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RESEARCH PAPER

# Quality by design enabled formulation development of regorafenib monohydrate loaded PEGylated PLGA polymeric nanoparticles: Enhanced oral bioavailability and biopharmaceutical attributes

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## ABSTRACT

***Objective(s):*** Using a quality-by-design methodology, the current research is aimed to prepare and enhance the PEGylated PLGA-loaded regorafenib monohydrate polymeric nanoparticles for enhancing oral bioavailability and biopharmaceutical attributes. The oral multi-kinase inhibitor inhibits VEGFR2-TIE2 tyrosine kinases on two separate targets, which results in anti-angiogenic activity. It also inhibits stromal and oncogenic receptor tyrosine kinases.

***Materials and Methods:*** The current study developed nanosized, biocompatible, and PEGylated PLGA polymeric nanoparticles to administer regorafenib monohydrate to patients with metastatic colon cancer. This was accomplished using a modified nanoprecipitation technique to make drug-encapsulated PEGylated PLGA nanoparticles with poloxamer 188 as a stabilizer.

***Results:*** The polymeric nanoformulations were characterized for zeta potential, distribution of particle size, entrapment efficiency, DSC, FT-IR, X-RD, and SEM. Both *in vitro* and *in vivo* experimental studies were performed for the pure drug and the improved nanoparticle formulation.

***Conclusion:*** The nanoparticles obtained from optimization studies were found to have smaller particle sizes, higher entrapment efficiency (%), drug loading capacity, spherical shape particles, amorphous drug embedded matrix, and a biphasic delayed release pattern. These findings suggest that drug-loaded PEGylated PLGA nanoparticles are a potent formulation for the treatment of colon cancer, with improved oral bioavailability and biopharmaceutical properties.

***Keywords:*** *Bioavailability potential, Polymeric nanoparticles, Quality by design, Regorafenib monohydrate, Zeta potential*

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## INTRODUCTION

In our study, we employed the modern, rigorous technique known as QbD (Quality by Design) in order to circumvent the limitations of the traditional formulation optimization methodologies. In addition to addressing the shortcomings of traditional optimization methods, QbD application offers some additional benefits, such as accounting for the interactions between several independent parts and how those interactions impact important quality attributes (CQAs). The QbD begins with defining QTPPs (Quality Target Product Profiles) of the product, which is entirely dependent on the product and

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process understandings and then the QTPP is used to identify critical quality attributes (CQAs) [1, 2].

Regorafenib monohydrate is a multi-kinase inhibitor that is taken orally. It is an antineoplastic agent and the recommended medication for advanced gastrointestinal tumors, hepatocellular carcinoma, and metastatic colorectal cancer. [3, 4]. Although the dosage of regorafenib monohydrate varies from patient to patient, a daily dose of 160 mg is advised for the treatment of colorectal cancer. The most common side effects include nosebleeds, hammering in the ears, sustained bleeding from cuts, bloody or cloudy urine, bleeding gums, vision problems, blood in cough, dyspnea or swallowing, lightheadedness, pyrexia or cold sweats, recurring eagerness to urinate frequently, headaches, severe menstrual flow, lower back or side pain [5]. Significant issues with regorafenib monohydrate oral bioavailability (21%), elimination takes 28 h to complete water solubility, and dissolution rate have been reported [6, 7].

Polylactic-co-glycolic acid (PLGA) has emerged as one of the most alluring polymeric possibilities for drug delivery and tissue-engineered things over the past 20 years. In addition to having a wide variety of erosion timescales, variable mechanical behaviour, and being a polymer that has received FDA approval, PLGA is also very biocompatible. PLGA has been extensively researched in the creation of devices for the controlled regime of proteins, small molecule therapies, and other macromolecules in the commercial and academic application sectors. [8, 9]. The exceptionally short circulation durations of PLGA-based formulations are reportedly caused by opsonins present in blood serum, which trigger phagocytes to phagocytose the formulations and remove them from the blood. In the current study, PEGylated PLGA was developed to increase blood circulation time [10, 11]. There are several uses for the non-ionic polymer poly-(ethylene)-glycol (PEG) in the food, chemical, and pharmaceutical sectors. The USFDA states that it is "Generally Regarded as Safe" (GRAS), biodegradable, and biocompatible. It is also non-toxic and non-immunogenic, and it is soluble in both aqueous and organic media. Nanoparticles (NPs) have changed how tumorigenesis and chemotherapies are conceptualized by altering the pharmacokinetic properties of the anticancer drugs as well as significantly protecting the encapsulated drug from unfavorable actions and suppressing unwanted effects of the confined therapeutic, facilitating co-encapsulation of potentially synergistic compounds, providing controlled drug release of the encapsulated drug, and thereby improving the therapeutic effect of active [12].

Xia D et al. investigated the use of self assembled lipid nanocarriers loaded with regorafenib to improve the drug's lymphatic absorption-mediated oral bioavailability and anti-colorectal cancer efficacy. Lipid based nanocarriers were found to have an oral bioavailability that was mostly dependent on the lymphatic route of absorption, with values 1.70 and 65.9 times greater, respectively, than that of solid dispersion (SD) and pure drug suspension. .In mice bearing colorectal tumors, the self-assembled lipid-based nanocarrier showed superior therapeutic efficacy, had a longer elimination half-life (9.34 ± 2.51 h) than the SD (3.51 ± 0.46 h), and increased drug distribution in the GI tract and tumor. The self assembled lipid based nanocarrier showed a promising clinical translation in the treatment of colorectal cancer, with the positive outcomes. [13].

Therefore, the objective of the current study was to use PEGylated PLGA nanoparticles to enhance regorafenib monohydrate oral bioavailability, and biopharmaceutical attributes via modified nanoprecipitation technique by increasing its water solubility and rate of dissolution.

## MATERIALS AND METHODS

### *Materials*

A free sample of the drug regorafenib monohydrate was obtained from M/s Aurobindo Pharma Ltd. in Hyderabad, Telangana. Other excipients from Sigma Aldrich in the US were poly(lactic-co-glycolic acid) (PLGA) (Lactide: Glycolide ratio 50:50), PEGylated PLGA, and poloxamer-188. All the other solvents and chemicals are analytical-grade were used for the studies.

### *Methods*

#### *Regorafenib-loaded PEGylated PLGA nanoparticles prepared by modified nanoprecipitation method*

The necessary amount of regorafenib monohydrate was first dissolved in 2.5 mL of distilled water and DMSO (50:50) using a magnetic stirrer running at 200 rpm. Similar to that, the above-mentioned procedures were used to produce the PEGylated PLGA polymeric solution. Dissolved the necessary concentration of poloxamer-188 (1% w/v) in 10 mL of ethanol while being stirred with a magnetic stirrer to generate the aqueous phase. All volatile solvents were evaporated after introducing the oil phase dropwise using a syringe gauze size #27 at a steady rate rotation of 1500

rpm, and the purity of the nano-emulsion became visible. For two minutes, the nano-emulsion was subjected to sonication at 60% amplitude to guarantee the desired transparency. After properly diluting the DMSO with water, the nano-emulsions were separated from the DMSO using a funnel. The emulsion was ultimately turned into powder using the lyophilization technique with trehalose as the cryoprotectant at 5%w/v of the solution for 6h each day for one week in order to achieve optimized freeze-dried samples. The similar procedure has been adopted for the preparation of blank PEGylated PLGA nanoparticles without drug. The obtained nano-formulations were optimized by using Stat-Ease Design Expert version 13.

