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Formulation and optimization of Retapamulin loaded PLGA nanoparticles for burn wounds

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Abstract

In the present study, PLGA nanoparticles (PLGA-NP) of retapamulin (RP) were prepared and optimized by studying the effect of various formulation and process variables for effective delivery at burn wound site. Drug loaded PLGA-NP were successfully prepared and characterized by TEM, XRD and DSC study. Formulation and process variables like surfactant concentration, drug concentration, polymer concentration etc. showed significant effect on the particle size, entrapment efficiency and drug loading. PLGA-NP exhibited prolonged drug release following Higuchi release kinetics (R2= 0.9907). *In vitro* study demonstrated systemic escape of drug from PLGA-NP which might eliminate side effects associated with topical

exposure through conventional treatment. Further retention of activity of entrapped drug was confirmed by *in vitro* antimicrobial assay._Optimized PLGA nanoparticle of Retapamulin was incorporated into PVA-Chitosan hydrogel slurry and casted into film to prepare multiphase hydrogel.Thus, present optimized system can be effectively used for delivery of drug at burn wound site in especially compromised wounds.

Keyword: Retapamulin, Process variables, PLGA Nanoparticles, Infected burn wound, MRSA.

1. INTRODUCTION

A wound is a defined gap in the barrier of skin and the exposure of barrierless tissue followed by loss of arranged skin layers provides a supportive moist environment to microbial growth (Shittu et al. 2002). On the basis of wound healing process there are two types of wound i.e. acute wounds and chronic wounds, caused by various environmental factors affecting the human body. Among them burn wound is a serious acute type of wound which damages the skin and underlying structures caused by excessive heat, caustic chemicals and electrical hazard. Further burn cases become worse during long treatment of patient in the hospitals due to secondary infections i.e. Methicilin resistant staphylococcus aureus (MRSA). Exposure of subcutaneous tissue is the common cause of MRSA infection on burn wound space increasing morbidity and mortality rate with almost 61% of deaths (Baker et al. 2016). It is important to combat all environmental factors inhibiting natural healing process by keeping the burn wound space free of such type of infections (Singh et al. 2008, 2009, 2011). The pathogens frequently identified for infection in burns are primarily gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Church et al. 2006, Madhan et al. 2014). The challenging task in treatment of burn wound is control and removal of MRSA microorganism from wound space which is further complicated by widespread bacterial resistance to antibiotics like methicillin (Eleftheriadou et al. 2010). Topical and prophylactic antibiotics are used in infection control especially for MRSA burn unit. Some authors have reported that antibiotic prophylaxis is one of the challenging steps that may prevent MRSA infected burn wound and protect from invasive infections (Hamblin et al. 2010). Topical Antibiotics are effective against the infection associated with MRSA *in vitro*; however, during infection the bacterium is localized inside the cell causing the incomplete wound healing (Singh et al. 2008). Thus, there is need of designing smart delivery vehicles that allow localized intracellular and controlled

delivery of antibiotics for preventing intracellular MRSA infections on burn wound space (Kalita et al. 2015).

Retapamulin (RP) is a hydrophobic broad spectrum topical antibiotic chemically derived from pleuromutilin, acts as bacterial protein synthesis inhibitor interacting with the 50S subunit of the bacterial ribosome. It is specially used for the treatment of methicillin resistant *Staphylococcus aureus* infected burn wound (Nalwade et al. 2014, Free et al. 2006). Conventional treatment requires twice administration for 5 days compromising patients' acceptance and compliance due to frequent and high dose administration (Cada et al. 2007). Parish et al. 2006 developed topical retapamulin ointment (1%, wt/wt) to be used twice for 5 days and compared with oral cephalexin twice daily for 10 days to treat infected dermatitis. Therefore, a reasonable need exists to develop a smart delivery system that occupies effective localization of drug and controlled release of retapamulin with extended period of time on infected burned wound space reducing frequency of application of antibiotic on burn wound site.

Various synthetic polymers are used for the preparation of drug loaded nanoparticles including Polyalkylcyanoacrylate(PACA),Poly(isobutylcyanoacrylates)(PBCA),Poly(methymethacrylates) (PMMA),Poly(hexylcyanoacrylates) (PHCA) etc (Saade et al. 2016, Stenzil et al. 2016, Samanta et al. 2012). In present study PLGA (poly lactide co glycolic acid) with 50: 50 monomers and molecular weight 17000 was taken in preparation of nanoparticle because of its biodegradability, biocompatibility and long shelf-life (Dangi et al. 2013, Dhas et al. 2015). Particulate carriers such as polymeric nanoparticles may deliver the antibiotics to the inside of the cell in a sustained manner, reducing the dosing frequency and drug toxicity (Singh et al. 2011). A variety of nanoencapsulation techniques are used for effective encapsulation of drugs (Madiha Jabeen et al. 2016). Among various nanoparticle preparation methods, nanoprecipitation method is a widely acceptable alternative method for the encapsulation of hydrophobic drugs. This method involves the dispersion of drug organic phase in a surfactant containing continuous aqueous phase. The organic solvent diffuses instantaneously to external aqueous phase to precipitate polymer. The free-flowing nanoparticle can be obtained under reduced pressure followed by complete removal of organic solvent (Abitha et al. 2015).

The 3^3 Factorial design technique is an efficient method to get the optimized best formulation manifesting the relative significance of a number of dependent and independent variables and

their interactions (Dhas et al. 2015). In the present study, we aimed to develop Retapamulin loaded PLGA nanoparticulate system for intracellular localization and controlled delivery of antibiotic in bacterium site of MRSA infected burn wound. The present work deals with the optimization of various formulation and processing parameters, such as stirring time (ST), sonication time (SNT), surfactant concentration (SC), polymer concentration (PC), drug concentration (DC) and stirring speed (SS) for the formulation of RP loaded PLGA-NP by nanoprecipitation method. The effects of the process variables on the particle size, poly dispersity index (PDI), zeta potential (ZP), drug entrapment efficiency (EE) and drug loading (L) of PLGA-NP were investigated. The PLGA-NP were subjected to physicochemical characterization by transmission electron microscopy, powder X-ray diffraction (PXRD) and Differential scanning calorimetry (DSC). Drug release studies were also performed to observe the drug release profile from the developed PLGA-NP. Antimicrobial activity was performed to evaluate the potency of encapsulated drug in PLGA-NP. Finally, stability of the developed formulation was evaluated.

2. MATERIALS AND METHODS

2.1 Materials: Retapamulin was purchased from Clearsynth Labs Ltd., Mumbai, India. Polyvinyl alcohol (PVA) and Dimethylsulphoxide (DMSO) were purchased from HiMedia Pvt. Ltd., Mumbai, India. PLGA (ratio 50:50) was kindly supplied by Evonik, degassa, Mumbai, India. The bacterial strains namely Bacillus subtilis (MTCC 441), Methicillin-resistant S. aureus (MRSA, ATCC 25923), Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853) were collected from the Central Lab for Microbiology and Biotechnology (CMBT), Bhopal. The dialyollsis membrane (Molecular weight cut off 10,000–12,000 Da) was purchased from HiMedia Laboratories (India). Purified water from ultra-pure water system (Synergy UV water purifier system, India) was used throughout the study. Other chemicals used were of analytical grade.

