QbD enabled optimization of solvent shifting method for fabrication of PLGA-based nanoparticles for promising delivery of Capecitabine for antitumor activity

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Abstract

The key objective of the current research was to fabricate and optimize Capecitabine (Cap)-loaded [poly(lactic-co-glycolic acid)] PLGA-based nanoparticles (NPs) by enabling quality by design (QbD) approach for enhancing antitumor activity by promising delivery of the drug at the colonic site. The current research was based on fabricating PLGA-based nanoparticles along with Eudragit S100 as enteric polymer employing solvent shifting method followed by optimization using QbD approach. This approach was found to be useful for understanding the multiple factors and their interaction influencing the product by utilizing Design of Experiment (DOE). Box-Behnken design (BBD) was adopted to achieve the required critical quality attributes (CQAs), i.e., minimizing particle size, maximizing entrapment efficiency, and minimizing PDI value. The optimized nanoparticles were lyophilized and characterized by FT-IR, DSC, TEM, DLS, MTT assay using HT-29 cell lines, and in vivo pharmacokinetic studies. The optimized PLGA-based nanoparticles were found to possess average particle size, PDI, zeta potential, and entrapment efficiency of 195 nm, 0.214, −6.65 mV, and 65%, respectively. TEM analysis revealed the spherical nature of nanoparticles. The FT-IR and DSC studies revealed no interaction. The bioavailability of Cap-loaded nanoparticles was found to be two fold increased than the pure drug, and also, it exhibited significantly more cytotoxic to tumor cells as compared to pure drug as confirmed by MTT assay. The optimized PLGA-based nanoparticles found to possess enhanced bioavailability and significantly more cytotoxic potential as compared to pure drug.

Keywords Capecitabine · PLGA · Quality by design (QbD) · MTT assay · Cytotoxic potential · DoE

Introduction

Capecitabine (Cap) is a prodrug of 5-Fluorouracil (5 FU) with antineoplastic activity. It is highly recommended for the treatment of advanced stage of colorectal cancer and metastatic breast cancer [1, 2]. It has short tenure of elimination half-life of about 2 h [3]. It is available in the market as film coated tablet. It is highly unstable in acidic medium. Its ultra-short elimination half-life may lead to increase in dosing frequency, side effect, and decrease in patient compliance [4].

This prodrug is tumor-activated to cytotoxic moiety as 5 FU by the enzyme thymidine phosphorylase. This enzyme is present more in tumor cells than in normal cells. The 5 FU is further activated to two active metabolites which cause cell injury by two different mechanisms firstly, by inhibition of DNA and secondly, by protein synthesis [5]. Our research was concentrated to fabricate and optimize the Cap-loaded [poly(lactic-co-glycolic acid)] PLGA-based nanoparticles by solvent shifting method implementing quality by design (QbD) platform which would boost drug availability at the target site with more cytotoxic potential to colon tumor cells.

PLGA is a US FDA-approved biodegradable and biocompatible co-polymer of lactic and glycolic acid [6]. The most common method for developing PLGA nanoparticles is by solvent shifting method, which may lead to low entrapment efficiency for hydrophilic drugs as reported in the literature.
[7], but of course it is not an issue for hydrophobic drugs. Hence, hydrophobic drug, i.e., Cap has been selected in our research.

The purpose of including Eudragit S 100 in the optimized batch is to prevent premature release in the gastric environment as it is a pH sensitive polymer.

Nanotechnology-based delivery of drugs has emerged as the most promising approach to address all the challenges associated with conventional delivery approach of drugs, and again, the polymeric nanoparticles have generated huge interest among researchers last few decades as an effective drug delivery carrier [8, 9]. There are different methods available for synthesizing nanoparticles, but solvent shifting method is widely accepted as it is the simplest technique of few steps [10, 11].