***Important quality characteristics and profiles of quality goal products***

QTPPs are crucial for identifying product CQAs and determining the proper quality while accounting for the medication product's safety and effectiveness. According to Table 1, the QTPPs were created after careful consideration of risk evaluations as well as regulatory, scientific, and practical considerations. CQAs are developed with reference to the QTPPs, which regulate the product and process development. Nanomaterials have connections to other parts of the formulation, materials and process, such as critical formulation variables, critical material attributes (CMAs) and critical process parameters (CPPs) [14, 15].

***To assess risk, the fishbone diagram is utilized***

To identify key material features and major process factors in the production of PEGylated PLGA (PEG-PLGA) nanoparticles and evaluate their impact on CQAs, a Fishbone diagram was developed. Particle size (PS), zeta potential (ZP), and polydispersity index (PDI) were chosen as CQAs due to their possible impact on the therapeutic potential of drug-loaded nanoparticles. The Fishbone analysis also discovered production and process variables that could influence the features of nanoparticles. [16, 17].

***Risk evaluation***

Risk was divided into three categories: low, medium, and high. These categories were then used to determine the proper formulation or process parameters for the chosen CMAs and/or CPPs. The frequency with which a failure mode affected the pharmaceutical product was calculated using FMEA (Failure Mode and Effects Analysis). To determine the RPN, or risk priority number, we ranked the severity, detectability, and incidence of each CMA on a scale from 1 to 10. Detectability (D), Severity (S), and Occurrence (O) form the RPN acronym (RPN). Using the Taguchi method, we conducted a series of trials to identify potential confounding variables associated with elevated RPN [3, 18, 19].

***Solubility studies***

A variety of solvents, including acetonitrile, methanol, ethanol, DMSO, DMF, acetone, low molecular weight PEGs (PEG 200 and PEG 400), distilled water, 0.1N HCl, and phosphate buffers with pH values of 6.8 and 7.4 were used to test the solubility of drug. The solubility study was conducted by using Rivotek bench top mechanical shaker set at a 37±0.5 °C to mix the required amount of drug with each solvent, and the process was repeated for 72 hr. The absolute solubilization of the drug was checked in the vials at predetermined intervals. The insoluble or immiscible drug was separated from the excipients by transferring them to Spinwin MC02 - Micro Centrifuge tubes [20].

***Analytical method development by ultra-fast liquid chromatographic technique***

A Shimadzu liquid chromatographic system with an ODS (Octadecylsilyl) C18 HPLC column (250 x 4.6 mm; 5 µm) and a photodiode array (PDA) detector was used to optimize the analytical procedure. A 250 mm x 4.6 mm internal diameter C18 column was used to perform the separation.

Table 1. Selected quality target product profiles (QTPPs) and critical quality attributes (CQAs) for developing regorafenib monohydrate loaded PEGylated-PLGA polymeric nanoparticles

| QTTPs | Target | CQAs | Pre-determined target | Justification |
|---|---|---|---|---|
| Dosage type | Sustained release dosage forms | Cumulative drug release at 24 hr (QT24 %) | ≥ 90-95% | Sustained release of drug is the objective of the study and is important for better absorption |
| Dosage form | PEGylated-PLGA polymeric nanoparticles | Zeta potential | ≥ ± 20mV | Highly critical factor as per the stability perspective of the PEGylated-PLGA polymeric nanoparticles |
| Drug release and absorption | $C_{max}$ and AUC higher compared to pure drug | Mean particle size(nm) | 100 nm-200 nm | Particle size in these ranges is highly critical and important for better absorption of drug |
| Dispersity | High Dispersity | PDI (Polydispersity index) | 0-0.4 | Uniformity in the particle distribution by their size is essential for therapeutic activity hence highly critical |

The pH of mobile phase was adjusted to 4.50 from its original value of 7.50 by using Phosphoric acid, and the stationary phase consisted of a 5 µm particle size column. For UV detection, a cutoff of $\lambda_{max}$ 261 nm was chosen. The retention time of regorafenib monohydrate was reported to be 3.35 min [20, 21].

***Taguchi design for primary component screening***

Taguchi design was used for the first screening of factors. In order to determine the most significant factor or variables impacting the CQAs, a screening design comprising seven components at two levels was devised. Based on Taguchi design, a set of eight formulation experiments was performed and the CQAs had been characterized. ANOVA was performed to verify each component's overall significance [22].

***Design of experiment for consistently enhancing formulation***

With three parameters and three levels, a Box-Behnken design, response surface methodoogy was used to optimize the composition of the polymeric nanoparticles. Three levels (-1, 0, and 1) were generated for the trials using the latest version of Stat-Ease Design Expert software to analyze the significance of the impact of these multiple components or replies. The concentration of Poloxamer (188% w/v) (X2), the stirring rate (X3), and the ratio of PEGylated PLGA (mg) (X1) were taken into consideration as independent variables. The zeta potential (mV), polydispersity index (Y1), and particle size in nanometers (nm) (Y1) were all stated directly. The 17 formulation runs were combined, followed by a review of the CQAs [23].

***Optimizing the solvent shift of PLGA-based nanoparticles with the Box-Behnken Design***

The Taguchi design was one of many qualitative and quantitative methods used to screen the two key components. Following the documentation of their primary impact on the chosen parameters using Box-Behnken Design, they were subjected to three rounds of optimization. The earliest experimental testing first determined the three levels (-1, 0, and 1). After reviewing the outcomes from ANOVA analysis and Pareto charts from the Taguchi screening model, it was established that the influence of the five assessed components on the CQAs was not statistically significant [1, 24, 25].

***Optimization of the design space***

The design space was verified using the prediction plots from the Taguchi design and the findings from the FMEA. The program suggested a batch that includes all of the right response values. The software calculated the feasibility of the proposed formula and evaluated the results. All expected responses are met by all observed ones [26-28].

***Characterization of regorafenib monohydrate-loaded PEGylated PLGA nanoparticles***

To further understand the homogeneity, applicability, and scalability, flow properties by Angle of repose, Carr's index and % moisture content of the regorafenib monohydrate polymeric nanoparticles were analyzed [29].

***Particle Size and Size distribution of the nanoparticles***

The particle size distribution of regorafenib PEGylaed PLGA nanoparticles were evaluated using the Malvern Zetasizer (Zetasizer Nano ZS90). Before deciding on the particle size and size distribution, the appropriate amount of dilution was made using distilled water. The information was given as mean S.D. Each determination was conducted in triplicate (n=3), as stated [30].

***Zeta potential and the polydispersity index***

The zeta-potential and PDI of the nanoparticles were measured using the Zetasizer Nano ZS90 following the proper dilution [31, 32].

***Percentage of drug entrapment efficacy and drug loading evaluation***

The indirect approach, significantly modified, was used to determine the % entrapment effectiveness of regorafenib monohydrate. In an ultra-cooling centrifuge, 5 mL of nanoemulsions were spun for 30 min at 15000 rpm. The concentration of free regorafenib present in the nanoemulsion was detected by UV analysis at a maximum wavelength of λmax 261nm after the supernatant was collected and filtered. Entrapment Efficiency (EE) is expressed in % and was calculated using Equation 1 [33].

$$\text{Entrapment efficiency (\%)} = \frac{(\text{Total amount of drug} - \text{Free drug})}{(\text{Total amount of drug})} \text{ x } 100 \ldots\ldots\ldots\ldots\ldots \text{[Eq. 1]}$$

25 mg of drug-loaded polymeric nanoparticles were accurately weighed in a 50 ml screw-cap container, and they were immersed in an ultrasonic field with frequent shaking for 15 min in order to assess drug loading. 2 mL of dichloromethane (DCM) was added to the mixture. After diluting the mobile phase (acetonitrile/water, 70:30, v/v) to volume, syringe filters (Nylon,0.22 µm) were used to filter the mixture. The appropriate analytical method was used to calculate the quantity of drug entrapped. By dividing the total weight of the formulation by the amount of drug loaded, the drug loading capacity (LC) was calculated. By dividing the loaded medication by the entire amount fed for encapsulation, the loading efficiency (LE) was calculated. [34].