2.2 Method of preparation: RP loaded PLGA nanoparticles were prepared by modified nanoprecipitation method (Yadav et al. 2010, Christine et al. 2008). Briefly, RP (10 mg) and PLGA (20mg) were co-dissolved in 10 ml DMSO by sonication. The organic phase containing RP and PLGA solution was added drop wise to aqueous phase containing 1 % PVA under stirring to form homogenous emulsion for 3 hr. The solution was kept aside for 6 h to allow

evaporation of DMSO. After solvent evaporation, 5 ml of resulting NPs was taken for size and zeta potential determination, and the rest of the suspension was centrifuged at 10000 rpm for 30 minutes and washed three times by distilled water to remove unentrapped drug. The final NP pellets were collected and stored in desiccator for further studies (Yadav et al. 2010).

2.2.1 Preparation of PVA-Chitosan hydrogel

PVA-Chitosan hydrogel was prepared by freeze thaw method with slight modifications reported by Michael et al (2007) and Cascone et al (2004). In a typical experiment, 10% PVA aqueous solution was prepared by adding 10g of PVA in 100ml of hot water with stirring at 80ºC for 20 min. Aqueous solutions of chitosan(Gel), were prepared adding 2.5g of chitosan to 100ml of distilled water under stirring at about 50°C. The PVA solution was blended with the chitosan to produce Gel/PVA blends with the following weight ratios: 10/90, 20/80 and 30/70 coded as H1, H2, and H3 respectively. 10ml of solution was placed in a petridish (Internal diameter 10cm Corning) and kept for freeze-thaw cycles. The samples underwent five freeze-thawing cycles to obtain hydrogels. Each cycle, with the exception of the first one, consisted of 1h at −20ºC and 30 min at room temperature. The first cycle differed from the others due to a longer standing time (overnight) at −20ºC. The prepared hydrogels were washed with triple distilled water and the petridish was sealed with the parafilm and kept under U.V light for sterilization.

2.2.2 Incorporation of PLGA-RP-NP in hydrogels

Pure drug-containing hydrogels (PDH) were prepared by adding a calculated amount of drugs (equivalent to RP 100mg) into the hydrogel. Similarly, an accurately weighed amount of drug-loaded PLGA nanoparticles were dispersed in a PVA-Chitosan slurry, followed by homogenization at 1000 rpm for 20 min for uniform distribution . A total of 10ml of respective solution was placed in a petri dish (internal diameter 10cm, Corning) and kept for freeze-thaw cycles. The samples underwent five freeze-thawing cycles to obtain hydrogels. Each cycle, with

the exception of the first one, consisted of 1h at 20 ° C and 30 min at room temperature. The first cycle differed from the others due to a longer standing time (overnight) at 20° C.The prepared hydrogels were washed with triple distilled water and the petridish was sealed with the parafilm and kept under U.V. light for sterilization. The prepared hydrogel films were used for subsequent characterization and *in vitro* release studies.

2.3 *In vitro* characterization

2.3.1 Mean particle size and polydispersity index

The assessment of mean particle size and PDI of RP loaded PLGA-NP were done by Malvern Zetasizer ZS 90 (Malvern Instruments Inc., UK). Samples were prepared by diluting PLGA-NP dispersion with sufficient amount of ultra-pure distilled (Millipore, Billerica, MA) water prior to use of instrument.

2.3.2 Zeta potential measurement

Zeta potential (ZP) reflects the electric charge on the surface of individual particle. It is used to study the physical stability of suspension (Patel et al. 2014). ZP was determined using Malvern Zetasizer Nano ZS (Malvern Instruments Inc., UK). The Helmoltz–Smoluchowski equation was used for the Calculation of zeta potential:

$$\xi = \frac{EM \times 4\pi\pi\eta}{\varepsilon}$$

Where, ξ, EM, η and ε denotes zeta potential, electrophoretic mobility, viscosity of the dispersion medium and the dielectric constant. Prior to the use of instrument for the measurment, all samples were diluted using ultra-pure distilled water.

2.3.3 Percent entrapment efficiency and drug loading

The percent entrapped drug was calculated from sedimented PLGA-NPs dispersion prepared by centrifuging the sample at 12,000 rpm, 4°C for 15 min and the supernatant was decanted and fixed volumes of supernatant were diluted in methanol and analyzed for unentrapped drug using UV spectrophotometer at 243.6 nm (PerkinElmer, Lambda 25, UK). % EE and % DL was calculated using the following formula.

$$\%\ EE = \frac{\text{Weight of retapamulin used (Total drug used)} - \text{Weight of free retapamulin (Free unentrapped drug)}}{\text{Weight of retapamulin used (Amount of drug used in preparation)}} \times 100$$

$$\%\ DL = \frac{\text{Weight of retapamulin used (Amount of drug used in preparation)}}{\text{Weight of polymers (Amount of polymer used in preparation)}} \times 100$$

2.3.4 Transmission electron microscopy

The size and shape of the prepared RP loaded PLGA-NPs was evaluated using Transmission Electron Microscopy (model TECNAI 200 KV TEM-Fei, Electron Optics, Japan). The diluted sample was taken on the surface of the carbon coated copper grid and stained by 1% (w/w) phosphotungstic acid (Aqueous solution) for 30s. Rest of staining solution was washed out using filter paper. The stained samples were dried leaving a thin aqueous film on the surface at room temperature for 10 min to carry out investigation (Rawat et al. 2007).

2.4 *In vitro* release study

In vitro release studies of RP loaded PLGA-NPs and plain RP suspension was performed using a modified Franz diffusion cell as reported by Khurana et al. (2013a). A dialysis membrane (molecular weight cut-off of 10,000–12,000 Da) was set between donor and acceptor compartment assembly. The membrane was pretreated in double-distilled water for about 12 h prior to the experiment. Phosphate buffer (pH 7.4) was used as the diffusion medium. *In-vitro* modified assembly was placed on a magnetic stirrer with stirring speed of 300 rpm (±) at 37.0±0.5°C. The samples (plain RP suspension and RP loaded PLGA-NPs dispersion) containing 2 mg drug were kept in the donor compartment holding dialysis membrane and magnetically stirred. Samples were withdrawn at specific intervals (0, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h) from the receptor compartment, filtered for the evaluation and the volume was replaced with the same quantity of fresh Phosphate buffer (pH 7.4). The samples were taken to analyse spectrophotometrically at 243.5 nm after appropriate dilutions. The % cumulative drug release was calculated with the help of standard calibration curve of Retapamulin pure drug and graph of % cumulative drug release verses time was plotted. Release studies were performed three times for each formulation.

