPMC also acts as lubricants which additionally help to improve patient comfort. Brown Larry (1997) has already proved the role of Poloxamer in dry eye treatment [8]. Additionally; formulation contains Polyquaternium-1 which is reported as safest preservative for dry eye disorder.

The dug loaded PLGA NPs were prepared by single emulsion solvent evaporation method. Initially; nanoparticles were characterized by particle size analysis, zeta potential, encapsulation efficiency, SEM, DSC, XRD, in-vitro drug release study, accelerated stability study. In later stage nanoparticles laden in-situ gel was characterized for viscosity, drug release, gelation temperature, gelling strength, pH, clarity, drug release kinetics. Effectiveness of formulation in treatment of dry eyes was evaluated by Schirmer test on mice.

2. Material and method

2.1. Materials

Pioglitazone was obtained as a gift sample from Glenmark Pvt. Ltd. Nashik, India. PLGA 50:50 was gifted by Evonik Pvt. Ltd. Mumbai, India. Poloxamer 407 and HPMC K4M was purchased from BASF Ltd., Mumbai, India and S.D. Fine Chemicals, Mumbai, India respectively. Dichloromethane, Tween 80, Potassium dihydrogen orthophosphate and Dipotassium hydrogen phosphate was obtained from SDFCL, Mumbai, India. Polyvinyl alcohol and Mannitol was procured from Thomas baker, Mumbai, India. Polyquaternium-1 was obtained from Pharmonix Biologicals Private Limited, Thane, India. All solvents and materials used were of analytical grade.

2.2. Analytical method development

UV–Visible Spectrophotometry (UV–Vis Spectrophotometer 1800 Shimadzu Co., Japan) method was developed and validated as per ICH guidelines Q2 (R1) to enumerate the content of Pioglitazone in samples. Stock solution of concentration 100 μg/ml was prepared by dissolving drug in 1 ml methanol and volume was adjusted by phosphate buffer pH 7.4. The absorbance was recorded at 238 nm.

2.3. Fabrication of PLGA nanoparticles

PLGA NPs were formulated by using single emulsion solvent evaporation method. The method involves mixing of an organic and aqueous phase together. PLGA dissolved in dichloromethane (DCM) and Pioglitazone (50 mg) in methanol acts as organic phase. The 1% Polyvinyl alcohol and 0.45% Polysorbate 80 in water acts as aqueous phase. The organic phase was added drop by drop into the aqueous phase during high speed homogenization (T25 IKA Digital Ultra Turrax) at 15,000 rpm. It leads to form o/w emulsion which further broken down in nano-droplets. Prepared emulsion was further subjected to High pressure homogenizer (NS 1001 L Panda Gia Niro Soavi, Italy) at 650 bar pressure for 15 min. Particle size and PDI of each batch was determined by using Zeta-sizer ZS 90, Ver. 7.01 Malvern Instruments. The organic solvent was evaporated at room temperature under magnetic stirrer. Then this nanosuspension was centrifuged by using cooling centrifuge for 20 min at 4 °C and 15,000 rpm. The supernatant was collected and evaluated for entrapment efficiency [14].

2.4. Experimental design

PLGA loaded surface modified nanoparticles were optimised by using 3^2 factorial design. This design was applied to quantify the influence independent variables on dependent variables. Concentration of PLGA (X1) and number of HPH cycles were selected as independent variable. These two factors were evaluated at 3 levels as higher, middle and lower levels with coding +1, 0 and −1 respectively. Levels of X1 were selected as 100, 200 and 300 mg and for X2 as 6, 7 and 8 cycles. The dependent or response variables included particle size (Y1), PDI (Y2), % entrapment efficiency (Y3) and zeta potential (Y4). The resulting data were fitted into Design Expert software v12 and analysed statistically using analysis of variance (ANOVA). The statistical significance of the data was performed in terms of regression coefficients [15,16].

Based on optimization, a batch which was found to be optimised, the sediment of same batch was washed with distilled water and subjected for lyophilisation. Initially; mannitol was added into the nanosuspension as cryoprotectant and freeze-dried using freeze dryer. This nanosuspension was lyophilized at the condition shows in Table 1. Lyophilized nanoparticles were further evaluated for various parameters.

2.5. Characterization of nanoparticles

2.5.1. Particle size and polydispersity index (PDI), zeta potential

The particle size, polydispersity index and zeta potential of nanoparticles were measured by using zetasizer (Zetasizer Nano ZS 90, Malvern Ltd., UK). Particle size, PDI and magnitude of zeta potential can be correlated with stability of colloidal dispersion. Particle size of optimised batch was again measured by re-dispersion of freeze dried product [15–17].

2.5.2. Entrapment efficiency

For determination of entrapment efficiency, nanoparticles were separated from aqueous part containing free drug by cold centrifugation at 4 °C, 15000 rpm for 20 min. The clear supernatant was collected and the concentration of Pioglitazone in the supernatant was determined by using UV spectrophotometer at 238 nm. Percent entrapment efficiency was further calculated by using following formula;

Table 1
Lyophilisation condition.

Segments	Temperature (^{0} C)	Ramp (^{0}C/min)	Vacuum (mbar)	Time (h)
Pre-freezing	−70	–	–	8
1	−30	0.25	0.040	6
2	−15	0.25	0.040	6
3	−5	0.50	0.040	6
4	5	0.50	0.040	4
5	15	0.50	0.040	4

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

2.5.3. Drug loading efficiency

For determination of drug loading efficiency, freeze dried product was subjected for weighing. Further it was calculated by using following equation;

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

2.5.4. Product yield

Product yield was calculated by dividing total weigh of freeze dried product by the initial total weight of material used in preparation i.e. drug, polymer and dispersing agent [15]. The percent product yield was calculated by using following formula;

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

2.5.5. Total drug content

Freeze dried nanoparticles equivalent to 10 mg of drug was weighed and dissolved in 100 ml of 7.4 phosphate buffer to obtained final concentration 100 μg/ml. Stock solution was further serially diluted to make 10 μg/ml. Total drug content was determined by taking absorbance at 238 nm.

2.5.6. Morphology of NPs

Morphological analysis of statistically optimised batch of drug loaded nanoparticles was performed by scanning electron microscopy (JEOL model JSM-6390LV) operated at an accelerated voltage of 5 kV [18].

2.5.7. X-ray diffraction studies (XRD)

Diffractogram (Brucker AXS D8 Advance) was used to record x-ray diffractogram of the pure drug, PLGA polymer and drug loaded PLGA nanoparticles. All samples were measured in the 2 θ angle range between 3 and 80°. Diffractogram was recorded by using Cu-Ka line as a source of radiation [18].

2.5.8. Thermal analysis of NPs

Thermal analysis of pure drug, PLGA polymer and freeze dried drug loaded nanoparticles was performed by using differential scanning calorimeter (PerkinElmer 4000). All samples were subjected to heating at the rate of 10 °C/min.

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 K15 M solution with continuous agitation. Mixture was stored at 4 °C 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 under constant stirring to obtained uniform solution. Final volume was adjusted to 100 ml with purified water. At last prepared formulations were subjected for terminal sterilization by autoclaving at 121 °C, 15 p. s.i. for 20 min. Prepared freeze dried nanoparticles were placed in UV chamber for 1 h. Nanoparticle (equivalent to 100 mg of drug) aseptically transferred into sterile in situ *gel* [19–21]. Composition of nanoparticle loaded in situ gel is shown in Table 2.

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 design was applied to study the effect of concentration of Poloxamer 407 and HPMC K4M on formulation. The amount (%) of temperature sensitive polymer, Poloxamer 407 (X1) and the amount (%) of viscosity modifier, HPMC K4M (X2) were selected as independent variables, in this study. These two factors were evaluated at 3 levels as higher, middle and lower levels with coding +1, 0 and −1 respectively. Levels of X1 were selected as 14%, 16% and 18% and for X2 as 0.5%, 0.75% and 1%. The dependent or response variables included viscosity at 37 °C and 20 rpm (Y1), cumulative % drug diffused at 10 h (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. The pH of all formulations was determined immediately after preparation as well as after 24 h [22].

2.8.2. Gelling capacity

Gelling capacity was determined by adding 100 μl of sample in 2 ml Simulated tear fluid (STF) present in a vial. Temperature was maintained at 37 ± 0.5 °C and gel formation assessment was done visually. Time required for sol-to-gel as well as for gel-to-sol transition was also noted. Different grades were allotted as (−): no gelation, (+): gelation after few minutes and remains for few hours, (++): gelation immediate and remains few hours and (+++): gelation immediate and remains for extended time [23].

2.8.3. Viscosity

Viscosity of formulation was measured by using small volume adapter of Brookfield viscometer in order to select best suitable in-situ gel composition. Viscosity was measured at 20 rpm and two temperature viz. 25 °C (non-physiological) and 37 °C (physiological) [24].