***Fourier transform Infrared spectroscopy (FT-IR)***

The chemical groups of regorafenib pure drug (A), blank formulation without polymers (B), PEG-PLGA (C), Physical mixture of pure drug and polymers (D), and optimized PEGylated PLGA polymeric nanoparticles (E) were determined using Fourier transform infrared (FT-IR) spectroscopy at a resolution of 2 $cm^{-1.}$ The samples' infrared absorption spectra (4000 to 400 $cm^{-1}$) were measured using the KBr disc method [34].

***Differential scanning calorimetry (DSC)***

DSC was used to examine the drug's physical condition in the PEGylated-PLGA NPs. A standard aluminium pan was filled with freeze-dried regorafenib monohydrate-NPs, PLGA, poloxamer 188, and a physical mixture of regorafenib monohydrate and placebo nanoparticles (Regorafenib monohydrate: blank nanoparticles 1:4). There were roughly 5 mg of each chemical in each test sample. An empty aluminium pot was utilized as a reference. The nanoparticles were monitored from 25 to 200 °C using a scan rate of 10 °C/min [35].

***X-ray diffraction***

Using an XPERT-PRO-MRD with a cu anode and a graphite monochromator, operating at a voltage of 35 kV and a current of 20 mA, the crystallinity of regorafenib monohydrate, PLGA, regorafenib monohydrate-loaded PLGA NPs, and empty PLGA nanoparticles was evaluated. The analysis was conducted in the 2θ angle range of 5-50 °, with the process parameters set at a scan-step size of 0.02 °(2θ) and a scan-step time of 25 s [36].

***Scanning electron microscopy (SEM)***

SEM was used to examine the morphology of regorafenib's PEGylated PLGA nanoparticles. (SEM; Jeol, Japan; JSM-6390LV) that can reach 30 kV and has high resolution. At first, the formulation sample stuck to the metal stub due to the carbon layer on its underside. SEM is useful for closely evaluating surface morphology because it employs a high-energy electron to scan across a specimen's surface with an Au and Pt coating to maximize contrast and signal-to-noise ratio. Regorafenib monohydrate surface shape and optimized PEGylated PLGA polymeric nanoparticles were the subject of additional research [37].

***In vitro drug diffusion studies***

Regorafenib monohydrate-loaded PEGylated PLGA nanoemulsions were subjected to in vitro drug diffusion studies using the dialysis sac method, aided by a dialysis membrane (Himedia; dialysis membrane-60; average flat width: 25.27 mm, average diameter: 15.9 mm, 10000 kDa–12000 kDa, 5 ml capacity). The dialysis membrane, which is impenetrable to nanoparticles is permeable to free drugs. According to the dialysis sac method (Tarson, Multispin, India), the nanoparticulate systems were first placed into 100 ml of diffusion medium (pH 6.8 phosphate buffer) that was set at a temperature of 37±0.5°C and rotated at a speed of 100–150 rpm. Regorafenib monohydrate, 25 mg, was present in each formulation. In order to maintain the sink condition, 5.0 ml of the samples were pipette-collected at the appointed time and replaced with freshly made phosphate buffer pH 6.8. Using a UV spectrophotometer (UV-1800 Shimadzu, Japan) with a maximum wavelength (λmax) of 261 nm, the sample was filtered and its drug concentration was assessed. [38].

***In vivo pharmacokinetic studies***

Male albino rabbits weighing between 1.5 and 2.5 kg were used in an in vivo pharmacokinetic investigation. The Institutional Animal Ethical Committee (IAEC) granted protocol permission to Jeeva Life Sciences in Hyderabad with approval number IAEC CPCSEA/IAEC/JAS/17/03/22/48, permission No. 48.

***Categorization and care of animals***

12 male albino rabbits with body weights ranging from 1.5 to 2.5 kg were selected from the animal house's stock. Each of the two groups contained six animals. Using a 8FG diameter Ryle's tube, the first group was given an oral suspension of the pure medication regorafenib monohydrate. The second group received enhanced regorafenib monohydrate-loaded PEGylated PLGA nanoparticles.

***Dose estimation***

Equation 2 was used to compute the dose for male albino rabbit in the manner shown below:

$$\text{Total dose (for humans)} \times 0.07 \text{ (factor for each rabbit)} \times 2.5 \text{ kg of male albino rabbit}/1.5$$
$$= (160 \times 0.07 \times 2.5)/1.5 = 18.666 \text{ mg}$$
$$= 19 \text{ mg} \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(Eq 2)}$$

As a result, 19 mg of regorafenib monohydrate was chosen as the total dose to be administered to the rabbit. A dose of 19 mg of regorafenib monohydrate, dissolved in 5 ml of distilled water, was administered to six rabbits of group-A animals, similarly, equivalent weight of optimized formulation was given to six rabbits of group-B animals as per the approved study design protocol. A pure drug and an improved nanoparticle formulation were administered to the animals simultaneously using wood and a feed tube (Ryle's tube). For up to 24 hr, the marginal ear vein was repeatedly poked with gauge size #24 needles, yielding 0.5 mL of blood each time into an Eppendorf tube.

***Study of pharmacokinetics***

0.5 ml of blood was drawn from the marginal ear vein of male albino rabbits, which was then placed into Eppendorf tubes at intervals of 0, 2, 4, 6, 8, 12, and 24 hr after the dosage was given. Before the blood was extracted for serum, it was left inert in a tilted position for 30 min to allow for coagulation. After that, the blood was centrifuged with a Remi R-8C plus for 20 min at 8000 rpm at room temperature. A micropipette was used to separate the supernatant layer. The Institutional Animals Ethics Committee (IAEC), the Animal Care Committee, and CPCSEA have all been asked to approve the study.

***Estimation of the drug in rabbit serum***

Following collection, the blood samples were processed in the same way as mentioned previously for the calibration curve construction. The solvent extraction method was used to assess the standard curve of the drug regorafenib monohydrate in blood serum. The peak area for the various concentrations at the maximum wavelength of $\lambda_{max}$ 261 nm was calculated. In the concentration range of 100-1000000 ng/mL, the linearity was observed.

***Pharmacokinetic parameter estimation***

The pharmacokinetic parameters were computed using the serum concentration versus time plot. The area under the curve (AUC), time to attain peak plasma concentration (Tmax), and maximum plasma concentration (Cmax) were computed as pharmacokinetic parameters. The elimination rate constants (k) for aqueous suspensions of pure regorafenib monohydrate and optimized PEGylate PLGA nanoparticles were calculated using semilogarithmic plots of serum concentration against time. The slope of the last linear segment of the curve was used to compute the elimination rate constant (k). Using 0.693/K, the first-order elimination half-life (t1/2) was determined. [37].

***Stability study***

The improved nanoparticle formulation was subjected to accelerated six-month stability testing using a stability chamber (TH 200G, Thermolab). The compositions were housed in glass containers with cotton closures, and the capsules were kept in a stability chamber that was kept at 40°C± 2°C and 75% relative humidity. The samples were subjected to initial and 1, 2, 3, and 6 months of stability tests, including those measuring particle size, zeta potential, and polydispersity index. [39].

## RESULTS AND DISCUSSION

***Results***

***Screening excipients based on solubility study***

According to estimate the saturation solubilities of regorafenib monohydrate (REGO) in DMSO, acetone, and ethanol are 2992.24 g/ml, 2358.11 g/ml, and 3256.33 g/ml, respectively.