2.5 XRD analysis

Sample was characterized for XRD analysis to study crystalline or amorphous nature of the RP loaded PLGA-NPs. X-ray powder diffraction studies of pure RP, PLGA , physical mixture of RP and PLGA equivalent to ratio used in formulation and RP loaded PLGA-NPs were carried out using powder X-ray diffractometer (PANalytical 3 kW X'pert Powder, UK). Samples were placed on the sample stage and scanned with an operating at 40 kW with 30 mA over the 2 h range of 5° to 60° degrees 2-theta to measure the XRD pattern of each sample.

2.6 DSC Analysis

DSC studies of pure RP, PLGA (50:50), PVA and physical mixture of RP and PLGA (at a ratio used equivalent to that in the formulation) and optimized formulation (NP6) of RP-PLGA-NPs powder were carried out. The minimum quantity of samples were accurately weighed and carefully placed in aluminum pan without further treatment and heating temperature were recorded in the range of heat conduction between 20–350°C at a heating rate of 20°C/min under a constant flow of sweeping dry nitrogen gas. The study was carried out using Differential Scanning Calorimeter (Perkin Elmer Jade, CA) equipped with a computerized data station TA-50WS/PC. Thermograms results obtained were further studied for evidence of Drug- excipient interactions.

2.7 Antimicrobial assay

2.7.1 Collection and maintenance of Microbial culture

The strains were collected from the Central Lab for Microbiology and Biotechnology (CMBT), Bhopal. The bacterial strains such as *MRSA, B.Subtilis, P.aerugenosa and E.coli* were inoculated in a nutrient broth at 37^0C for 24 hour in incubator. The 36g of Muller Hinton agar (Himedia) was mixed with distilled water and then stabilized in autoclave at 15lbs pressure for 15 min. The sterilized media was poured into Petri dishes; the solidified plates were bored with 5mm diameter cork bearer. The plates with wells were used for the antimicrobial studies. The Retapamulin were tested against the *MRSA, B.Subtilis, P.aerugenosa and E.coli* for antimicrobial activity. Wells of equal size were cut and the antibiotic was added into it for positive control; respective solvents acting as a negative control. The plates were incubated at 37^0 C, overnight.

2.7.2 Antibacterial sensitivity

The antibacterial activity of retapamulin was determined by well diffusion method. Plates were prepared by pouring sterile Mueller Hinton agar (Himedia) into sterile petri dishes that were previously autoclaved. Sterilized cotton swabs were dipped in the bacterial culture in nutrient broth and then swabbed on the agar plates. Wells of equal size were cut with proper gaps in the medium and the samples were added into it. Then the plates were incubated at 37°C and observed for zones of growth inhibition after 24 hours.

The antibacterial activity of retapamulin loaded PLGA nanoparticle and free retapamulin was examined against four bacterial strains namely Bacillus subtilis (MTCC 441), Methicillin-resistant S. aureus (MRSA, ATCC 25923), Escherichia coli (ATCC 25922) and Pseudomonas

aeruginosa (ATCC 27853). All the cultures were sub-cultured on nutrient agar. The inoculum size of each strain was standardized to 10^4 bacterial cells/ ml for each test organism by adjusting the optical density of the bacterial suspension to a turbidity corresponding to spectrophotometric absorbance of 0.05 at 600 nm. The antimicrobial activity of the retapamulin loaded PLGA nanoparticle and free retapamulin was evaluated using well diffusion method, sustained antimicrobial activity of PLGA-RP-NP was determined by counting the colony forming units (CFU). At a time interval of 24 h and 72 h, 0.1 ml of the sample were withdrawn for diluting two fold with normal saline. From the diluted sample, 0.1 mL was taken and spread on Mueller–Hinton agar plates with incubation time for 24 h and 72 h at 35 ± 2^0C. Bacterial colony forming units were counted in order to evaluate the sustained antibacterial activity.

2.8 Stability studies

The accelerated stability studies of the optimized RP-PLGA-NPs were carried out for 3 months as per ICH Guidelines (Q1AR2) at temperatures of 25±2^0 C and 60 ±5 % RH. The formulations were sealed to 5 ml glass vials using plastic caps and were kept in stability chamber with temperature of 25±2°C and RH 60%±5%. The formulations were monitored for changes in in the particle size, ZP, PI, EE and DL (Jain et al.2010).

3.*In vivo studies*

An infected burn wound model was selected to evaluate the performance of the delivery system. An studies were conducted according to the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA) Government of India and permission was taken from animal ethical cornittee (Approval no. 13334/PG/16/CPCSEA). The Experiments were carried out on random albino rat maintained in the animal house of Patel College Of Pharmacy **,**Bhopal, M.P. The animals were transferred to the laboratory at least 1h before the start of the experiment. Animals were acclimatized to laboratory conditions before testing. Each animal were used once. The colony was maintained under controlled conditions of light (12:12, light and dark,. temperature (22±3^0C) and humidity (50±10%). The animals were housed in sterile polypropylene cages containing autoclaved paddy husk as bedding material. They had free access to standard autoclaved mice food and to filtered acidified water. Healthy, 8-10 weeks old male albino rat, weight 120 ± 5g were used for experiments.

The selected animals (Albino rats) were divided no.five groups of six animals to each group. Group 1 (control group) consisted of animals not treated topically with any formulation. Group II consisted of animals treated with PLGA nanoparticle of RP. Group III consisted of animals

treated topically with prepared PVA –Chitosan hydrogel. Group IV- consisted of animals treated topically with formulation (Multiphase hydrogel containing PLGA nanoparticle of RP). Group V- consisted of animals treated topically with marketed product of retapamulin (Retarel 1 % cream). Percentage wound contraction was measured using the formula.

$$\text{Wound contraction\%} = \frac{\text{initial wound size} - \text{specific day wound size}}{\text{Initial wound size}} \times 100$$

3.1 Histological analysis

For histological analysis, On days 0,4, 8, 12, and 16, three animals from each group were sacrificed with anesthesia overdose to collect the granulation/healing tissue.The harvested samples were fixed in 4% formaldehyde in PBS at 4^0C, dehydrated in a graded series of ethanol, and then embedded in paraffin for routine haematoxylin–eosin (H&E) staining and Masson's trichrome staining for collagen fibres. H&E- and Masson's-trichrome-stained sections were observed with a light microscope (Sung et al, 2010, scott et al, 1965).

4 Results and discussion

RP loaded PLGA-NP were prepared by the modified nanoprecipitation method varying different parameters as given in Table 1. These parameters were evaluated in terms of the particle size, ZP, PI, EE and DL. Based on *in vitro* characterization, the final batch was prepared with desirable properties (selected batch).

4.1 Effects of studied variables

The modified nanoprecipitation method was found to be efficient and quick to produce PLGA-NP. Influence of different variables on particle size, PDI, ZP, EE and DL are discussed in the following sections and presented in Table 2.