2.8.4. In-vitro drug release

The drug release study was carried out in triplicate as per procedure given by Rajesh Kesarla (2016). A dialysis membrane was soaked for overnight in the diffusion medium (phosphate buffer pH 7.4) and tied

Table 2
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[a]	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

[a] Used in v/v.

from one of the end. A 1 ml volume of the formulation was filled into dialysis bag and remaining end was tied. Formulation loaded dialysis bag was suspended in a beaker containing 100 ml of freshly prepared phosphate buffer. Whole assembly was placed on magnetic stirrer at 50 rpm. Temperature was maintained to 37 ± 0.5 °C. Sample (5 ml) withdrawn at specified time interval for 10hrs and analysed at 238 nm. Fresh release medium was added to replenish withdrawn samples. From obtained data % Cumulative drug release (%CDR) was calculated. Simultaneously optimised nanoparticles were also subjected for drug release study by using same procedure. Drug release of optimised nanoparticle loaded in situ gel was compared with drug release of simple nanoparticles. Drug release from optimise 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. Gelation temperature was recorded at which stiffed gel formed which should not move after tilting test tube by 90° [20].

2.8.6. Sterility testing

Sterility testing of prepared formulation was carried out as per Indian Pharmacopoeia (2010) by direct inoculation method. Testing was carried by incubating formulation for not less than 14 days in alternate thioglycolate medium at 30–35 °C and soyabean casein digest medium at 20–25 °C to find bacteria and fungi respectively [26].

2.8.7. Preservative efficacy testing

Preservative efficacy study of optimised in situ gel was performed by challenging the formulation with *Pseudomonas aerugenosa and Staphylococcus aureus* as per Indian Pharmacopoeia (2010). Serial dilution method was used to adjust microbial count to about 1×10^5 to 1×10^6 CFU with sterile saline solution. 0.1 ml of microbial suspension was separately mixed with each 20 ml of formulation. Formulation (0.1 ml) was then withdrawn from container at 7, 14, 21 and 28 days which was inoculated by spread plate method. Petri plate containing soyabean casein digest agar media was kept for 24 h in incubator and viable count was determined by plate count method [26].

2.8.8. Histology study

Ocular irritation potential of optimised nano-particle loaded in situ gel was evaluated by histology study of goat eye cornea. Eyes of freshly sacrificed goat were taken from local slaughter house. Corneas were removed from the eyes and incubated at 37 °C for 5 h in formulation, Sodium dodecylsulfate solution 0.1% w/w (positive control) and saline solution (negative control). After incubation, corneas were washed with saline solution and immediately fixed in 8%, w/w formalin. Dehydrated tissue in an alcohol gradient were placed in melted paraffin and solidified to form block. Cross sections were cut, stained with haematoxylin and eosin and observed under motic microscope [27].

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.

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 × 3.0 mm was positioned under the lower lid within each eye close to the medial canthus and, after 2 min, the length of the wetting strip was quantified at 10× magnification by a micrometre on the dissecting microscope. During Schirmer test, the animal was slightly sedated with pentobarbitone sodium (45 mg/kg) [28–31].

3. Result and discussion

3.1. Fabrication of drug loaded PLGA nanoparticles

Nine formulation sample of pioglitazone loaded surface modified PLGA NPs were prepared by single emulsion solvent evaporation method. Lyophilisation technique was implemented for optimised batch. This technique was selected to avoid material loss.

3.2. Data analysis, statistical optimization and validation

Two independent variables viz. PLGA concentration (mg) and Number of HPH cycles at constant pressure with lower, middle and upper design points in their actual and coded form is shown in Table 3.

Responses particle size (Y1), PDI (Y2), Entrapment efficiency (%EE) (Y3) and Zeta potential (Y4) were found to be in the range of 163.51 ± 19 nm to 300.06 ± 09 nm, 0.281 ± 0.084 to 0.456 ± 0.042, 80 ± 2.0% to 95 ± 2.0% and −5.18 ± 1.5 to −10.8 ± 1.33 respectively. Detail results are depicted in Table 3. Software suggested best fitted model as Quadratic for Y1 and Liner for Y2, Y3 and Y4. As value of probability is

Table 3
Design parameters, formulation composition, experimental conditions and characterization of nanoparticles.

Run	FC	Coded levels of variables		Particle size (nm) (Y1)	PDI (Y2)	%EE (Y3)	Zeta potential (Y4)
		Factor X_1 (PLGA)	Factor X_2 (No of cycles)				
1	F1	100 (−1)	6 (−1)	189.0 ± 08	0.309 ± 0.057	80 ± 2.0	−5.18 ± 1.5
2	F2	100 (−1)	7 (0)	170.12 ± 12	0.291 ± 0.058	83 ± 1.5	−6.22 ± 2.12
3	F3	100 (−1)	8 (+1)	163.51 ± 19	0.281 ± 0.084	84 ± 1.0	−6.96 ± 0.65
4	F4	200 (0)	6 (−1)	232.19 ± 07	0.381 ± 0.063	85 ± 2.5	−7.49 ± 1.25
5	F5	200 (0)	7 (0)	209.02 ± 14.5	0.356 ± 0.045	86 ± 1.0	−7.87 ± 2.56
6	F6	200 (0)	8 (+1)	199.14 ± 11	0.321 ± 0.060	89 ± 1.3	−7.83 ± 1.66
7	F7	300 (+1)	6 (−1)	300.06 ± 09	0.456 ± 0.042	91 ± 1.0	−9.74 ± 1.26
8	F8	300 (+1)	7 (0)	278.75 ± 17	0.421 ± 0.061	93 ± 1.9	−10.1 ± 2.19
9	F9	300 (+1)	8 (+1)	250.47 ± 22	0.401 ± 0.043	95 ± 2.0	−10.8 ± 1.33

less than 0.05 in each model, results of ANOVA and regression analysis confirmed that suggested models were significant for operational parameters (Table 4).

Software suggested following polynomial equations for dependent variable;

$$Y1 = +211.22 + 51.00\ X1\ -18.17 \times 2\ -6.00\ X1\ X2 + 11.67\ (X1)^2 + 3.17\ (X2)^2 \quad (1)$$

$$Y2 = +0.3574 + 0.0662\ X1\ -0.0238\ X2 \quad (2)$$

$$Y3 = +87.33 + 5.33 \times 1 + 2.00\ X2 \quad (3)$$

$$Y4 = -8.00 - 2.05 \times 1\ -0.4967 \times 2 \quad (4)$$

From equation (1) it can be concluded that impact of X1 is positive while X2 is negative which means that as PLGA concentration increases the particle size also increases and as the number of cycles increases the particle size decreases. X1 and X2 have negative combined effect on particle size, while exponential form of X1 and X2 has positive effect on particle size.

Factor X1 and X2 showed positive and negative impact on PDI (equation (2)). Therefore; as polymer concentration increases PDI increases while increase in HPH cycle number lead to decrease in PDI. Ideally PDI of nano system should be less than 0.3. On the other hand X1 and X2 showed positive impact on percent entrapment efficiency and negative impact on zeta potential. Further impact of X1 and X2 on Y1, Y2, Y3 and Y4 can also be 3D response plot (Fig. 1.).

Desirability search approach was implemented to select optimised nanoparticle system. Formulation showing less particle size, PDI less than 0.3, good entrapment efficiency and less negative potential was the criteria for selection of optimised batch. Additionally; while selecting optimised batch its possible impact on drug release was also taken into consideration. Various research studies already showed that as polymer concentration increases drug release decreases. Based on selected criteria, batch F3 having 100 mg PLGA concentration, 8 HPH cycles possesses desirability near to 1 was selected as optimised batch.

3.3. Characterization of nanoparticles

3.3.1. Particle size, PDI and zeta potential

Particle size for all formulated batches was found to be in the range of 163.51 ± 19 nm to 300.06 ± 09 nm. Optimised formulation was subjected for freeze drying and again investigated for particle size which showed slight increase in particle size (170.41 ± 11 nm). Ophthalmic formulation should not contain particles greater than 10 μm which otherwise leads to scratching as well as sensitization to protective mechanism of eye [15]. After lyophilisation, particle size was less than 200 nm which made it suitable for ocular administration.

PDI i. e Polydispersity Index is use to identify the degree of non-uniformity of particle size distribution. PDI below 0.3 indicates uniform particle size distribution [32]. For prepared formulation batches, PDI was observed in the range of 0.281 ± 0.084 to 0.456 ± 0.042. Optimised batch showed PDI 0.281 ± 0.084 which further supports the acceptance criteria.

Measurement of zeta potential of nanoparticles is another factor which provides information about charges on fabricated particles and magnitude of it can be correlated with stability. Colloidal system showing zeta potential in the range of −30 to +30mv considered as stable formulation [15]. Fabricated nanoparticle system showed zeta potential in the range of −5.18 ± 1.5 to −10.8 ± 1.33. Zeta potential of optimised batch was found to be −6.96 ± 0.65. Even though the zeta potential is negative, presence of P80 as surface modifier will help for cellular association of particle with negatively charged cornea [33]. Additionally; particles are further loaded in temperature triggered system, which will further help to retain nanoparticles for longer time at eye site.