***Making use of a fishbone diagram for uncertainty analysis***

Regorafenib monohydrate-loaded PEGylated PLGA-based nanoparticles' important quality aspects were analysed using a fishbone diagram to identify the potential risk factors associated with formulation and manufacturing process of nanoparticles. The Taguchi design was used to identify the impact of seven potential risk variables on the CQAs and these were then examined using FMEA method. Polydispersity index, cumulative percentage of drug release, zeta potential, and particle size are all examples of these CQAs. There are seven factors considered in the Taguchi design for factor screening. Table 2 lists the following parameters: Drug: PLGA concentration; Concentration of Poloxamer-188, Ultrasonication time; Stirring speed; Stirring type; Temperature and Time etc.

***Experiment planning, tweaking, and analysis***

Simply put, the drug: The ratio of PLGA to

poloxamer 188 concentration changed as a function of study design. Following preliminary investigation and Pareto-chart analysis data, three levels (-1, 0 and 1) were chosen for each component. The results of 17 rounds of an experiment with three components and three levels of variation are displayed in Table 3. The impacts of various parameters on the CQAs were studied in greater depth for each formulation.

Table 2. Design matrix for factor screening as per Taguchi design along with the experimental results of various CQAs

| RUNS | A | B | C | D | E | F | G | Particle size PS (nm) | ZP (mV) | PDI |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 2 | 1 | 1 | 2 | 2 | 259.45 | -9.5 | 0.452 |
| 2 | 2 | 2 | 1 | 2 | 1 | 1 | 2 | 435.46 | -4.1 | 0.562 |
| 3 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 365.36 | -5.4 | 0.541 |
| 4 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 202.9 | -15.2 | 0.292 |
| 5 | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 315.23 | -6.5 | 0.531 |
| 6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 232.56 | -12.5 | 0.399 |
| 7 | 2 | 2 | 1 | 1 | 2 | 2 | 1 | 385.22 | -4.2 | 0.671 |
| 8 | 1 | 2 | 2 | 2 | 2 | 1 | 1 | 295.47 | -7.5 | 0.481 |

| Factors | Codes | Low level (1) | High level (2) |
|---|---|---|---|
| Drug: PLGA conc. (mg) | A | 40 | 80 |
| Poloxamer-188 conc. (gm %) | B | 1% | 2% |
| Ultrasonication time (mins) | C | 5 | 15 |
| Stirring speed (rpm) | D | 1000 | 2000 |
| Stirring type | E | Magnetic | Mechanical |
| Temperature ($^0$C) | F | 25 | 40 |
| Stirring time (h) | G | 1 | 2 |

Where; QT24% cumulative % drug release at 24 hr; PS, Particle size; ZP, Zeta potential; PDI, polydispersity index

Table 3. Design Expert software predicted different combinations of independent variables in coded forms to execute possible experimental runs and their outcomes

| Runs | Factor 1 | Factor 2 | Factor 3 | Response 1 | Response 2 | Response 3 |
|---|---|---|---|---|---|---|
| | A: PEGylated-PLGA (mg) | B: Poloxamer 188 (gm%) | C: Stirring Speed (rpm) | Particle size (PS) in (nm) | Polydispersibility index | Zeta potential (mV) |
| 1 | 0 | 0 | 0 | 215 | 0.192 | -21.76 |
| 2 | 0 | -1 | 1 | 155.5 | 0.198 | -22.03 |
| 3 | 1 | -1 | 0 | 153.5 | 0.204 | -21.99 |
| 4 | -1 | 0 | -1 | 152.5 | 0.211 | -21.99 |
| 5 | 0 | -1 | -1 | 171.5 | 0.346 | -21.85 |
| 6 | -1 | 0 | 1 | 152.5 | 0.538 | -22.2 |
| 7 | 0 | 0 | 0 | 156 | 0.206 | -20.99 |
| 8 | 1 | 0 | -1 | 415 | 0.499 | -11.78 |
| 9 | 1 | 0 | 1 | 455 | 0.207 | -17.76 |
| 10 | -1 | 1 | 0 | 155.5 | 0.367 | -22.03 |
| 11 | 0 | 0 | 0 | 157 | 0.296 | -11.09 |
| 12 | -1 | -1 | 0 | 151.1 | 0.398 | -21.2 |
| 13 | 0 | 0 | 0 | 159.9 | 0.209 | -22.04 |
| 14 | 0 | 0 | 0 | 160 | 0.395 | -19.05 |
| 15 | 0 | 1 | 1 | 317 | 0.197 | -15.6 |
| 16 | 1 | 1 | 0 | 615 | 0.536 | -10.98 |
| 17 | 0 | 1 | -1 | 315 | 0.389 | -20.09 |

Where PS, Particle size; ZP, Zeta potential; PDI, polydispersity index; here the independent factors and their respective low, medium and high levels are coded below:

| Independent Variables | Levels | | |
|---|---|---|---|
| | LOW ( -1) | MEDIUM (0) | HIGH (1) |
| X1: Drug: PEG-PLGA ratio (mg) | 1:1 (40 mg) | 1: 2 (80 mg) | 1:3 (160 mg) |
| X2: Polaxomer-188 Conc. % | 1% | 1.5% | 2% |
| X3: Stirring speed (RPM) | 1000 | 1500 | 2000 |

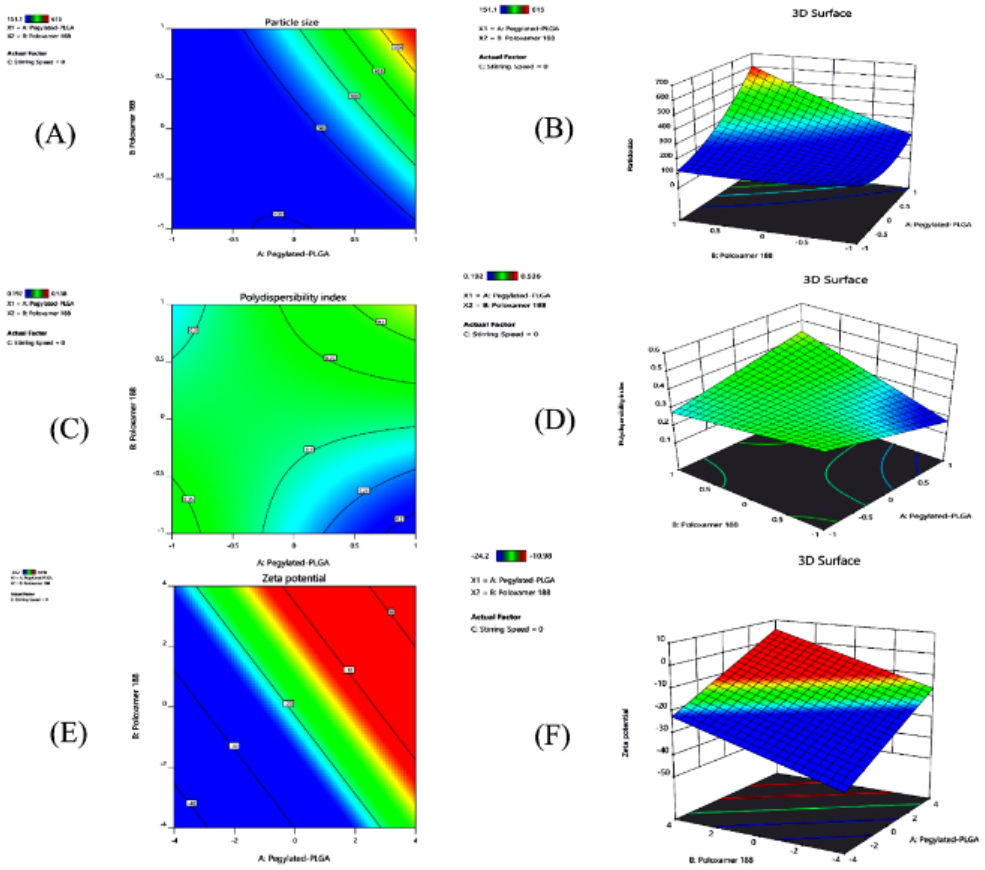


Fig. 1. Contour plots (2D) and response surface plots (3D) of selected independent factors on selected dependent factors particle size (A) & (B), Poly dispersibility index (C) & (D), Zeta potential (E) & (F)

***Examining 2D and 3D response surfaces***

***Effect on PS in terms of CQAs***

Fig. 1(A) and 1(B) show a 2D and 3D plots for the CQA (Particle size), respectively. Particle size of the nanoformulations of run 12 and 16 showed 151.1 nm and 615 nm, respectively. The drug release pattern was prolonged and the dissolution was impacted with the increase in particle size and aggregation which is influenced by increased concentration of the polymer [39].