4.1.1 Stirring time (ST)

ST did not show any significant influence on the particle size. Further it is an intermediate step for emulsification of organic phase containing drug and polymer in the aqueous phase with surfactant. Generally, ZP value correlates to the degree of repulsion betweent close and similarly charged particles in the PLGA-NPs dispersion with prediction of physical stability of the dispersion (Lason et al. 2013). High ZP (negative or positive) indicates that highly charged

particles produce stable nanoparticle dispersion causing prevention of aggregation of the particles due to electric repulsion. In this case, highest ZP (-16±0.24 mV) was observed in the formulation with stirring time of 2 hr. EE was observed minimum and maximum in case of 0.50 hr ST and 2hr ST respectively, which might be due to better dispersion of nanoparticles in 2 hr as compared to poor dispersion of nanoparticles at 0.5 hr ST. However, with 3 hr ST the highest PDI and particle size was achieved due to development of charge on the surface of nanoparticles leading to aggregation of smaller nanoparticles into larger sized particles. Based on the the *in vitro* results of the particle size, PDI, zeta potential and % DL, stirring time for 2 hr was selected as optimum ST for RP loaded PLGA-NP formulation.

4.1.2 Surfactant concentration (SC)

Concentration of the surfactant in formulation had significant influence on the particle size and PDI. The particle size decreased on increasing surfactant concentration (SC) from 0.5% to 1.5% w/v due to better stabilization of internal structure of dispersion preventing coalescence at higher surfactant concentration. PDI decreased on increasing SC up to 1%w/v. These observations might be due to production and stabilization of smaller polymeric droplets at higher SC as enough surfactant was present to stabilize the nanodroplets. However, in this case PDI was higher surprisingly at 1.5 % w/v SC. According to the literature published, the nanoparticles having PDI lower than 0.25 are considered relatively homogeneous, with minimum tendency for aggregation (Mitri et al. 2011). SC at 1% w/v and 1.5 %w/v showed ZP value of -13±0.11 mV and -11±2.05 mV, respectively, which predicted poor physical stability of the PLGA-NP dispersions. In our observation, EE and DL increased with increase in concentration of SC. This could be due to the presence of sufficient SC which helped the drug to remain within the polymer particles and/or on the surface of the particles. Low concentration of RP in PLGA-NP and high compatibility between the drug and the polymer further contribute to high EE. Dhas et al. (2015) also reported similar results for delivery of bicalutamide loaded folic acid conjugated-chitosan functionalized PLGA nanoparticle (Dhas et al. 2015). They demonstrated decrease in the particle size and increase in % EE with increase in the concentration of surfactant. Based on the results of investigation 1% w/v SC, which produced particles with optimum particle size, PDI, zeta potential, EE and % DL was chosen as the optimum SC required for preparation of RP loaded nanoparticles.

4.1.3 Polymer concentration (PC)

The particle size significantly increased with increasing polymer concentration (PC). This may be correlated to better distribution of sonication energy in the dilute (low PC) dispersion as compared to the concentrated dispersion, which was responsible for more efficient particle size reduction at lower PC. Numerous studies have reported that increasing polymer content results in larger particles and broader particle size distribution (Trotta et al. 2001, Subedi et al. 2009). In this case, the particle size was lowest in case of 1% w/v PC with very high PDI (0.505) and highest at 10% w/v PC with 0.306 PDI. In all PCs, ZP was more than -11 mV. As expected, EE increased with increasing PC. This might be due to availability of higher amount of polymer for drug encapsulation at high PC which led to higher EE. Opposite tendency was noticed for low PC. In contrary, % DL significantly decreased with increasing PC. DC remained same in spite of increase in PC as it led to reduction of drug to polymer ratio (i.e., loading) with increase in PC. This observation was in accordance with Hao et al. (2012). They developed baicalin loaded solid lipid nanoparticles and reported positive influence of high lipid concentrations on EE. By this observation, 2% w/v PC was selected to achieve best results because with 2%w/v PC a poorly stable system with high PDI was observed whereas at higher PC of 5%w/v and 10%w/v very large sized particles with lower % DL were observed. However, with PC of 2%w/v optimum particle size, PDI, zeta potential, higher EE and % DL were observed.

4.1.4 Sonication time (SNT)

Sonication time (SNT) showed marked influence on the particle size and PDI. The particle size and PDI significantly decreased with increasing SNT. These observations are reasonable as sonication was responsible for the final particle size of PLGA-NPs, which breaks the coarse drops to nano droplets. Longer sonication time puts more sonication energy to the PLGA-NPs dispersions, which reduced size of the nano droplets and decreased size distribution. This observation was in accordance with sahana et al (2008). They prepared PLGA nanoparticle for hydrophobic drug and reported that on increasing SNT reduced both the particle size and PDI , but with further increase in SNT Particle size increased due to higher surface free energy of particles causing them to clump larging size of NPs. In the present study, we observed ZPs to be -18.1±0.61mV and -17.4±0.8 mV for 15 min and 20 min SNT, respectively, but for 10 min

sonication time ZP was -18.2±0.17 mV indicating a stable system at 10 min sonication time. SNT of 5 min produced particles with comparatively lowest EE. Otherwise, EE and % DL were not significantly different among the groups. Samein also reported less impact of sonication time on EE of nystatin loaded SLN prepared using glyceryl monostearate as the lipid constituent (Samein et al. 2014). Based on the observation of the particle size and stability aspect, 10 min SNT was chosen as the optimum value for preparation of RP loaded nanoparticles as with SNT less than 10 min (5 min SNT) very large particle size was observed and with SNT more than 10 min (15 min and 20 min SNT) poor stability of formulation was observed.

4.1.5 Drug concentration

There were remarkable differences in particle size among different batches prepared with drug concentrations (DC) from 2.5 mg to 15 mg. PDI also increased with higher DC. This observation can be correlated to the presence of high amount of drug. ZPs were observed to be more than 14.1 ± 0.1 mV in all cases, which suggests good stability of the PLGA-NP dispersions. EE decreased with increasing DC whereas % DL increased with increase in DC upto 15 mg. EE directly depends on the amount of drug added pertaining to the entrapment capacity of the polymer used (Patil et al. 2014). As the polymer has a certain drug loading capacity, addition of excess drug led to increase of the unencapsulated drug (i.e. decrease of EE). Similarly, % DL cannot increase beyond maximum drug loading capacity signifying that loading has reached its maximum concentration of 10 mg DC. But at 10 mg DC the highest particle size with high PDI was observed indicating a poor stability of formulation. Therefore, on the basis of the particle size, PDI, EE and % DL, DC with 10 mg was selected for the preparation of the final drug loaded PLGA-NP formulation. Similar observation was observed by Das et al. (2012).

4.1.6 Stirring speed

In this study, increase in the stirring speed led to decrease in the particle size but above 3000 rpm there was slight reduction in the mean particle size. Moreover, effect of the stirring speed was found insignificant in case of EE and DL. Similar result was reported by Kharia and Singhai

They reported a decrease of the nanoparticle mean size with an increase of the strirring speed. But above 3000 rpm, there was no significant reduction of the particle size (Kharia and Singhai 2013). In our study, 3000 rpm was selected for further preparation of drug loaded PLGA-NP.Therefore, the final formulation was prepared using 10 mg DC, 2% w/v PC, 1% w/v SC, 3hr ST, 10 min SNT and 3000 rpm SS as final values of variables.