3.3.2. Entrapment efficiency

Entrapment efficiency of prepared batches was determined by indirect method in which amount of drug in supernatant (obtained after cold centrifugation) was recorded. Percent entrapment efficiency was found in range of 80 ± 2.0% to 95 ± 2.0%, whereas; optimised batch showed 84 ± 1.0% EE. Although few other batches showed more entrapment efficiency, F3 was selected as optimised batch by considering the release retardation property of PLGA polymer. At high concentration of PLGA, maximum amount of drug get entrapped in polymeric system but at such high concentration it can hold drug for long time period. Also, we took into consideration further slow release of drug due to temperature triggered polymeric system. Several researchers have proved the sustained release property of Poloxamer and HPMC together. Therefore; to obtain maximum drug release, batch F3 was selected during optimization process.

3.3.3. Drug loading efficiency, product yield and total drug content

Freeze dried nanoparticle product was evaluated for drug loading efficiency. Product yield and total drug content. Drug loading efficiency was found to be 6.31, whereas; product yield and drug content was found to 67.47% and 89.61% respectively. This loss may be result of various operations involved in fabrication of colloidal system.

3.3.4. Surface morphology

Surface morphology of freeze dried nanoparticles was studied by using scanning electron microscopy (Fig. 2). Particles were found to be spherical and with smooth surface which would help to avoid rapid clearance of particles along with its uniform distribution at ocular cavity. Moreover; non-spherical particles will lead to scratching effect of cornea [15,34].

3.3.5. X-ray diffraction studies (XRD)

X-ray diffraction spectra of Pioglitazone, Polymer and surface modified drug loaded nanoparticles were recorded (Fig. 3). Pioglitazone X ray diffractogram showed sharp peak 2 theta value 13.125° and 15.334°, indicating crystalline nature of drug. No sharp peak observed for polymer which indicates its amorphous nature. In contrast, sharp peaks of drug were absent in X ray diffractogram of surface modified nano particles which indicates the conversion of drug into amorphous state as well as molecular dispersion of drug in polymer.

3.3.6. Thermal analysis

Pioglitazone, PLGA and drug loaded nanoparticles were subjected for thermal analysis. Thermogram of drug showed sharp endothermic peak at 198.19 °C, which is melting point of drug as confirmed in literature. Characteristic peak of drug was disappeared in thermogram of drug

Table 4
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	0.9974	0.9931	0.9716	292.28	2	146.14	9.44	<0.005	Significant
Y2	Liner	0.9846	0.9795	0.9596	0.0297	2	0.0148	191.92	<0.0001	Significant
Y3	Liner	0.9832	0.9776	0.9721	194.67	2	97.33	275.20	<0.0001	Significant
Y4	Liner	0.9541	0.9388	0.8915	26.61	2	13.31	63.34	<0.0001	Significant

Y1: Particle size; Y2: PDI; Y3: %Entrapment efficiency; Y4: Zeta potential; SS: Sum of Square; DF: Degree of freedom; MS: mean square.

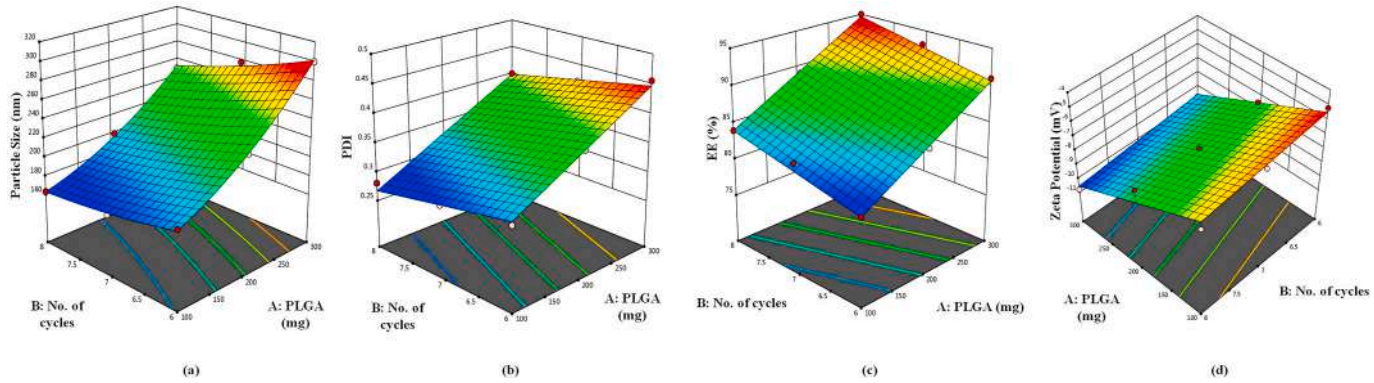

Fig. 1. Three dimensional (3 D) response surface plot for response a) Y1 (Particle Size) b) Y2 (PDI) c)Y3 (%EE) and d)Y4 (Zeta Potential).

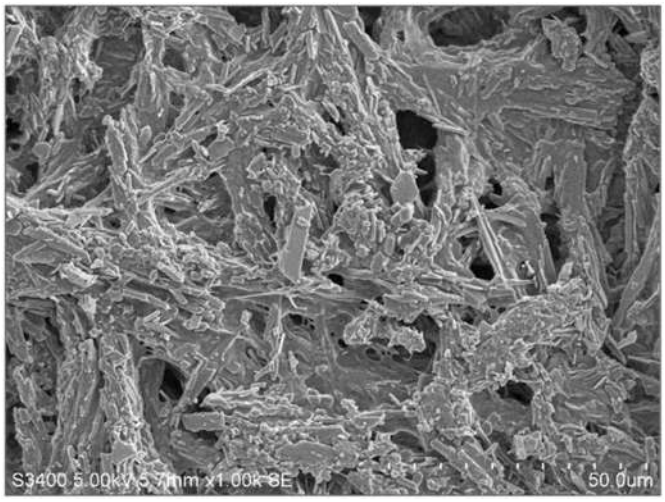

Fig. 2. SEM images of pioglitazone loaded surface modified PLGA nanoparticles.

loaded NPs (Fig. 4). It further ensures that drug was molecularly dispersed in polymer.

3.4. Preparation of nanoparticle loaded in-situ gel

Nanoparticles loaded temperature sensitive in situ gel was prepared by cold method. Lyophilized 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 5.

Responses Viscosity (Y1), Percent cumulative drug release (CDR) (Y2), Gelation temperature (Y3) were found to be in the range of 7220 ± 137 to 15,441 ± 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 6).

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;

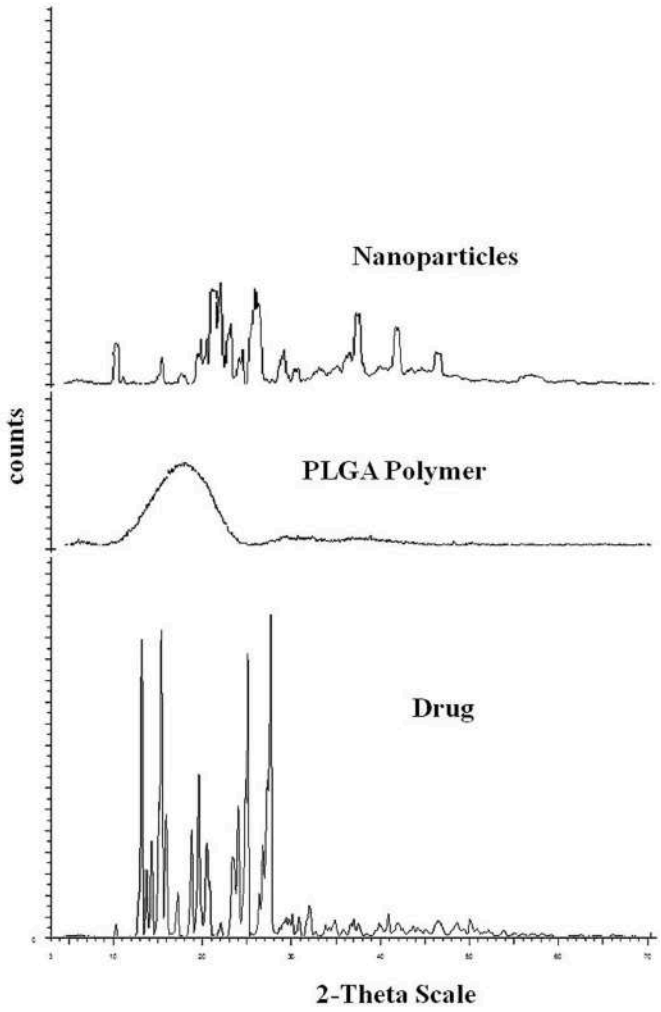

Fig. 3. XRD diffractogram.

$$Y1 = +12131.89 + 3322.50\ X1 + 802.50\ X2 - 101.75\ X1\ X2\ -692.83\ (X1)2–6.83\ (X2)2 \quad (5)$$

$$Y2 = +46.19–8.32\ X1\ -3.58\ X2 + 0.6300\ X1\ X2 \quad (6)$$

$$Y3 = +32.41–5.32\ X1 – 1.33\ X2 \quad (7)$$

Equation (5) 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 which might be due to

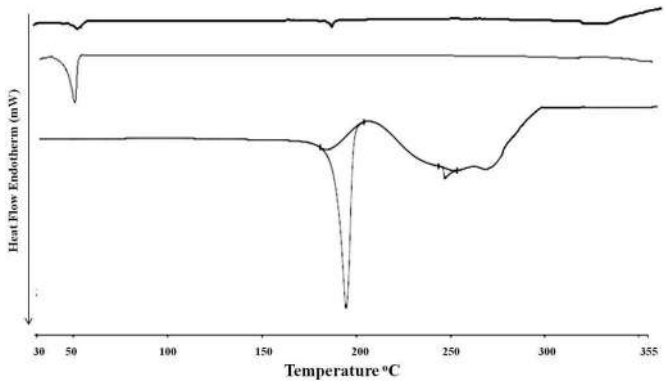

Fig. 4. DSC thermogram.

high concentration of Poloxamer than HPMC. X1 and X2 showed negative effect in combined as well as exponential form.