***Consequences for CQAs of factor and PDI***

The contour plot and 3D plot for the CQA PDI are shown in Fig. 1(C) and 1(D), respectively. The PDI seems to respond similarly to the ratio of DRUG: PLGA (factor A) and Poloxamer 188 concentrations (factor B). It varies from 0.398 to 0.536 for runs 12 and 16, respectively [40].

***Influence of the variable on zetapotential as CQAs***

The contour plot and 3D plot for the CQA ZP are displayed in Fig. 1(E) and 1(F), respectively. At a lower level, it fluctuates between -21.1 mV for run 12 and -10.98 mV for run 16. Both of the parameters seem to have a considerable impact on the CQA ZP. PLGA concentration for drug (factor A) and poloxamer 188 concentration (factor B) [41].

***The analysis of variance used in the experiment***

Table 4 displays the ANOVA summary for each component and its importance in connection to the quadratic model. After applying the design matrix to the model, the F-values for the particle size, zeta potential, and polydispersibility index are found to be 10.15, 9.91, and 6.43. P values (<0.05) for the quadratic model are all under for a variety of CQAs, demonstrating that the model is statistically significant. Zeta Potential, Polydispersibility Index, and Particle Size all have computed p-values of 0.0487, 0.090, and 0.0138, respectively. A p-value less than 0.05 indicates that the model terms are meaningful. The predicted R2 (0.5308) and the actual R2 (0.6408) are quite congruent. However, the predicted R2 of 0.0678 is not as close as to

Table 4. Summary of ANOVA for different factors and its significance with respect to quadratic model

| Source | PS | | ZP | | PDI | |
|---|---|---|---|---|---|---|
| | F value | P-value | F value | P-value | F value | P- value |
| Model | 10.05 | 0.0043* | 4.44 | 0.0384* | 13.35 | 0.0018* |
| A-DRUG: PLGA Conc. | 10.15 | 0.0154* | 9.91 | 0.0162* | 6.43 | 0.0389* |
| B-Poloxamer-188 Conc. | 3.83 | 0.0912 | 1.20 | 0.3100 | 2.31 | 0.1724 |
| AB | 4.91 | 0.0623 | 1.60 | 0.2460 | 5.48 | 0.0518 |
| $A^2$ | 28.07 | 0.0011* | 8.01 | 0.0254* | 17.19 | 0.0043* |
| $B^2$ | 0.1131 | 0.7465 | 0.0021* | 0.9650 | 15.34 | 0.0058* |
| Lack of fit | 6.70 | 0.0487* | 4.50 | 0.0900 | 13.96 | 0.0138* |

Where PS, Particle size; ZP, Zeta potential; PDI, polydispersity index; *Significant levels, i.e., less than α value (0.05)

the Adjusted R2 of 0.7874 in case of Particle size obtained from the BBD model summary for optimizing the nanoparticles of REGO, which could indicate a strong block influence. Fig. 2 (A-F) showed the perturbation plots and the predicted vs. actual responses for the observed responses. The signal is sufficiently robust, though, as shown by the accuracy ratio of 8.138. The adjusted R2 of 0.5860 for the aggregate mean indicates that it might be a stronger predictor of ZP than the predicted R2 of -0.5011. The signal appears to be sufficient, as the precision ratio is 5.597. Table 5 displays that while the estimated R2 for PDI is just 0.2574, the precision ratio of 11.3013 indicates there is enough signal to warrant further investigation.

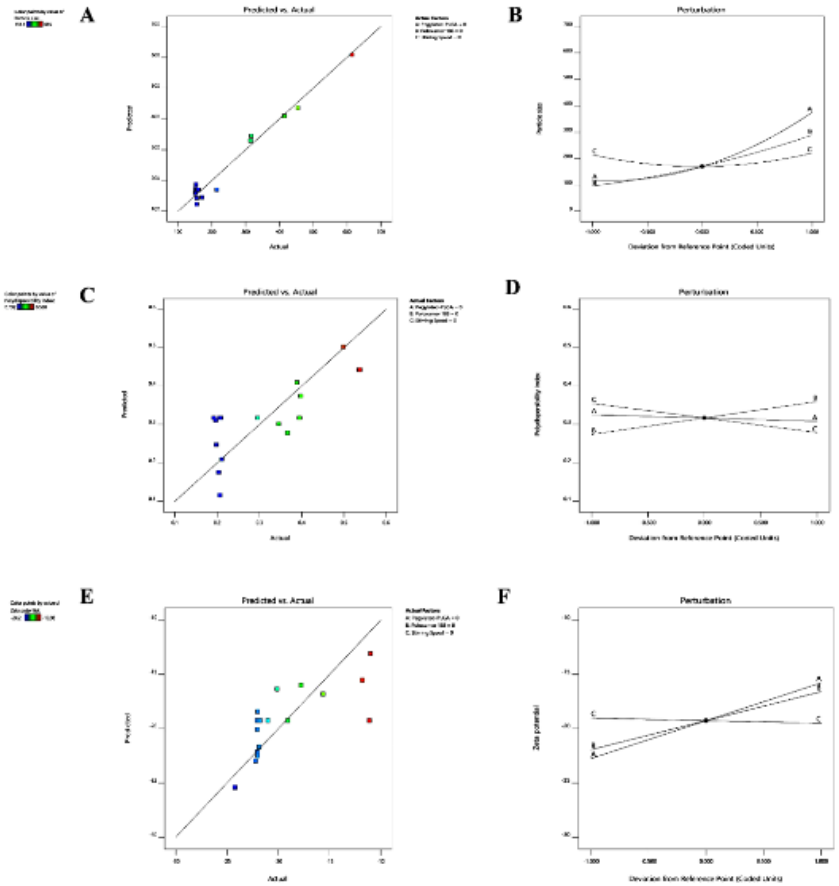


Fig.2. Normal v/s predicted and perturbation plots for the observed responses of factors on selected dependent factors particle size (A) & (B), Poly dispersibility index (C) & (D) Zeta potential (E) & (F)

Table 5. Constraints for the process of optimization of polymeric nanoparticles of REGO using DoE model

| Responses | PS | ZP | PDI |
|---|---|---|---|
| $R^2$ | 0.8778 | 0.7603 | 0.9051 |
| Adjusted $R^2$ | 0.7874 | 0.5860 | 0.8373 |
| Predicted $R^2$ | 0.0678 | -0.5011 | 0.2574 |
| Adeq Precision | 8.138 | 5.597 | 11.3013 |
| Std. Dev. | 110.07 | 5.99 | 0.0543 |

Where PS Particle size; ZP, Zeta potential; PDI, polydispersity index; $R^2$ Correlation coefficient; Std. Dev; Standard deviation

Table 7. Summary of Design of Experiment with Various Parameters Fitting to Quadratic Model

### *Determine the design space by analyzing overlay plots*

Using the Box-Behnken Design, 40 mg of regorafenib monohydrate, 40 mg of PLGA, and 1% w/v poloxamer 188 were combined to create the optimal regorafenib monohydrate polymeric nanoparticles. Table 6 presents an overview of the optimization process together with the expected and actual values for the responses of the improved formulation.