4.2 Mean particle size and polydispersity index

The mean particle size of RP loaded PLGA-NP were found to be in the range of 94.32± 1.92 nm to 308.21 ± 1.03 nm with PDI of 0.505 to 0.306. However, the mean particle size for the selected batch was obtained to be 110.5 ±1.22 nm with PDI of 0.025 (Table 2).

4.3 Zeta potential

The zeta potential of drug loaded PLGA-NPs were found to be in the range of -11±0.53 mV to -19.8±0.31 mV. The zeta potential of the selected batch was obtained to be - 20.8 ± 0.21 mV (Figure2b). The zeta potential value of the selected batch indicated the stable formulation (Table 3).

4.4 Percent entrapment efficiency and loading

% EE of the prepared drug loaded PLGA-NP was found to be in the range of 61.02±0.11 to 82.20±1.10. For the selected batch % EE and % DL was obtained to be 82.31 ± 0.12 and 2.21 ± 0.042. High entrapment can be attributed to the lipophilic nature of the drug having higher affinity for the selected polymer matrix (Table 3).

4.5 Transmission electron microscopy

Shape and size of the selected batch of nanoparticles were evaluated by TEM. TEM images of the PLGA-NP confirmed that the particles were nearly spherical or oval in shape with narrow size distribution and were non-aggregated (Figure 1). The diameters of the particles observed were in good agreement with the data obtained from the Malvern particle size analyzer.

4.6 X-ray diffraction study

XRD was performed to assess the state of encapsulated drug and to confirm whether it was dispersed in microcrystalline form, without polymorphic change or transition change in amorphous form. Pure RP showed a sharp diffraction peak at 9.6, 12.8, 13.9 and about 19.6±0.2 degrees 2° [Figure 2 (A)]. In the physical mixture, the crystalline peak for RP was clearly evident [Figure 2 (c)], whereas the PLGA-NP formulation showed a suppressed peak for RP, indicating the solubility of drug in the polymer solution and higher polymer concentration with relative reduction in the diffraction intensities. The peaks of PLGA-NP formulation were found to be similar to pure polymer peaks, indicating that the drug was completely entrapped within the polymer matrix mixing with polymer. This could be further predicted due to reduction in the crystallinity of the precipitated RP, and this change in diffraction pattern supports the enhancement of solubility of the drug [Figure (2 D].

4.7 Differential scanning calorimetry

DSC is a tool to investigate the drug–excipient interaction in the formulation (Sah et al. 2015). The DSC thermogram was obtained by running samples of pure drug RP, PLGA (50:50), physical mixture of RP and PLGA at 1:1 ratio and RP loaded PLGA NPs (Figure 3).

The melting point of RP was observed in the range of 121–124^{0}C which indicated crystalline amorphous nature of the drug. Pure RP showed a sharp endothermic peak at 121°C corresponding to its melting point, indicating its characteristic crystalline nature. The DSC thermogram presented the glass transition temperature of PLGA (50:50) as 50^{0}C. PLGA exhibits less intense and broader peaks at the position of endothermic event, ascribing to the melting, around 50°C. These broader melting endothermic peaks of bulk Polymer indicate that the starting materials were crystalline. There were less intense and broader peaks at the position of endothermic peaks with physical mixture of RP–PLGA-NPs in the ratio of 1:1 and showed peak point at 121^{0}C. Thus, no chemical interaction was evident between RP and PLGA. The peak for RP was completely absent in RP loaded PLGA-NPs while it was clearly evident in the physical mixture of RP (121.54°C) and PLGA (50°C) as shown in Figure 3c. It has been reported that when the drug does not show its endothermic peak in the nanoparticulate formulations, it is said to be in the amorphous state (Liu et al. 2005). Hence, it could be concluded that the drug was

present in the amorphous phase and entrapped in polymeric matrix or may have been homogeneously dispersed in the PLGA-NPs. It indicated stability of the nanosystem.

4.8 *In vitro* release study

The cumulative percentage release of RP from RP suspension and RP loaded PLGA-NP were investigated *in vitro* over a period of 12 h. Each sample was analysed in triplicate and release curves are shown in Figure 4. It was indicated that plain RP suspension released almost 96.72 ± 5.46% of the drug at the end of 7 h, while in RP loaded PLGA-NP 67.19 ± 3.23% drug release was observed after 24 h. RP loaded PLGA-NP showed a biphasic drug release pattern with an initial burst release phase followed by a sustained drug release phase. RP loaded PLGA-NP suspension depicted a biphasic release pattern characterized by relatively faster initial burst release followed by comparatively slower release with 67.19 ± 3.23% of RP released at 24 h. Burst release can be useful to improve the penetration of the drug, while sustained release supplies the drug for a prolonged period of time (Gupta et al. 2013). On the other hand, the drug release from plain RP suspension was faster with 96.72 ± 5.46% release of RP within 7 h. Regarding the drug release profiles, RP loaded PLGA-NP suspension followed Higuchi release kinetics (r2 = 0.9909) and zero order release kinetics (r2 = 0.994), respectively. Similar results for novel and conventional formulation were obtained by Khurana et al. (2013b).

5 *In vivo* studies

In vivo performances of the prepared optimized formulations were evaluated on infected burn wound model on Albino rat. Wound contraction, epithelization time, and histopathological study were selected as parameters to evaluate the prepared delivery systems.

5.1 *Wound contraction*

Wound contraction was analyzed in each group as a percentage of the reduction in wounded area at days 4, 8, 12 and 16 [Table 4]. In this study, the Hydrogel containing PLGA-RP-NP treated animals completely healed the wound of animals on 16 days. The marketed formulation-treated animals showed complete wound contraction on 20 days. Hydrogel-PLGA-RP-NP showed complete contraction on the day of 14^{th}(……..) Among the treated groups, animals treated with formulation optimized Hydrogel-PLGA-RP-NP showed more significant ($P < 0.001$) wound contraction than animals having treatment with other formulation. The mean percentage of wound contraction of the optimized Hydrogel-PLGA-RP-NP treated group was significantly

(99.96% at day 14, $P < 0.001$) higher compared with those of the control (97.56% at day 14, $P < 0.01$) and marketed formulation-treated (99.98% at day 14, $P < 0.01$) groups after wound creation.

5.2 *Histopathological observations*

Histological studies play significant role in investigating the mechanism of the wound healing. As result revealed that Figure 8 exhibited that healing of burn wound was due to beginning of remodeling of skin, treated with optimized Hydrogel-PLGA-RP-NP after 12 days. Figure 8,9 indicates albino rate treated with marketed formulation after 16 days. Moreover, the collagen was noticeably seen in control groups.

Histopathological studies conceived that accumulation of keratinocytes in the basal lamina of epidermis. The whole *in vivo* outcomes suggested that the animals treated with optimized Hydrogel-PLGA-RP-NP exhibited higher burn wound healing activity compared to control and marketed formulation treated group of animals.