X1 and X2 showed negative impact of %CDR (equation (6)). 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. However; impact of X1 is more significant than X2. As the concentration of X1 increases, gelation temperature decreases. Formulated system with high concentration of Poloxamer may lead to gel formation even below to normal room temperature. These factors were taken into consideration while selecting optimised batch.

Impact of X1 and X2 on Y1, Y2 and Y3 can be further explained by 3D response surface plot as shown in Fig. 5.

Process was optimised for responses Y1, Y2 and Y3. Optimised nanoparticle loaded in-situ gel was selected by desirability search approach. Formulation having gelation at physiological temperature, maximum drug release and optimum viscosity at 37 °C were the selection parameters. Additionally; gelling capacity was also taken into consideration while selecting optimised 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.

Table 5
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	11,221 ± 154	49.97 ± 0.41	35.10 ± 0.26	+++
5	F5	16	0.75	12,132 ± 122	46.37 ± 0.31	33.70 ± 0.40	+++
6	F6	16	1.0	13,029 ± 117	42.31 ± 0.39	31.35 ± 0.70	+++
7	F7	18	0.5	14,141 ± 104	40.71 ± 0.24	27.36 ± 0.85	+++
8	F8	18	0.75	14,689 ± 168	37.77 ± 0.32	26.76 ± 0.75	+++
9	F9	18	1.0	15,441 ± 121	35.07 ± 0.21	25.10 ± 0.10	+++

Table 6
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

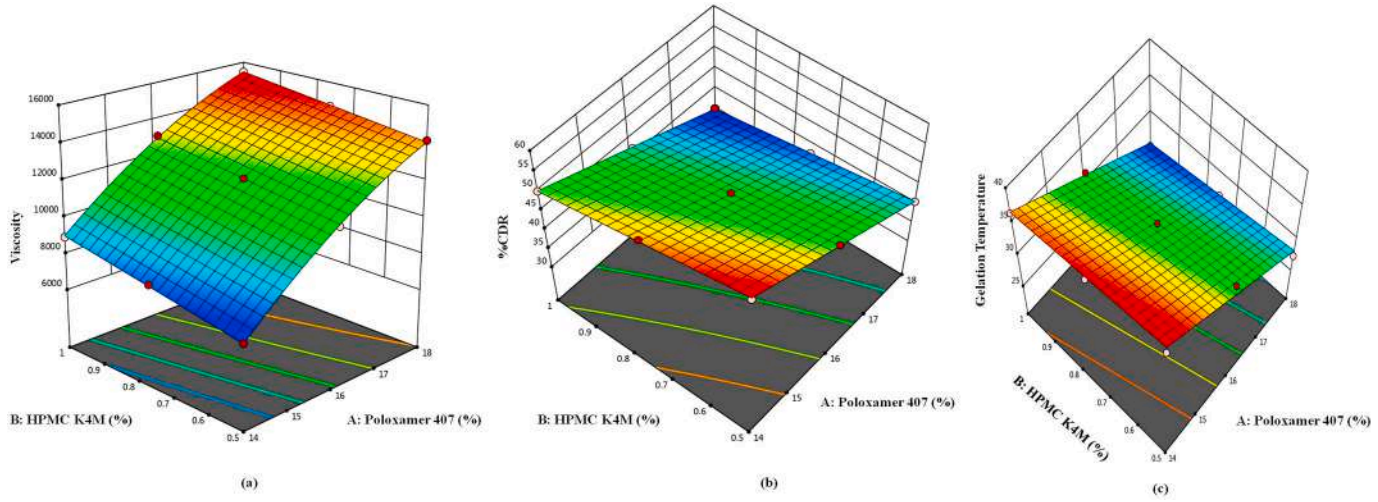

Fig. 5. Three dimensional (3 D) response surface plot for response a) Y1 (Viscosity) b) Y2 (%CDR) c)Y3 (Gelation temperature).

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 6.4–6.9. Ocular site can tolerate pH in the range of 3.5–8.5 without sensitization to patient significantly [20].

3.6.2. Gelling capacity

Gelling capacity of all prepared batches was determined in simulated tear fluid at 37 °C. None of the batch failed to gel at physiological pH. However; batch F1 and F2 showed less stable gel (++) in comparison to rest of the batches. This effect might be due to less concentration of polymer which failed to hold gel structure for longer duration.

3.6.3. Viscosity

Viscosity of all prepared batches was found in range of 7220 ± 137 to 15,441 ± 121 (cP) when measured at 37 °C and 20 rpm. Viscosity of formulation after instillation into eye is key factor which determines residence time of formulation at ocular site. It should not be too height which otherwise may cause patient discomfort and to less which otherwise affect the residence time of formulation.

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 10 h 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. Optimised nanoparticle loaded in situ gels showed 50.21 ± 0.16% drug release in comparison to simple nanoparticles which showed 74.17 ± 1.38%. This indicates more sustained (prolong) drug release from in situ gel than simple nanoparticles. This is due entrapment of nanoparticles into gel matrix formed by Poloxamer and HPMC at physiological temperature. Therefore; drug travels from PLGA to gel matrix and then into dissolution medium. Although; the drug release was less, presence of Poloxamer and HPMC together can show effect on DED due to lubrication property [8,25,35]. Comparative drug release profile is shown in Fig. 6.

Data obtained from in vitro drug release study was further subjected to mathematical treatment to determine drug release kinetic profile. The release constant was calculated from the slope of the appropriate plots and the regression coefficient (R^2) was determined which is summarized in Table 7. The drug release from simple nanoparticles and NPs loaded in-situ gel was best explained by Higuchi kinetic with highest R^2 value. Korsmeyer-Peppas equation indicated a good linearity of regression coefficient (R^2) for both system N value above 0.5 for nanoparticles and below 0.5 to NPs loaded formulation (Table 8.) indicating the drug transport mechanism is non-fickain and quasi fickian respectively.

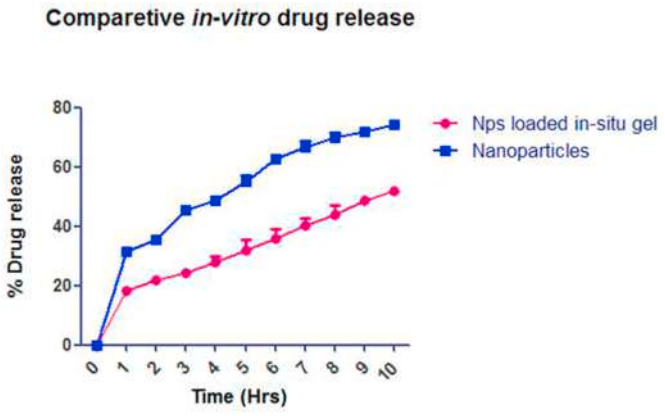

Fig. 6. Percentage drug release from nanoparticle loaded in situ gel and simple nanoparticles (n = 3, mean ± SD).

Table 7
Model fitting for release profile.

Formulation	Coefficient of determination (R^2)				Best fit model
	First order	Zero order	Higuchi	Hixon-Crowel cube root	
Nanoparticles	0.9578	0.9262	0.9979	0.9762	Higuchi
Nps loaded in-situ gel	0.9578	0.9155	0.9879	0.9374	Higuchi

Table 8
Korsmeyer-Peppas drug release kinetics.

Formulation	R^2	N value	Mechanism
Nanoparticles	0.9895	0.510	Non fickian
NPs loaded in-situ gel	0.9895	0.455	Quasi fickian

3.6.5. Gelation temperature

Temperature triggered formulation must get convert into stiff gel at or slightly below to physiological temperature. Temperature at which sol form converts into gel form is known as the gelation temperature. Prepared all batches showed gelation at temperature in the range 25.10 ± 0.10 °C to 38.43 ± 0.20 °C. If system showing gelation above to physiological temperature, then it may get fail to convert in gel form after instillation at eye site. On the other hand if gelation temperature is too less then it will get convert into gel form during storage. Therefore; batch F3 was selected as optimised batch which showed gelation at temperature 36.43 ± 0.32 °C.

3.6.6. Sterility testing

Optimised 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.