### *Solid-state characterization of PNs*

### *Micrometric characteristics*

The micrometric properties data of the optimized polymeric PEGylated PLGA nanoparticles are listed in Table 7. The results indicate that the moisture content (%), angle of repose (ɵ) and Carr's index values are 2.95±0.44, 24.62±0.03, and 16.36±0.25, respectively.

### *Zeta potential and measuring of particle size*

As can be shown in Fig. 3(A), the size range discovered to be best for regorafenib monohydrate polymeric nanoparticles for run 12 is (151.1nm). The produced regorafenib-loaded PLGA-NPs were 200 nm in size, spherical, and had a homogeneous size distribution. Run 12's zeta potential was (-21.1 mV). Each formulation's particle size was evaluated using the zeta-sizer tool in accordance with Fig. 3(B).

### *Entrapment efficiency* and drug loading capacity

Poloxamer-188 1% w/v at 2000 rpm had the highest entrapment efficiency (73.25%), according to the results of the 12 experiments. The effects of

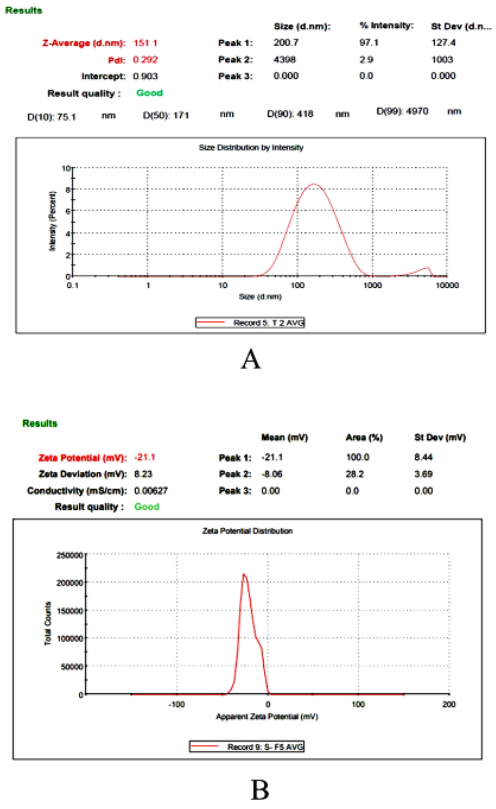


Fig. 3 (A) Particle size distribution and (B) Zeta potential curve optimized PEGylated PLGA polymeric nanoparticles

Table 6. Summary of design of experiment with various parameters fitting to quadratic model

| Run 12 Response | Predicted mean | Predicted median | Observed | Std Dev | SE Mean | 95% CI low for Mean | 95% CI high for Mean | 95% TI low for 99% Pop | 95% TI high for 99% Pop |
|---|---|---|---|---|---|---|---|---|---|
| Particle size | 158.325 | 158.325 | 151.1 | 32.7772 | 28.3859 | 91.203 | 225.447 | -47.1852 | 363.835 |
| Polydispersibility index | 0.373316 | 0.373316 | 0.398 | 0.099842 | 0.0746364 | 0.207016 | 0.539616 | -0.171659 | 0.918291 |
| Zeta potential | -25.4206 | -25.4206 | -21.1 | 3.27833 | 1.82183 | -29.3564 | -21.4848 | -41.1903 | -9.65084 |

Table 7. Carr's index, angle of repose, moisture content of pure drug and optimized polymeric formulation

| Formulations | Carr's index | Angle of repose (ɵ) | Moisture content (%) |
|---|---|---|---|
| Pure drug (Regorafenib monohydrate) | 27.69±0.05 | 30.25±0.22 | 3.52±0.55 |
| Optimized formulation | 16.36±0.25 | 24.62±0.03 | 2.95±0.44 |

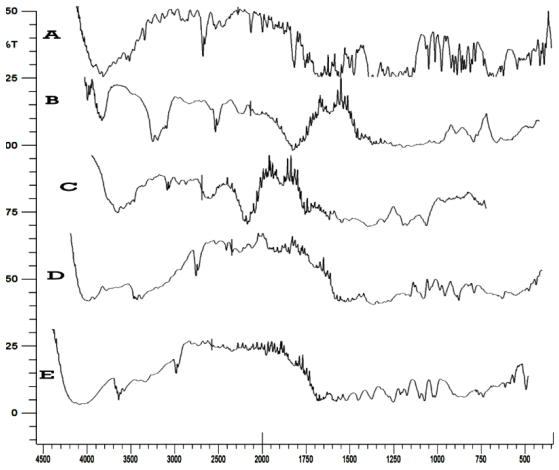


Fig. 4. FT-IR spectrum of (A) Pure drug, (B) Blank formulation without polymers (C) PEG-PLGA, (D) Physical mixture of pure drug and polymers (E) Optimized PEGylated PLGA polymeric nanoparticles

various process variables highlight the technique, solvent, and excipient choices. After preparation, the optimized polymeric nanoparticles provided high drug loading capacity of 30 % as compared to the other formulations.

### *FT-IR*

Regorafenib monohydrate -polymer interactions were studied using Fourier transform infrared spectroscopy. FT-IR spectra of regorafenib monohydrate and physical mixes (PM) including PLGA and poloxamer-188 were acquired using a 4 $cm^{-1}$ resolution in the 4000-400 $cm^{-1}$ region. Pure regorafenib monohydrate was subjected to FT-IR analyses, which identified the following bands: C-N stretching band at 1226 $cm^{-1}$, C-O vibration at 1548 $cm^{-1}$, N-H band at 3256 $cm^{-1}$, O-H vibration at 3336 $cm^{-1}$, and C=C band at 3215 $cm^{-1}$. FT-IR studies indicates

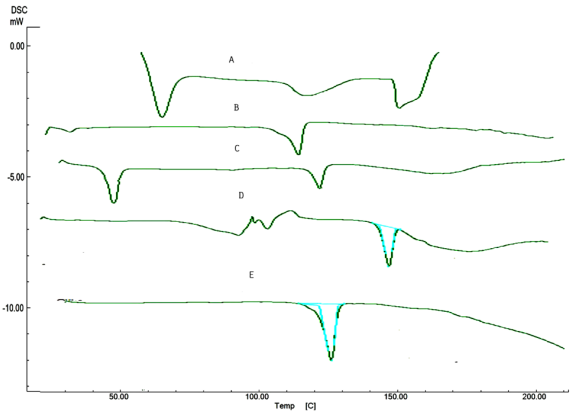


Fig. 5. DSC thermogram of (A) Pure drug, (B) Blank formulation without polymers (C) PEG-PLGA, (D) Physical mixture of pure drug and polymers (E) Optimized PEGylated PLGA polymeric nanoparticles

that the combined activity of the drug regorafenib and polymers has not altered considerably. Utilizing FT-IR spectroscopy, interactions between rogorafenib and polymers were studied. Based on the findings, Fig. 4 (A-E) depict the compatibility of REGO with various additives.

### *DSC*

The DSC thermogram of (A) regorafenib monohydrate, (B) blank formulation without polymers (C) PEG-PLGA, (D) physical mixture of pure drug and polymers (E) optimized PEGylated PLGA polymeric nanoparticles as shown in Fig. 5 (A-E). The DSC thermograms of physical mixtures also revealed a unique endothermic peak, and the regorafenib monohydrate thermograms demonstrate the drug's crystallinity.