The optimization of formulation and process parameters for synthesis of nanoparticles is very complex when classical optimization technique was being adopted. In classical optimization process, one factor at a time was being considered for optimization which was very complicated, time-consuming, and tedious. In order to overcome the barriers raised due to classical optimization technique, a systematic, modern technique was adopted in our research known as QbD technique. This technique not only solves the problems of classical optimization technique but also has some additional benefits like taking into account the interaction among different independent variables and their impact on the critical quality attributes (CQAs). In the QbD technique, at first QTPP (Quality Target Product Profile) is fixed based on the desired quality to be built into the product. Based on the QTPP, CQAs are identified [12, 13]. The factors which influence the CQAs known as CMAs (critical material attributes) and CPPs (critical process parameters) are decided, and the impacts of CMAs and CPPs on CQAs are investigated [14, 15]. The different steps involved in QbD approach are summarized in Fig. 1. In our research from the preliminary investigation and knowledge gained through the literature reports, seven factors are identified and screened using Taguchi orthogonal array method to investigate the main effects by using licensed Design Expert version 8.0.6. From the Pareto chat and ANOVA table obtained from Taguchi method, three independent variables and three dependent variables are selected. Then the Box–Behnken design (BBD) is adopted to study the impacts of CMAs (Conc, of PLGA and Conc. of Poloxamer 188) and CPPs (amplitude of sonication) on CQAs (particle size, entrapment efficiency, and polydispersity index) and to optimize the nanoformulations. In BBD, seventeen nanoformulations are fabricated using solvent shifting technique [16, 17], and evaluated, and the results are analyzed. From the counter plots as well as 3D surface plots, the effects of independent variables on dependent variables are interpreted, and finally, the formulations are optimized using numerical as well as graphical technique. The overlay plot obtained during optimization revealed that the operating conditions are coming under the design space. The optimized formulation is used for cytotoxicity study using HT 29 cell lines and in vivo study by using Rabbit model.

![Fig. 1 Outline for stepwise elements of QbD for fabrication of PLGA based nanoparticles](image)

### Table 2 Factors under investigation coded value and corresponding actual value as per BBD

| Factor under investigation | Levels          | Low (−1) | Medium (0) | High (1) |
|---------------------------|-----------------|----------|------------|----------|
| PLGA conc. (X1)           |                 | 30 mg    | 40 mg      | 50 mg    |
| Conc. of Poloxamer 188 (X2) |                 | 0.5%     | 1.0%       | 1.5%     |
| Amplitude of Sonication (X3) |                 | 40       | 50         | 60       |
Materials and methods

Materials

Capecitabine, Poloxamer 188, and PLGA (Lactide: Glycolide 50:50) were received as a gift sample from Ra Chem Pharma Ltd, Hyderabad, India. The acetone and mannitol were purchased from Sigma Aldrich, India. All other chemicals used in the experiment were of high purity. The double distilled water was used throughout the experiment.

Method of preparation of PLGA-based nanoparticles

The nanoparticles were prepared by modified solvent shifting method [18–20]. Briefly, required amount of Cap and PLGA as depicted in Tables 1 and 2 were

| Sl no | Batch code | Conc. of PLGA (mg) | Conc. of Poloxamer 188 (%) | Amplitude of Sonication (%) | Particle Size (nm) | %EE | PDI |
|-------|------------|-------------------|---------------------------|---------------------------|-------------------|-----|-----|
| 1     | NP1        | 0.00              | −1.00                     | 1.00                      | 218.6±5.5         | 65.08±5.25 | 0.662±0.022 |
| 2     | NP2        | −1.00             | 1.00                      | 0.00                      | 198.5±3.5         | 58.09±3.51 | 0.711±0.012 |
| 3     | NP3        | 1.00              | 0.00                      | −1.00                     | 230.04±6.5        | 70.55±2.25 | 0.255±0.015 |
| 4     | NP4        | −1.00             | 0.00                      | −1.00                     | 199.95±3.2        | 59.01±3.55 | 0.69±0.005  |
| 5     | NP5        | 1.00              | 0.00                      | 1.00                      | 219.55±5.8        | 71.55±4.55 | 0.184±0.023 |
| 6     | NP6        | 1.00              | 1.00                      | 0.00                      | 220.05±4.9        | 70.08±3.65 | 0.195±0.013 |
| 7     | NP7        | 1.00              | −1.00                     | 0.00                      | 199.85±5.0        | 72.01±4.26 | 0.17±0.019  |
| 8     | NP8        | 0.00              | −1.00                     | −1.00                     | 219.01±5.5        | 66.98±3.15 | 0.573±0.025 |
| 9     | NP9        | 0.00              | 0.00                      | 0.00                      | 218.45±5.2        | 65.02±4.05 | 0.515±0.014 |
| 10    | NP10       | −1.00             | −1.00                     | 0.00                      | 194.75±6.0        | 57.98±3.09 | 0.915±0.025 |
| 11    | NP11       | 0.00              | 1.00                      | 1.00                      | 217.5±5.8         | 59.01±3.35 | 0.88±0.015  |
| 12    | NP12       | 0.00              | 0.00                      | 0.00                      | 218.09±3.9        | 58.11±4.15 | 0.538±0.032 |
| 13    | NP13       | 0.00              | 0.00                      | 0.00                      | 234.27±4.6        | 56.95±2.95 | 0.592±0.018 |
| 14    | NP14       | 0.00              | 0.00                      | 0.00                      | 217.98±5.9        | 57.75±2.99 | 0.851±0.021 |
| 15    | NP15       | 0.00              | 0.00                      | 0.00                      | 219.99±4.0        | 58.22±3.26 | 0.577±0.023 |
| 16    | NP16       | −1.00             | 0.00                      | 1.00                      | 200.05±4.5        | 59.09±3.28 | 0.566±0.013 |
| 17    | NP17       | 0.00              | 1.00                      | −1.00                     | 218.02±3.5        | 59.08±2.27 | 0.161±0.011 |