3.6.8. Histology study

Ocular itchiness capability of prepared nanoparticles loaded with in situ gel was evaluated by a goat eye corneal histolopathology. Cornea framework was well conserved in saline solution and cornea-treated with the formulation. Even so, the corneal structure was seriously compromised by control group (SDS treated). There was no haemorrhage or necrosis in the formulation of treated cornea. Therefore, the prepared formulation was found to be suitable for the further in vivo investigations. Similar results were observed by Ameeduzzafar et al. (2018) [36] as shown in Fig. 7.

3.7. In vivo characterization

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 min (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 9) which stated the DED in BAC-treated mice. Mice from the

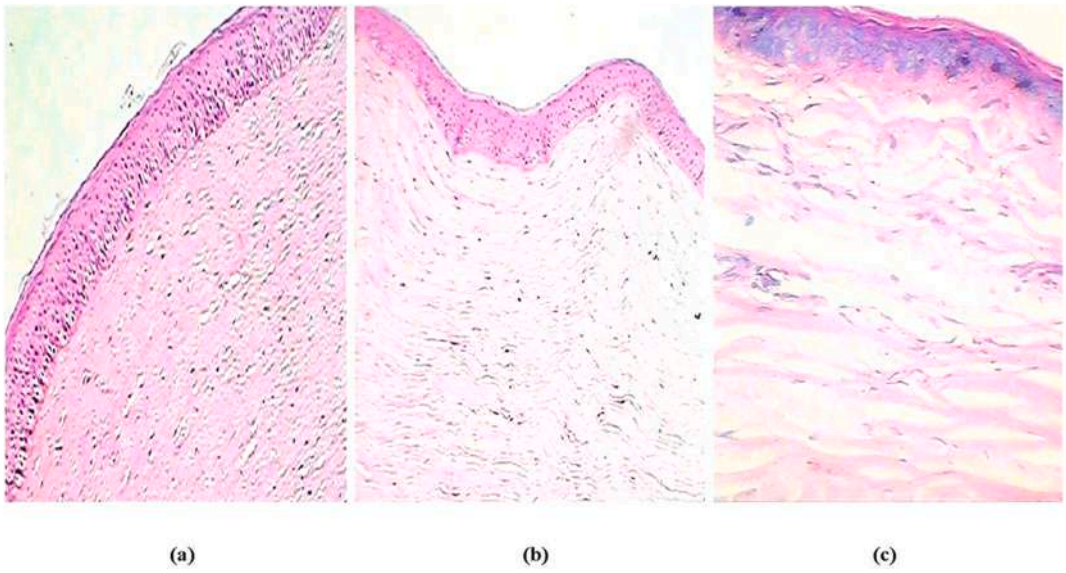

Fig. 7. Histological section of goat eye cornea a) negative control: untreated cornea, b) test specimen: formulation treated cornea, c) positive control: SDS treated cornea.

Table 9
Tear secretion measurement by Schirmer's test.

Group	Filter paper wetting (mm) at the 7th day of BAC treatment	After 15 min of formulation administration	Before second dose administration	After 15 min 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).

nanoparticle group, in situ formulation and standard treatment received 1 drop of formulation. Upon 15 min, 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 h before the formulation was administered and 15 min after the formulation was administered. Results are shown in Table 9 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.

4. Conclusion

In present research, we prepared PPAR-γ agonist PLGA nanoparticles loaded in situ gel by using Poloxamer 407 and HPMC K4M for treatment of dry eye disease. Nanoparticles as well as in situ gel were optimised for composition and process parameter by using 3^2 factorial design. Particles were found to be spherical during SEM study which proved non-scratching characteristic of particle. XRD and DSC study confirmed the molecular dispersion of drug in polymer. *In vitro* drug release study showed more sustained release of drug. Formulation was found to be non-irritant in histology study on goat eye cornea. Further, the effectiveness of formulation in dry eye disease was checked by Schirmer's test on benzalkonium choride dry eye induced mice. In comparison to marketed formulation, prepared formulation showed marked increase in tear secretion and further tear film stabilization. Moreover; drug loaded nanoparticle also showed promising results which supports the involvement of PPAR-γ in dry eye disease. Therefore; based on evidence, developed formulation can be consider as remarkable substituent for present treatment of dry eye disorder.

Declaration of competing interest

There is no any conflict of interest.

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Research article

Formulation of PPAR-gamma agonist as surface modified PLGA nanoparticles for non-invasive treatment of diabetic retinopathy: *in vitro* and *in vivo* evidences

Umesh D. Laddha *, Sanjay J. Kshirsagar

MET's Institute of Pharmacy, Bhujbal Knowledge City, Affiliated to Savitribai Phule Pune University, Nashik, 422003, MS, India

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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. The release profile of pioglitazone from nanoparticles appeared to fit best with Higuchi model. 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.

1. Introduction

Diabetes mellitus is recognized as a global epidemic, has already affected 382 millions of people worldwide and it is projected that by year 2035 this number will be 559 millions [1]. Management of diabetes mellitus associated complications along with primary disease is the biggest challenge to researcher and health care provider [2]. Diabetic patients are at high risk of development of the most devastating microvascular complication known as diabetic retinopathy [3, 4]. Diabetic retinopathy (DR), leads to blindness in a patient aged from 20-65 years [5]. 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 [6]. Still, the complete cure is not available for diabetic retinopathy, for a sake the loss of vision can be prevented or extended either by laser treatment or by vitrectomy, which is associated with pain and further fear of loss of vision [7, 8]. Recently US-FDA has approved drugs like ranibizumab, aflibercept for the management of diabetic retinopathy, administered in the form of intravetrous injection [9, 10]. Although intravetrous injection helps to deliver therapeutic amount of drug at the same time, it results in pain and increased risk of bleeding as well as chances to have secondary infection. Moreover; this current treatment is applicable at advanced stage of diabetic retinopathy, therefore; there is surge to develop alternative method for the treatment of diabetic retinopathy in its early stage. 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) reported the presence of peroxisome proliferator-activated receptor-γ (PPAR-γ) in the mammalian eye, prominently in the photoreceptor outer segments, retinal pigmented epithelium (RPE), choriocapillaris and retina. The PPAR-γ agonist is also well known to treat the diabetes and able to resolve crucial issues associated with diabetic retinopathy [4]. Increased level of VEGF can be acts as marker of progress of DR as reported by Saravia M. *et al.* (2017) [11]. It was found that the vitreous level of vascular endothelial growth factor (VEGF) in patient suffering from DR was markedly increased. Moreover; researchers from National Institute of Health and American Society of Retina Specialist concluded that formation of abnormal blood vessel, which results in blindness in diabetic retinopathy, is spurred by VEGF. Involvement of PPAR-γ receptors in diabetic retinopathy, current research involves preparation of topical formulation of PPAR-γ

* Corresponding author.
E-mail address: umeshladdha698@gmail.com (U.D. Laddha).

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agonist with aim to regulate VEGF. Since the target is posterior segment of eye, the delivery of drug towards the back of eye is challenging task in view of protective barriers and defence mechanism of eye. We have prepared the surface modified nano-particles of pioglitazone which is a PPAR-γ agonist by using two different grades of PLGA [poly (D,L-lactide-co- glycolide)] viz. PLGA 75:25 and PLGA 50:50 by single emulsion solvent evaporation method with the application of Box-Behnken statistical design. PLGA is a biocompatible and biodegradable polymer, which degrades hydrolytically into nontoxic oligomer and monomer as lactic acid and glycolic acid respectively. Moreover; it is approved by US-FDA for clinical use [12]. Surface modification was achieved by using non-ionic surfactant polysorbate 80 (P80) to improve interaction with eye. We investigated the drug delivery by PLGA after topical administration with measurement of VEGF by ELISA based on *in vivo* study.

2. 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 Ltd., Evonik Mumbai, India. dichloromethane, polysorbate 80, Potassium dihydrogen orthophosphate and dipotassium hydrogen phosphate was obtained from SDFCL, Mumbai. Polyvinyl alcohol and mannitol was procured from Thomas baker, 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. Stock solution of concentration 100 μg/ml was prepared by dissolving 10 mg Pioglitazone in 1 ml methanol and volume was adjusted to 100 ml by phosphate buffer pH 7.4.

2.3. Preparation of PLGA nanoparticles

2.3.1. Preliminary studies

Trial batches were performed for selection of concentration of polymer, concentration of surface modifier (Polysorbate 80) and number of cycles of high pressure homogenizer given in Table 1. From these trial batches low, medium and high levels of individual parameters were fixed. Particle size and PDI were observed for selection of experimental batches of PLGA NPs.

2.3.2. Procedure for preparation 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 is 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 nanodroplets. Further to achieve more 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. Further all batches were subjected for 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 fltrate 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 applied to reconnoiter the variables and experimental trials performed at all 15 possible combinations. Design was applied to enumerate the impact of independent variables viz. polymer concentration, surface modifier concentration and number of cycles on the dependent variables viz. particle size, polydispersity index (PDI) and percent entrapment efficiency (%EE) of nanoparticles. The resulting data were fitted into Design Expert software and analysed statistically using analysis of variance (ANOVA). The statistical significance of the data was performed in terms of regression coefficients [14, 15].