### *In vitro drug permeation studies*

Fig. 6 showed that after 12 hr of in vitro

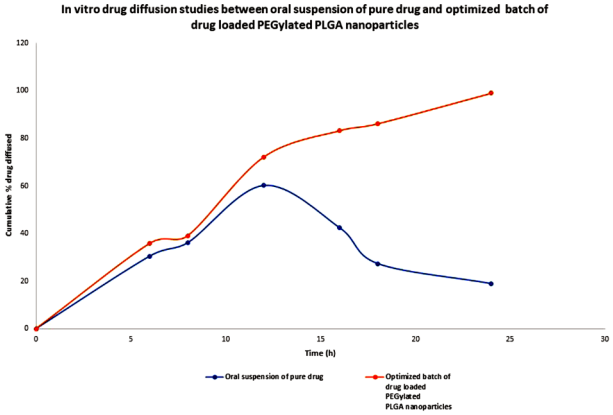


Fig. 6. *In vitro* cumulative % drug permeation of pure drug vs optimized PEGylated PLGA polymeric nanoparticles

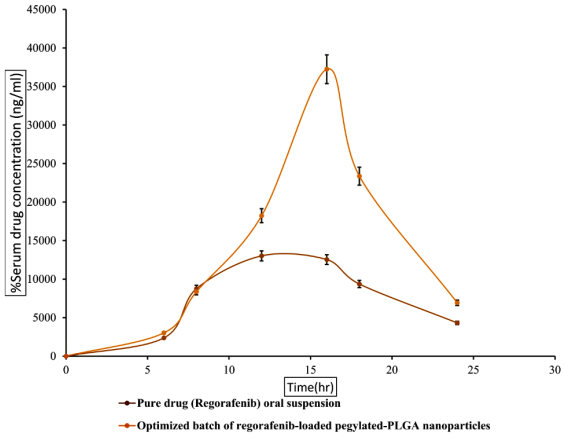


Fig. 7. Serum-drug concentration (ng/ml) v/s time (h) curve of optimized PEGylated PLGA polymeric nanoparticles vs pure drug oral suspension of regorafenib monohydrate

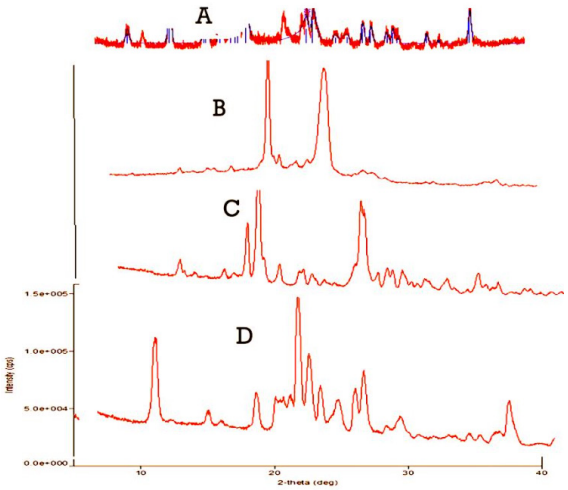


Fig. 8. P-XRD curves of (A) Pure drug and (B) Drug-loaded formulation (C) Blank formulation (d) Optimized PEGylated PLGA polymeric nanoparticles

drug permeation tests, the optimized batch's cumulative drug permeation percentage was 72.19%, which was higher than the pure drug's 60.15 percent. Additionally, after 24 hr, the drug release from nano formulation's is increased and observed about 98.99%.

### *In vivo pharmacokinetic estimation*

Based on pharmacokinetic study results, for both optimized PNs of REGO and pure drug, mean plasma concentration of drug vs. time plot was obtained which is displayed in (Fig. 7) following the conclusion of the pharmacokinetic investigation. Table 8 displays the estimated values for a number of pharmacokinetic parameters. Tmax, which indicates continuous drug release, was found to be 24 hr for the enhanced formulation compared to 8 hr for the pure medication. The Cmax of the improved REGO PNs was measured as 37254.27 ng/mL, while the pure drug's Cmax was 13023.06 ng/mL. Compared to the pure drug's AUC of 20838.55 (ng.hr/L), the optimized REGO PNs' AUC was reported to be 46529.50 (ng.hr/L).

### *P-XRD*

Fig. 8 (A-D) shows the X-RD patterns of pure drug, drug-loaded formulation, blank formulation, and optimized PEGylated PLGA polymeric nanoparticles. Large peaks found in pure drug (regorafenib monohydrate) at diffraction angles of 16.90, 18.30, 19.90, 20.30, 21.40, 25.6 0, and 26.20 revealed the presence of a distinct crystalline pattern. Optimized polymeric nanoparticles showed a reduction in the relatively narrow characteristic peak at these angles, suggesting an amorphous structure and molecular confinement of the drug in the polymeric form.

### *SEM*

As a result, (Fig. 9) provides SEM pictures of the optimal PNs for REGO.

### *Accelerated stability*

Table 9 shows the P-values for the accelerated stability study's ANOVA design. Each CQA has a

Table 8. *In vivo* pharmacokinetic parameters data for the pure drug (Regorafenib) and optimized batch of regorafenib-loaded PEGylated-PLGA nanoparticles

| Pharmacokinetic parameters | Pure drug (Regorafenib) | Optimized batch of regorafenib-loaded pegylated-PLGA nanoparticles |
|---|---|---|
| $K_E$ | 0.47 | 0.57 |
| $C_{max}$ (ng/mL) | 13023.06 | 37254.27 |
| $_{Tmax}$ (h) | 12 | 16 |
| $AUC_{(0-\infty)}$ (ng.hr/L) | 20838.55 | 46529.50 |

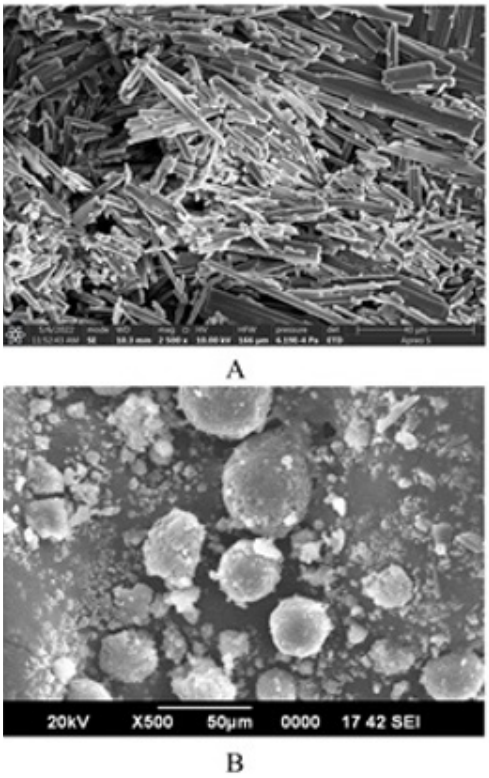


Fig. 9. SEM image of (A) Pure drug (Regorafenib monohydrate) (B) Optimized PEGylated PLGA polymeric nanoparticles

P-value greater than 0.05, indicating no significant variation.