6 Antimicrobial assay

Antibacterial activity was examined against four pathogenic strains, which are more prevalent in infected burn wound. The retapamulin loaded PLGA nanoparticle has been found to be most active against Gram-positive bacteria compared to Gram-negative strain. The developed optimized formulation of PLGA nanoparticle (NP6) exhibited efficient antimicrobial activity in sustained manner over the period of 72 h. The test material showed lesser CFU (colony forming unit) count at 72 h compared to 24 h against all the tested wound pathogens indicating the prolonged antibacterial activity (Figure 5, 6, 7). The retapamulin loaded PLGA nanoparticle had shown excellent activity against MRSA strain which further emphasizes the therapeutic benefit in treating infected burn wounds. Overall, PLGA nanoparticle containing retapamulin exhibited significant activity against MRSA and *B. subtilis*, moderate activity against *E. coli* and non-significant against *P. aeruginosa.*

7. Stability study

The result of accelerated stability studies of optimized RP-PLGA-NPs is shown in Table 3. Nanoparticle formulations increase the surface area by many folds and this may lead to very high aggregation of particles after 90 days of storage (Pignatello et al. 2002). Thus the particle sizes incresead from 112.4±1.02 to 128.5±0.16 upon 90 days storage. In general, zetapotential of more than +30mV or less than −30mV is considered as a standard value in providing enough

repulsion forces to avoid particle aggregation (Qi et al. 2004). The zeta potential (*z*- average) of formulation changing from -13.8± 0.61 to -15.1± 0.24 upon 90 days storage confirms the stability after storage for 3 month. Percent change in DL and EE were 2.71 ± 0.012 to 2.07± 0.049 and from 80.21 ± 1.32 to 78.11±1.41 respectively, on 3 months storage of PLGA-NP formulations. The change in particle size and the EE were assessed as the prime parameters. There was a alteration in size distribution with a slight increment as compared with initial particle size (Pignatello et al. 2002). Thus the RP loaded PLGA-NP showed minor enhancement of the particle size and PI with slight reduction of ZP, EE, and DL after 3 month storage at accelerated conditions of temperature (25±2°C) and relative humidity (60±5 %)

(Table 3). The changes found on results of selected *invitro* parameter were insignificant which indicate good physical stability of the PLGA-NP during their storage at accelerated conditions of temperature (25±2°C) and relative humidity (60±5 %) .

8. Conclusion

In this study, PLGA-NPs were successfully prepared by the nanoprecipitation method. Highly lipophilic drug RP was efficiently entrapped into the polymeric matrix of PLGA nanoparticles which were further confirmed by TEM study. Most of the process variables such as polymer concentration, surfactant concentration, drug concentration and sonication time showed significant effect on the chosen responses. The particle size remarkably increased on increasing the concentration of polymer and drug while opposite result was observed on increasing the sonication time. The particle size of the selected batch was found to be 114.5 ± 1.32 nm. Stirring time significantly influenced entrapment efficiency while there was no significant influence on the particle size on increasing stirring time. The drug release study exhibited prolonged release from the PLGA-NP following Higuchi release kinetics (R^2=0.9909) as compared with pure drug suspension (Zero order kinetics, R^2=0.994). After 3 months of storage in accelerated conditions, RP-PLGA-NP were found to be stable at 25 ±2 ° C and 60±5 % RH. The antimicrobial study was performed against some gram positive strain and gram negative strain confirmed that PLGA-NP preserves the potency of antimicrobial action of encapsulated drug.

On the grounds of improved patient compliance and efficacy due to reduced frequency of application, Hydrogel containing Antibiotic loaded PLGA nanoparticle-based formulations will

also have significantly better role in treatment of infected burn wound. The results obtained in *in vivo* studies, could propose this system for future especially for delivery of other antibiotic, useful in treatment of various infected burn wounds.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

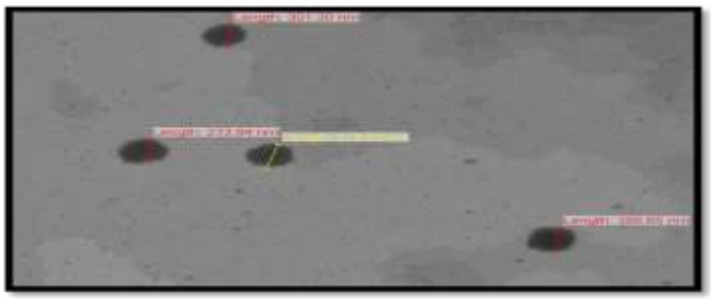


Figure 1: TEM image of the selected batch of RP loaded PLGA nanoparticle

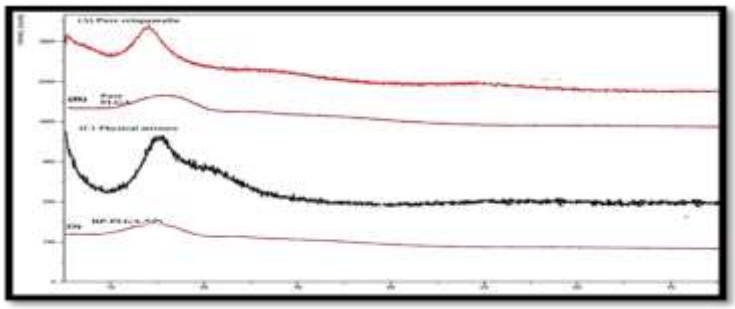


Figure 2: XRD Analysis (a) Pure drug (b) Pure PLGA (c) Physical mixture of pure drug,PLGA and PVA (d) RP loaded nanoparticle

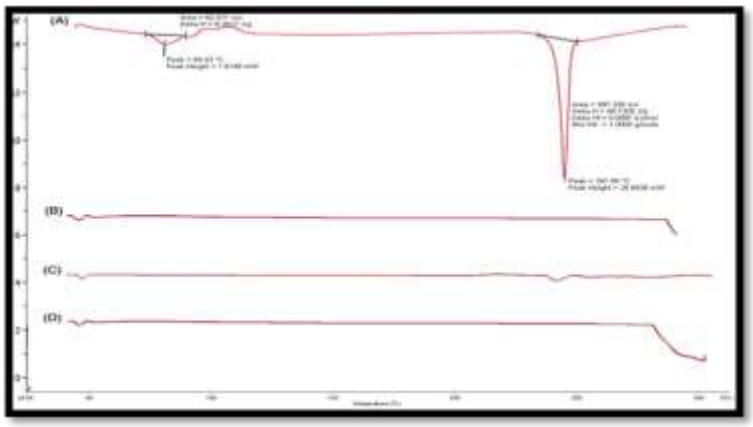


Figure 3: DSC Analysis (a) Pure drug (b) Pure PLGA (c) Physical mixture of pure drug, PLGA and PVA (d) RP loaded nanoparticle