The general polynomial equation for 3^3 Box-Behnken statistical design is:

$$Y = \beta_0 + \beta_1 * A + \beta_2 * B + \beta_3 * C + \beta_{12} * AB + \beta_{13} * AC + \beta_{23} * BC + \beta_{11} * A^2 + \beta_{22} * B^2 + \beta_{33} * C^2$$

The equation was applied to the responses, to describe the principal effects and interaction among the identified independent variables A, B, C with coded levels as -1, 0 and +1 (Table 2). These limits were selected on the basis of previous studies and the optimization was carried out within this domain. In general polynomial equation, Y is associated with the predicted responses; β_0 is the intercept; β1, β2, β3 are coefficients for variables A, B, C respectively; β_{12}, β_{13}, β_{14} representing coefficients for interaction of variables A and B, A and C, B and C respectively; β_{11}, β_{22}, β_{33} representing coefficients for interaction A and A, B and B, C and C respectively.

The three-dimensional (3D) response surface plots were used to show graphically the relationship and interaction between the coded variables and the response. The optimum concentrations of independent variables based on the responses constrained in their minimum levels were selected. Furthermore, formulations were selected by point prediction and chosen experimental domain as well as polynomial equation was validated by comparing the experimental responses with the predicted values. Then, finally optimized batch was prepared and evaluated for the

Table 1. Composition of trial batches.

PLGA Concentration	P80 concentration (mg)	Cycles (numbers)	PVA Concentration (%)
300	600	4	1
100	300	4	1
300	300	4	1
100	600	4	1
100	300	6	1
100	300	4	2
500	300	4	1

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

Where,

Coded levels	Indicates
-1	Low level of independent variable
0	Medium level of independent variable
+1	High level of independent variable

particle size, PDI and entrapment efficiency. A 3^3 Box-Behnken statistical design layout is shown in Table 3.

The results obtained were analysed by Design Expert Software 11.0.5.0. All the formulations were prepared as per the procedure used for the preliminary formulations.

Based on optimization, a batch which was found to be optimized, the residue of same batch was subjected for spray drying (Labultima LU222, India) after 2–3 times washing with distilled water. Prior to spray drying residue was dispersed in distilled water containing manitol which act as cryoprotectant. During spray drying inlet and outlet temperature was set at 150 °C and 90 °C respectively. Aspirator rate was adjusted at 35Nm^3/hr while sample feed rate was 2 ml/min [16]. With same procedure one batch of PLGA 50:50 was also prepared at optimized condition of PLGA 75:25 and it was also evaluated for same parameters simultaneously along with 75:25 grade nanoparticles.

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). Dynamic light scattering measures the fluctuations in light due to Brownian motion of particles, which is then correlated with the size of particles. The analysis was performed at 25 °C with an angle of detection 90° [15].

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

2.5.2. Entrapment efficiency

To determine the amount of Pioglitazone entrapped in nanoparticles, an indirect method was used. 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 238nm [17].

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

2.5.3. Zeta potential measurement

Zeta potential is the electrostatic potential that exists at the share plane of a particle, which is related to both; surface charge and the local environment of the particle. 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).

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

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].

The percent yield was calculated as:

$$\%\text{Yield} = \frac{\text{Total weight of obtained NPs}}{\text{Drug} + \text{polymer weight} + \text{dispersing agent}} \times 100$$

2.5.6. Total drug content

Weigh accurately spray dried PLGA NPs equivalent to 10 mg of pioglitazone. It was dissolved in 100ml of phosphate buffer pH 7.4 to give 100 ug/ml. From prepared stock solution, 1ml solution was withdrawn and further serially diluted to make 10ug/ml. Concentration 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 previously soaked overnight in the diffusion medium was tied from one end and 1ml of formulation containing equivalent to 1mg of drug was accurately pipetted into dialysis sac and it's another end was closed tightly. The dialysis membrane sac was suspended in 100 ml diffusion medium in a beaker and temperature was maintained 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 analysed by spectrophotometrically at 238 nm. Fresh release medium was added to replenish withdrawn samples. Cumulative percentage drug released was calculated. Drug release from optimize batch was also 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) operated at an accelerated voltage of 15 kV [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 °C/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. Experiment was designed and conducted in accordance with the guidelines laid by CPCSEA, New Delhi. 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 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).

3. Results and discussion

3.1. Preliminary studies

Preliminary studies were performed in order select the concentration of polymer, concentration of surface modifier and number of cycles of high pressure homogenizer. Based on literature survey, PLGA concentration was varied in the range of 100 mg–500 mg, whereas; surface modifier concentration and HPH cycles were changed as 300 mg–600 mg and 4 to 6 respectively [12]. Prepared trial batches were evaluated for particle size, PDI and EE. Results of the preliminary studies are represented in Table 4.

From results of preliminary study it was clear that, as concentration of polymer increases particle size and entrapment efficiency also increases. Increase in PLGA concentration led to an increase in the viscosity of the organic phase, thereby reducing the net shear stress and promoting the formation of droplets with larger size and uneven surface. NPs prepared with lower concentration of PLGA presented spherical shape without any agglomeration. On the other hand as the concentration of surface modifier increases, particle size and entrapment efficiency also increases. Molecules of the Polysorbate 80 adsorb onto the surface of PLGA nanoparticles and hence it increases the particle size. However; particle size does not increases significantly as compared to PLGA concentration. It was also observed that as the number of cycles of HPH increases particle size decreases along with PDI. Increase in number of cycles of HPH causes reduction in particle size due to very high shear stress which causing the formation of very fine droplets with uniform distribution of particles. Moreover; it has been observed that as the concentration of the PVA increases, there is increase in particle size as well as PDI.

Based on the preliminary studies result above mentioned factors were selected with three levels for optimization by the statistical design. Similar kind of results were obtained to Wagh and Apar, 2014; Jose et al, 2016 [15,23].

3.2. 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 min. Prepared system 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.

3.2.1. Statistical evaluation of experimental design

The best fit model obtained by design expert for each response was evaluated on the basis of ANOVA by calculating the F value and Statistical significance of the data was performed in terms of regression coefficients. It was observed that the best fitted models were linear for the PDI and EE whereas quadratic for the particle size. The regression analysis for responses R1, R2, R3 and ANOVA of each model has shown in Tables 6 and 7 respectively.

3.2.2. 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, 23]. 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. In this case, A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. The polynomial Eq. (1) of response R1 showing the combined effect of PLGA

Table 4. Result of the preliminary studies.

PLGA concentration (mg)	P80 concentration (mg)	Cycles (numbers)	PVA concentration (%)	Particle Size (nm)	PDI	Entrapment Efficiency (%)
300	600	4	1	600	0.546	92
100	300	4	1	412	0.408	81
300	300	4	1	488	0.489	89
100	600	4	1	465	0.454	86
100	300	6	1	323	0.378	83
100	300	4	2	578	0.562	82
500	300	4	1	700	0.621	90

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

Table 6. Summary of results of regression analysis for responses.

Response	Model	Sequential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2
Particle size (R1)	Quadratic	0.0001	0.2484	0.8880	0.8468
Polydispersity index (R2)	Linear	0.0053	0.5924	0.5807	0.3685
Entrapment efficiency (R3)	Linear	0.0001	0.1371	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

concentration, polysorbate 80 concentration and number of cycles of HPH on particle size. In this equation, impact A and B is positive while C is negative. However; magnitude of A significantly high in comparison to B. Therefore PLGA significantly affects to size in comparison to concentration of P80. Magnitude of A and B is high as compare to A & C and B & C but it showing the negative effect. Magnitude of A and C is high but as compare to B and C is low. Magnitude of exponential of A is high as compare to B^2 and C^2, it showing the negative effect on particle size.

The impact of A, B and C can be further interpreted with the help of counter plot and 3D response surface plots (Figure 1).

3.2.3. Polydispersity index

Polydispersity Index (PDI) which is alternatively known as the heterogeneity index is correlated with the uniformity of particle size distribution. It ranges from 0.0 to 1.0 which represents uniform to polydrisperse sample in context to particle size. Ideally, nanoparticles should possess PDI below or equal to 0.3. Particle size and size distribution are important factors for evaluating the stability of a colloidal dosage form upon storage [24]. 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 Eq. (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. Therefore; as concentration of polymer increases there is increase in PDI; as concentration of surface modifier increases there is slight increase in PDI and as number of cycles of HPH increases there is decrease in PDI [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. The impact of A, B and C on PDI can be further interpreted with the help of counter plot and 3D response surface plots shown in Figure 2.