**DISCUSSION**

Ethanol was shown to have the highest solubility when DMSO and acetone were contrasted with it (quantitative solubility for other co-solvents). The first stage of factor identification based on the FMEA method was completed by identifying the majority of risk factors related to the variables used in the manufacture of regorafenib-loaded PLGA-based nanoparticles using fishbone analysis and the Taguchi design for screening of factors. The p-values of the regression coefficients were used to determine the relative weights of each variable in each response. Low (1 level) and high (2 levels) denote the number of formulations with coded and real values for each factor created and characterized for the various CQAs. The Taguchi design was used to reduce the most significant elements following several iterations for each component. The model terms are deemed significant when the P-values for model factors A, B, and C are less than 0.05. If the model terms' corresponding p-values are less than 0.05, they can be considered significant. Based on the results of the factor screening study, the parameters A-Drug: PLGA concentration and B-Poloxamer-188 concentration were identified as relevant factors for additional optimization. The number of formulations with coded and real values for each factor developed and characterized for the various CQAs is represented by low (1 level) and high (2 levels). The Taguchi design was used to reduce the most significant elements following several iterations for each component. If the p-values for the model terms are less than 0.05, they can be considered significant. Following a factor screening study, the concentrations of A-Drug's PLGA and B-Poloxamer-188 were shown to be significant since the p-values for the model components A, B, and C is found less than 0.05. Research on the effect of the factor on PS as CQAs at low level (-1) has demonstrated that A-Drug: PLGA concentration and intermediate level (-1) of B-Poloxamer-188 concentration are examples of the blue region, or for obtaining a lower particle. Additionally, it is anticipated that the particle size will decrease at low A-Drug: PLGA concentrations. The dark yellowish-red zone indicates that this will drastically decrease as the concentration rises. The particle size is a function of the shear force needed to cause droplet breakup. The viscous forces that prevent stirring and sonication-induced

Table 9. Accelerated stability data for the optimized batch of regorafenib-loaded PEGylated-PLGA nanoparticles

| | Parameters (Accelerated stability data) | | |
|---|---|---|---|
| Time (In months) | Particle size (nm) | Polydispersibility index | Zeta potential (mV) |
| 1 | 152.06 | 0.292 | -21.1 |
| 3 | 152.09 | 0.291 | -21.1 |
| 6 | 154.05 | 0.289 | -21.1 |
| P-value α ≤ (0.05) | 0.061 | 0.072 | 0.085 |
| significant difference exists | | | |

droplet disintegration are amplified by PLGA concentration. According to the CQAs' examination of the factor's effect on the PDI, the PDI value stays below 0.4 only when the concentrations of both the factors A-Drug: PLGA and the B-Poloxamer 188 are in the light green region (between -1 and 0.5). The stabilizer mechanism maintains the size distribution, critical for efficient drug absorption across the gastrointestinal (GI) membrane. The factor's impact on ZP as CQAs suggested a larger value of zeta potential for the concentrations of A-Drug: PLGA and B-Poloxamer 188 at a low level (-1), which is anticipated to have a significant impact on the CQAs mentioned above. Adjusted R2 values for CQAs under observations are generally consistent with the predicted values. The ultimate objective was to build a set of potential CQAs in response to the QTPP need and then optimize it per the purpose stated in "Identification of different QTPPs and CQAs." The study found that the suggested increased formulations' average PS, ZP, and PDI values were 151.1 nm, -21.1 mV, and 0.398, respectively. Furthermore, drug-enhanced PNs demonstrated their capacity to reach the optimal QTTP composition. Run 12 was determined to be the formulation composition with the best flow characteristics based on these micrometric measurements. Micrometric characteristics of polymeric nanoparticles showed that the particles flowed well and contained an appropriate amount of moisture (% moisture content). The presence of particles larger than 250 nm in numerous formulations suggests a lesser possibility of including polymeric nanoparticles. The higher polymer content potentiated the particle size, which facilitated aggregation and gave the material a hazy look. The outcome was a considerably better entrapment efficiency in the optimized composition. Information about the FT-IR spectra of PLGA and poloxamer 188 are components of a physical mixture (PM) and a selected regorafenib monohydrate. There are no interactions between the drug substance and the polymers, as indicated by these peaks in the FT-IR spectra of the physical mixes. This investigation did not infer any interactions between the drug and the excipients because studies have demonstrated that the peak properties of the formulation and the pure drug are identical. Based on the R2 values found for several kinetic models, it was determined that the Higuchi model provided the greatest fit for both pure REGO and augmented PNs of REGO. Therefore, a better dissolving profile can be achieved when drug and PLGA is combined with poloxamer-188. To fit a suitable kinetic model and better understand the drug release mechanism, different kinetic governing equations are used. These include Higuchi models, zero-order models, and first-order models. Each kinetic model's R2 values for regorafenib monohydrate and drug-loaded PNs were independently determined using these models. The release exponents for the REGO formulation with enhanced PNs and the pure drug were 0.659 and 0.448, respectively. Consequently, the drug release from pure drug displays Fickian diffusion kinetics, but REGO-optimized PNs have non Fickian diffusion kinetics. The improvement in solubility and absorption of drug through the gastrointestinal barrier can be attributed to its increased bioavailability. In vivo tests show that using modified PNs of REGO dramatically increased the drug penetration and absorption capability, as shown by the significantly better pharmacokinetic criterion than pure drugs. The pure drug's notable peaks verified its distinctive crystalline structure. In optimized regorafenib monohydrate polymeric nanoparticles, the comparatively slight characteristic peak reduction at such angles revealed the amorphous nature of the drug and its containment at the nanoscale level in the polymeric form. While the SEM shows spherical particles in the freeze-dried formulation to have a smooth surface and crystalline components, the regorafenib monohydrate appears to have a rough surface. The optimized regorafenib monohydrate-loaded PEGylated PLGA polymeric nanoparticles were found to meet the stability requirement since their CQAs did not significantly change over time, indicating the stability features of the formulation in an accelerated condition.

## CONCLUSION

The QbD-enabled methodology was used to systematically develop regorafenib monohydrate-loaded PEGylated-PLGA polymeric nanoparticles to improve the formulations and increase the drug's oral bioavailability. The original QTPPs and CQAs were recognized and supported during the QbD process. While the Taguchi screening design was used for preliminary formulation screening, the Box Behnken Design (BBD) was used for systematic optimization of nano formulation. Both the response surface and the regression equation were examined. ANOVA analysis was carried out

to determine the most beneficial model term. The upper and lower bounds of specific CQAs were chosen in order to optimize the PEGylated-PLGA PNs. The optimized freeze-dried polymeric nanoparticle formulation showed a 24 hr in vitro drug release efficiency of over 90%. In PNs with BBD, the ideal ratio was 40 mg regorafenib monohydrate: 40 mg PLGA: poloxamer-188 (1.0% w/v) with a PS of 151.1 nm, ZP of -21.1 mV, a PDI of 0.398, and EE of 73.25%. In-vivo studies demonstrated that the ideal formulation exhibited a three-fold rise in oral bioavailability accompanied by increased AUC and Cmax, in comparison to an aqueous suspension of pure drug. An expedited stability analysis of regorafenib monohydrate-optimized PNs revealed no appreciable changes in the CQAs over six months storage, as shown by the p-values for each CQA. Based on the current investigation, 40 mg regorafenib monohydrate, 40 mg PEGylated PLGA, and 1.0% w/v poloxamer-188 was shown to be the optimal ratio to achieve the drugtargeted goals of improved bioavailability and efficient drug release..

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**ETHICAL ISSUES**

The current study's protocol was approved before any of the animal experiments were completed. The Animal Care Committee Jeeva Life Sciences, Hyderabad, Institutional Animals Ethics (CPCSEA/IAEC/JAS/17/03/22/48, Approval No. 48) gave their approval for the pharmacokinetic investigation. For animal study, the UK Animals (Scientific Procedures) Act, 1986 and its implementing regulations and EU Directive 2010/63/EU were adhered to. In addition, these regulations and the ARRIVE guidelines were also adhered to.

**CONFLICT OF INTEREST STATEMENT**

The authors assert that the work presented in this study does not seem to have been influenced by any of their known financial or intimate relationships.

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