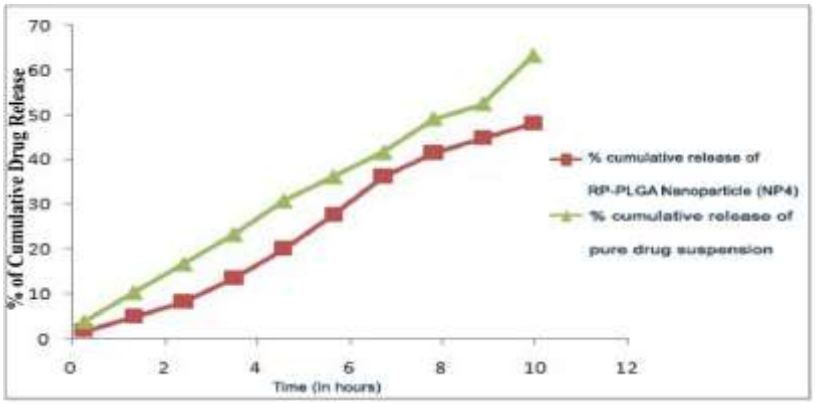


Figure 4: Percent cumulative release of RP from PLGA Nanoparticle and pure drug suspension

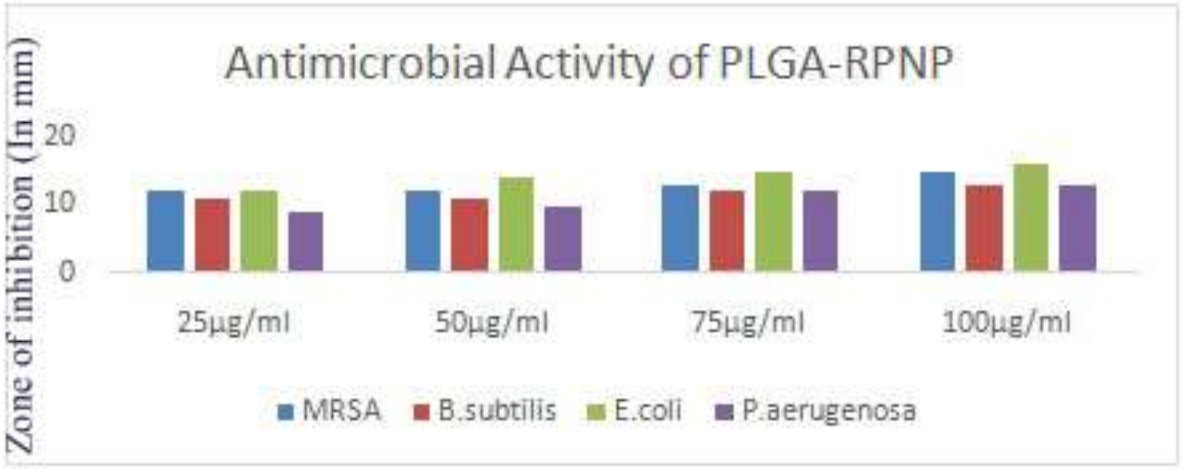


Figure 5: Antimicrobial activity of Retapamulin loaded PLGA nanoparticle

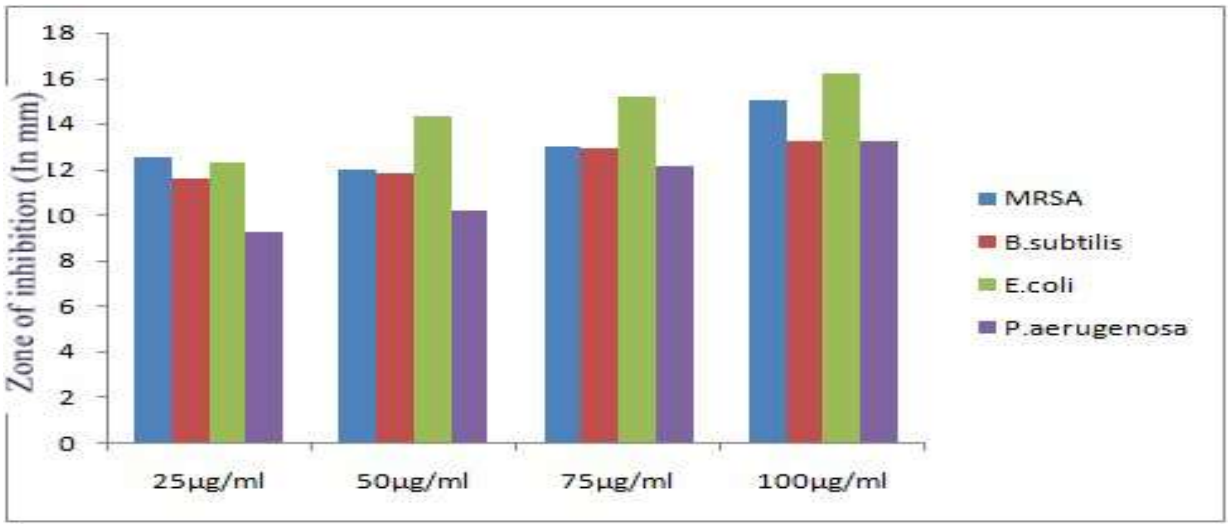
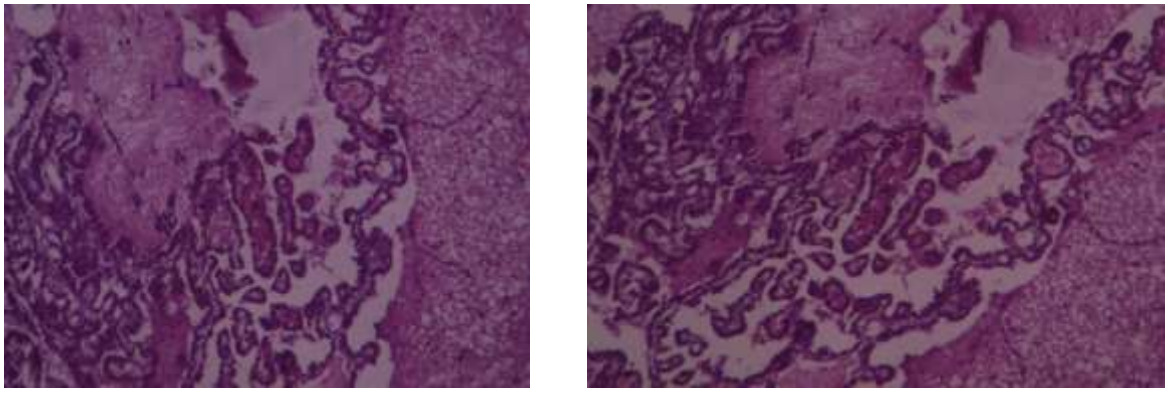


Figure 6: Antimicrobial activity of Pure Retapamulin

Figure 7: The zone of inhibition of different concentration of Retapamulin on cultured E.coli, MRSA using agar plate a) and b)