3.2.4. 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. Above Eq. (3) clearly

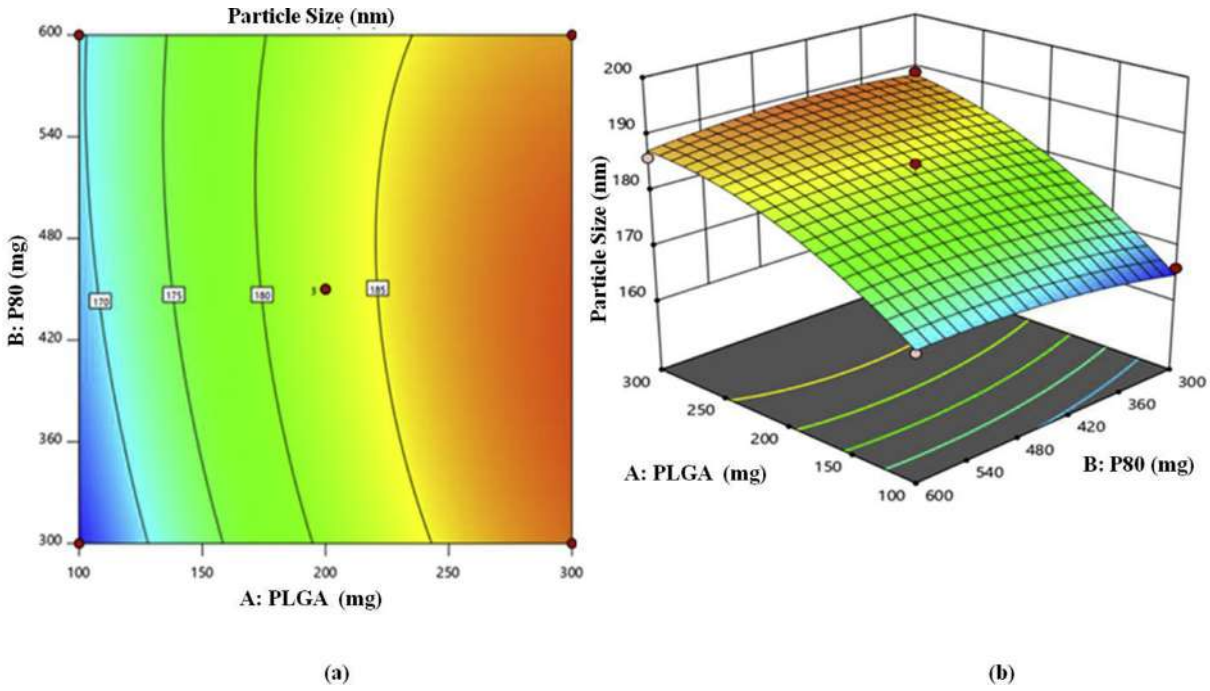

Figure 1. (a) Two dimensional counter plot, (b) three dimensional (3 D) response surface plot for response R1 (Particle Size).

indicates the significant impact of A on entrapment efficiency than B and C. The impact of A, B and C on entrapment efficiency can be further interpreted with the help of counter plot and 3D response surface plots given in Figure 3.

3.2.5. Development of the optimize batch

Based on the statistical evaluations the software gave further 31 possible solutions for the optimization of the batches. The minimum particle size and PDI and higher percent entrapment efficiency were the selection criteria for optimized batch. Formulation B_2 was selected as an optimized batch having lesser particle size, less PDI and more EE. A new optimized formulation was prepared according to the predicted model and evaluated for the responses as shown in Table 8.

The experimental values were compared with the predicted values & residual error calculated from Equation which given below and it was seen that the low percentage error which indicates that result given by software are validated.

$$\% \text{ Error} = \frac{|\text{Experimental value} - \text{Predicted value}|}{\text{Predicted value}} \times 100$$

3.3. Characterization of optimized batch

3.3.1. Particle size and PDI

The average particle size and polydispersity index of nano particles of PLGA 75:25 (with and without surface modification) and PLGA 50:50 (with surface modification) were determined. Results are summarized in below Table 9. Results clearly indicates size of nano particle is less than 180 nm and comparatively same for both grade of polymers.

3.3.2. Zeta potential measurement

Zeta potential measurement provides information about charges on particle and magnitude of it can be correlated with stability of nano-particles. Surface properties as well as eventual modification of nano-partilces can be evaluated by Zeta potential measurement. Zeta

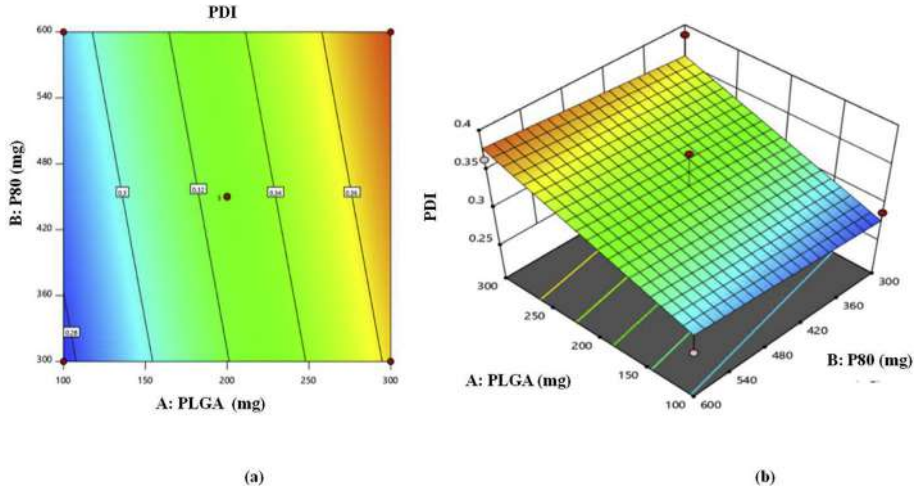

Figure 2. (a) Two dimensional counter plot, (b) three dimensional (3 D) response surface plot for response R2 (PDI).

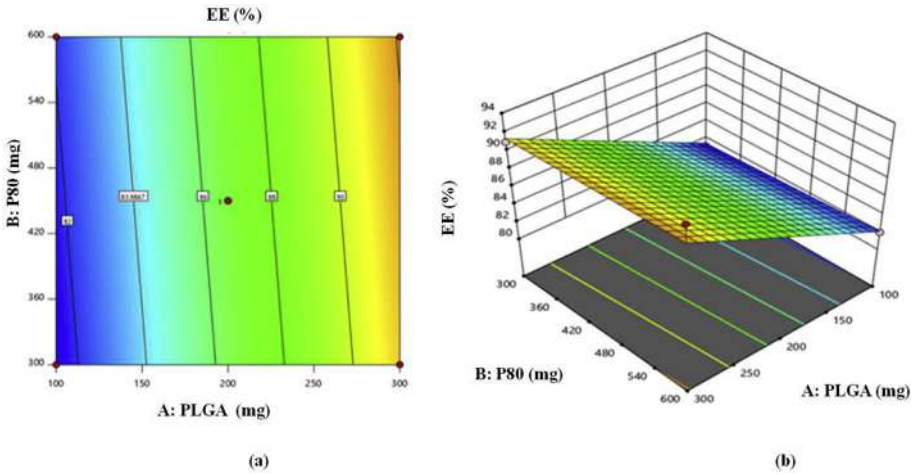

Figure 3. (a) Two dimensional counter plot, (b) three dimensional (3 D) response surface plot for response R3 (EE).

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

Response	Target	Predicted value	Experimental value	Residual error (%)
Particle size	150	162	163.23	0.759
PDI	0.250	0.284	0.286	0.70
Entrapment Efficiency	95	90	91	1.11

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

potentials indicate existence of repulsion between suspended particles due to which particles remain in Brownian motion. Zeta potential within in the range of is -30 to +30mv indicates the stability of the formulation [15].

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. Although the zeta potential of surface modified PLGA nano particles is more negative as compared to unmodified particles but Tahara K, Yamamoto H, Kawashima Y. (2010) has already proved the use of P80 as surface modifier increases the cell association and ultimately uptake and movement of drug towards retina [25]. Literature also revealed that presence of surface modification is a key step to target nanoparticles to back of eye [12]. Moreover; unmodified PLGA nano particles suffer from major limitation as short residence time when applied on mucosal tissues. It may be due to negative charges on cornea which repels the negatively charged PLGA particles. However; surface modification of PLGA particles using functional materials such as P80 results into improvement in the interaction with eye tissues and ultimately increases delivery efficiency. Polysorbate 80 has already proved its involvement in improved cellular association at corneal site due to its mucoadhesive property. It has been also observed that P80 modified NPs have a higher uptake into cells as compared with unmodified NPs. Additionally Polysorbate 80 also acts as a surfactant which help to reduce the surface tension at the ocular site [12]. Therefore; surface modified PLGA 75:25 and 50:50 nanoparticles were subjected for further evaluation.

Table 10. Entrapment efficiency and drug loading efficiency of optimized batch.

Nano Particles	Entrapment efficiency (%)	Drug loading efficiency (%)
Surface modified PLGA 75:25	91	7.89
Surface modified PLGA 50:50	93	8.12

3.3.3. *Entrapment efficiency and drug loading efficiency*

Entrapment efficiency and drug loading efficiency of optimized surface modified PLGA 75:25 and PLGA 50:50 nano particles was determined. Results are listed in following Table 10. Similar results were observed by Salama, Mahmoud & Kamel, (2016) [18].

3.3.4. *Product yield and total drug content*

Product yield and total drug content of the surface modified PLGA 75:25 and PLGA 50:50 was determined. The percentage yield and drug content of the PLGA 75:25 nano particles was found to be 67% and 93% respectively. On the other hand percentage yield and drug content of the PLGA 50:50 nano particles was found to be 66% and 90% respectively. This may be due to the loss of drug during the various operations of preparation of nano particles [18].