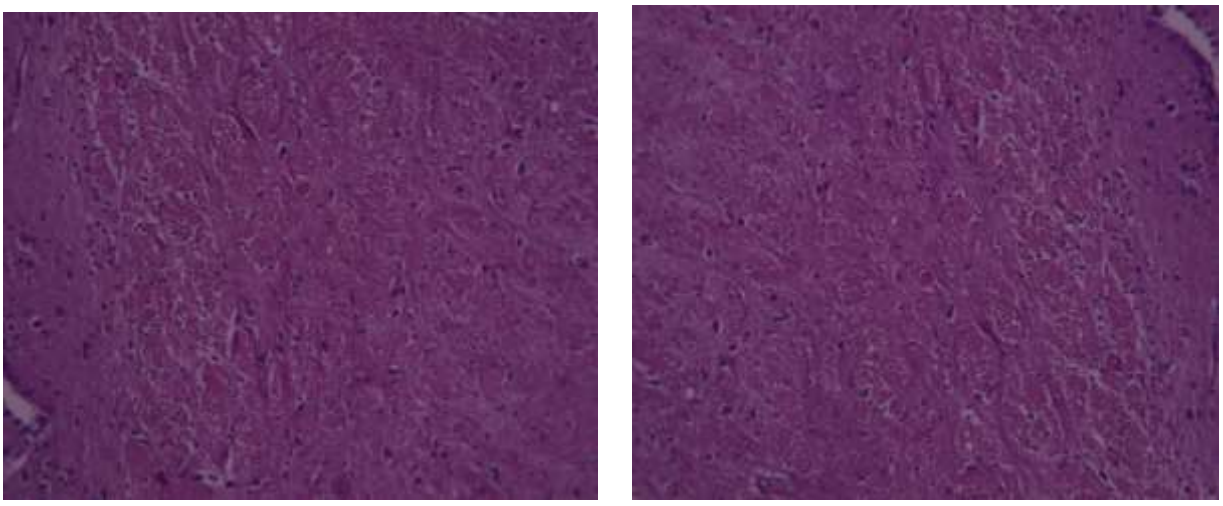
(a) **(b)**

Figure 8: Photomicrograph of albino rate skin on zero day (a) and 14th day (b) after post wounding with hydrogel treated (Without incorporated PLGA-RP-NP) in burn model, stained with Hematoxylin and eosin at 40X (C-Collagen; ed-Epidermis; bv-blood vesseels).



Photograph 8 (a) TS of skin at Zero day **Photograph 8 (b) TS of skin at 14th day**

Figure 9 Photomicrograph of albino rate skin on zero day (a) and 14th day (b) after post wounding with hydrogel treated (Multiphase hydrogel containing PLGA nanoparticle of RP) in burn model, stained with Hematoxylin and eosin at 40X (C-Collagen; ed-Epidermis; bv-blood vesseels).



Photograph 9(a) TS of skin at Zero day Photograph 9 (b) TS of skin at 14th day

Table no.1 Formulation design for PLGA-NP

Parameters						
S.no.	Stirring Time (min/hr)	Surfactant concentration (%w/v)	Polymer Concentration (%w/v)	Sonication time(min.)	Drug concentration (mg)	Stirring speed (rpm)
1	30min,1 hr.,1.5 hr.,2hr	1	2	10	5	3000
2	2 hr	0.5, 1 and 1.5	2	10	5	3000
3	2 hr	1	1,2,5 and 10	10	5	3000
4	2 hr	1	2	5,10,15 and 20	5	3000
5	2 hr	1	2	10	2.5, 5,10 and 15	3000
6	2 hr	1	2	10	5	2000,3000 and 4000

Table no.2 Size, PDI, ZP, EE and DL of the drug loaded PLGA-nanoparticle (data represent mean ±SD)

Variable	Particle size	PDI	ZP (-mV)	EE (%)	DL (%)
Stirring time (hr)					
0.30 hr	119.9±0.021	0.211	-11±0.53	72.20±1.39	2.925±0.011
1 hr	111.53±0.156	0.210	-12±0.37	74.34± 1.31	2.90±0.142
1.5hr	115.34±0.213	0.213	-13±0.78	75.21±2.23	3.0±0.123
2hr	124.51±0.114	0.540	-16±0.24	77.28±1.10	3.02±0.146
Surfactant concentration (%w/v)					
0.5	210.2±1.120	0.259	-12±1.34	77.23±1.21	2.950±0.016
1	111.2±2.010	0.211	-13±0.11	79.23±2.21	3.01±1.134
1.5	103.4±1.123	0.434	-11±2.05	82.20±1.10	3.81±0.076
Polymer concentration (%w/v)					
1	94.32±0.11	0.505	-11.2±0.11	61.02±0.11	3.0±0.023
2	106.22±0.131	0.205	-17.2±0.21	76.26±0.11	2.2±0.023
5	221.38±2.10	0.218	-19.1±0.81	78.15±1.07	0.90±0.012
10	388.41±1.13	0.316	-19.8±0.31	81.12±2.12	0.73±0.012
Sonication time (Min.)					
5	208.31±2.14	0. 601	-19.1±0.16	66.21±1.03	2.72±0.021
10	106.32±0.231	0.205	-14.2±0.17	78.16±0.11	3.0±0.013
15	100.54±0.29	0.201	-18.1±0.61	80.06±1. 72	3.02±0.014
20	90.61±2.82	0.210	-17.4±0.8	81.52±0.39	3.05±0.026
Drug concentration (% w/v)					
2.5	92.01±1.02	0.035	-14.1±0.1	80.11±1.19	2.02±0.012
5	100.36±2.76	0.086	-15.3±11	80.16±0.11	3.0±0.013
10	135.25±2.73	0.289	-15.1±0.26	72.28±3.54	4.08±0.029
15	204.11±0.70	0.223	-17.2±0.23	60.19±1.18	4.00±0.013
Stirring speed (rpm)					
2000	124.11± 0.65	0.303	-13. 1 ± 29	81.42 ± 1.56	3.02 ± 0.015
3000	107.11± 1.23	0.208	-14.8 ± 51	80.35 ± 2.13	3.02 ± 0.011
4000	105.41± 1.25	0.217	-15.8± 0.26	79.02± 2.24	3.0 ± 0.0

Table 3 Results of stability study conducted on the RP-PLGA-NPs for 90 days at 25 ± 2° C and 60 ± 5% RH

Time (Days)	Particle size(nm)	PDI	ZP (-mV)	EE (%)	%DL
0	112.4±1.02	0.028	-13.8± 0.61	80.21 ± 1.32	2.71 ± 0.012
30	118.3±2.12	0.039	13.78±0.67	80.10±2.12	2.40± 0.013
60	124.5±3.12	0.189	14.45±0.23	79.13±3.12	2.14±0.010
90	128.5±0.16	0.201	-15.1± 0.24	78.11 ± 1.41	2.07± 0.049

Table 4 Effect of formulation and standard preparation on % wound contraction and epithelization period on infected burn wound model in albino rat

Group	0 Days	4 Days	8 Days	12 Days	14 Days	16 Days	Epithelization period
Group 1	0%	±37.38%	39.49%	46.83%	49.56%	49.81%	26
Group 2	0%	50.48%	84.49%	97.42%	99.88 %	99.95%	15
Group 3	0%	52.26%	85.93%	98.93%	99.96%	100%	16
Group 4	0%	50.53%	82.81%	96.10%	97.55%	98.77%	20

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