3.3.5. *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 as per the procedure R. Kesarla et al 2016 [19]. 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. In both case drug release pattern was found to be biphasic which might be due to initial burst release [26, 27]. Difference in drug release between two polymers reveals the more sustained release property of PLGA 75:25 polymer in comparison to PLGA 50:50. Drug release through different grades of PLGA polymer governed by the hydrophilicity and rate of degradation of polymer which depends on composition. PLGA 50:50 undergoes rapid degradation than PLGA 75:25 due to high proportion of glycolic acid which determines the hydrophilicity. Comparative plot of cumulative percent drug release by surface modified PLGA 75:25 and PLGA 50:50 nano particles are shown in Figure 4.

Data obtained from *in vitro* drug release study was further subjected to mathematical treatment to determine drug release kinetic profile. The release constant was calculated from the slope of the appropriate plots and the regression coefficient (R^2) was determined which is summarized in Table 11. The drug release from both grades of PLGA was best explained by Higuchi kinetic with highest R^2 value. Korsmeyer-Peppas equation indicated a good linearity of regression coefficient (R^2) for

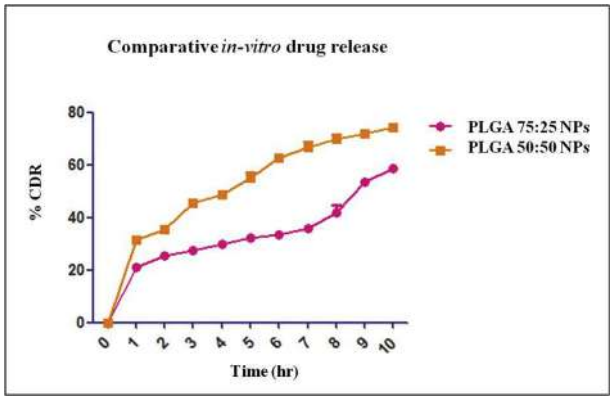

Figure 4. Percentage drug release from PLGA 75:25 and PLGA 50:50 NPs (n = 3, mean ± SD).

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

nano particles of both grades of PLGA with N value above 0.5 (Table 12) indicating the drug transport mechanism is quasi fickian. Similar results were observed by Wagh and Apar, 2014 [15].

3.3.6. 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 5. SEM analysis of particles reveals that all particles were spherical and possessed smooth surface without any fracture. This morphology further helps in uniform deposition of particles in ocular site and also facilitate movement of particles towards the retina.

3.3.7. Thermal analysis

The DSC thermogram of Pioglitazone showed sharp characteristic endothermic peak at 190.58 °C which is also reported by Faruksha & Vetrichelvan, 2013 [28]. 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 6. This study further confirmed the molecular dispersion of drug in polymer.

3.3.8. 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. *In-vitro* release study showed the more slow release of drug from PLGA 75:25 nanoparticles in comparison with PLGA 50:50 polymer. Therefore; drug loaded PLGA 50:50 nano suspension was evaluated at three different level of concentration to check impact of dose on VEGF level. 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. This difference might be due

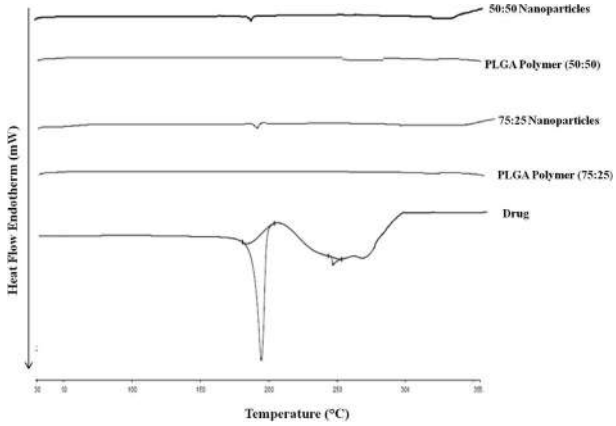

Figure 6. Comparative DSC

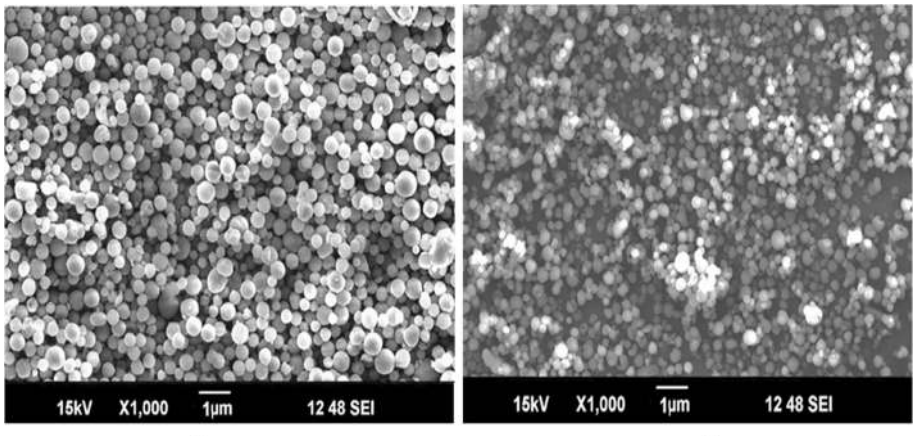

Figure 5. SEM image of surface modified Pioglitazone loaded nano particles of a) PLGA 75:25 and b) PLGA 50:50.

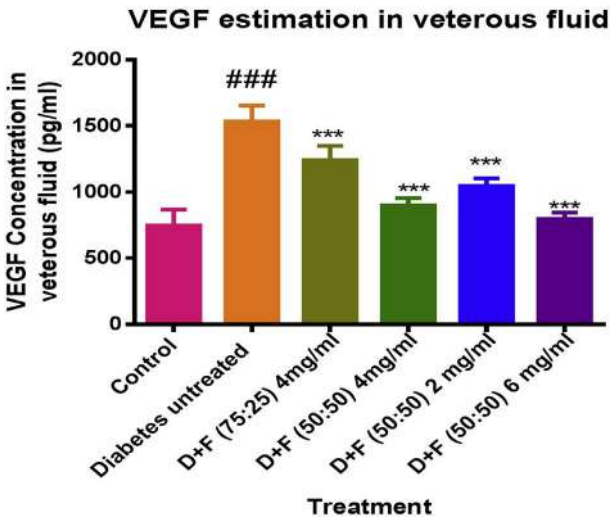

Figure 7. Effect of formulation on VEGF in rats.

to slight difference in permeation of particle towards the targeted size and further more slow release of drug by PLGA 75:25. The group treated with 6 mg/ml of nanosuspension showed decreased in VEGF as compared to 4 mg/ml. Whereas; level of VEGF was more in 2 mg/ml treated group than in 4 mg/ml treated group which clearly indicates the dose dependent activity of formulation given in Figure 7. *In vivo* study reveals the movement of drug loaded surface modified nano particles towards back of eye and its significance in management of diabetic retinopathy by topical instillation of formulation. Additionally; as the study proves the down regulation of VEGF by a PPAR-γ agonist, there is also hope to cure diabetic retinopathy by some other possible mechanisms which are associated with PPAR-γ receptors like inhibition of formation of AGE, decrease in angiogenesis, inflammation, retinal leakage and retinal leukostais as well as protection from apoptosis and oxidative stress on RPE.

4. Conclusion

Surface modified PLGA nanoparticles of Pioglitazone were successfully prepared and characterized for various parameters. Two grades of PLGA (75:25 and 50:50) were used to investigate ability to deliver drug towards posterior segment of eye after topical administration. Both grade nanoparticles showed small particle size as 163.23 nm (PLGA 75:25) and 171.7 nm (PLGA 50:50) which may help in penetration of particle and further haulage towards the retina site by different pathways of transportation. Zeta potential of surface modified PLGA 75:25 nanoparticle was different (-10.8 mV) in comparison to unmodified PLGA 75:25 nanoparticles (-6.22 mV) which confirmed the surface modification by polysorbate 80. Zeta potential of surface modified PLGA 50:50 nanoparticles was found to be -7.49 mV. SEM study showed all particles were spherical and with smooth surface. DSC study confirmed the molecular dispersion of drug in polymer. *In vitro* release study showed the initial burst release of drug which was later on controlled due to less solubility of polymer.*In vitro* study also reveals the more controlled and slow release by PLGA 75:25 in comparison to PLGA 50:50. Further effectiveness of formulation in treatment of DR was evaluated in diabetes induced rat. 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; VEGF level was significantly less in PLGA 50:50 nano suspension than PLGA 75:25 nano suspension. PLGA 50:50 nano suspension further proved dose dependent reduction in VEGF level. Since; increased VEGF is indicator of DR, its down regulation proves movement of surface modified nano particle toward the posterior segment of eye. These results indicates that pioglitazone loaded surface modified PLGA nano particles possesses great potential to treat DR. It can be consider as feasible breakthrough avenue in management of DR which is free from any invasive and painful process.

Declarations

Author contribution statement

U. Laddha and S. Kshirsagar: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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