All observed results were presented as mean ± standard deviation, where n = 3

Table 3 Quality target product profile (QTPP) for nanoformulations

| QTPP                                | Target                          | Justification                                |
|-------------------------------------|---------------------------------|----------------------------------------------|
| Dosage form                         | Nanoformulations                | To improve stability and bioavailability    |
| Route of administration             | Oral                            | Self medication, non-invasive                |
| Physical form                       | Lyophilized powder              | Elegant appearance and stable                |
| Physicochemical characterization     | Entrapment efficiency           | To ensure drug loading                       |
|                                     | Particle size                   | To enhance bioavailability                   |
|                                     | Zeta potential                  | To ensure stability                          |
|                                     | PDI                             | Uniform dispersibility                       |
|                                     | Drug release                    | Influence pharmacokinetics of drug           |
| API solubility in carrier system    | High up to 50%                  | Influence on drug release pattern along with therapeutic effect |
| Stability                           | Quality requirement             | Influence on quality of the product          |
| Container and closer system         | Appropriate for the dosage form | Ensuring target shelf life                   |
| Pharmacokinetics                    | Absorption                      | Required for desired efficacy of the drug    |
|                                     | Distribution                    |                                              |
|                                     | Metabolism                      |                                              |
|                                     | Targeting                       |                                              |
dissolved in 10 ml of acetone which constitutes the organic phase. Then 1 mL of organic phase was directly injected into the 19 mL of aqueous phase containing 1% Poloxamer 188 as stabilizer and followed by stirring using a magnetic bead at required rpm for 1 h in order to remove acetone followed by sonication at 60% amplitude for 3 s for producing uniform dispersion and size reduction. Then the nanosuspension was filtered and subjected to lyophilization using mannitol as cryoprotectant at −50 °C temperature and 0.045 Torr of pressure. The lyophilized products were stored in desiccator until further used.

The optimized batch was prepared same as the above mentioned method except Eudragit S100 was added as same quantity as PLGA.

### Quality Target Product Profile and critical quality attributes

QTPP is the predestined expected attributes of drug product in a nutshell, prerequisite to establish desired quality with respect to safety and efficacy as well as promotes diagnosing CQAs of the product [21]. QTPP was resolved based on regulatory aspects, scientific aspects, practical aspects, and risk assessments as portrayed in Table 3. CQAs are evolved from the QTPP and are employed to navigate the product and process development and also associated with in process materials, i.e., CMAs as well as process parameters, i.e., CPPs for fabricating nanomaterials [22, 23]. List of most commonly critical quality attributes (CQAs) for nanoformulations was presented in Table 4.

**Table 4** List of most commonly critical quality attributes (CQAs) for nanoformulations

| CQAs                     | Target          | Is this a CQA | Justications                                                                 |
|--------------------------|-----------------|---------------|-----------------------------------------------------------------------------|
| Particle size            | <250 nm         | Yes           | Small size of particles ensure more absorption and different cancer cell targeting |
| Zeta potential           | >± 25 mV        | Yes           | Ensures stability of globules                                                |
| PDI                      | <1              | Yes           | Ensures uniform dispersibility                                               |
| Entrapment Efficiency    | As high as possible | Yes     | Required for desired dose delivery, sustain release, reduce volume of administration and maximize therapeutic efficacy |
| Drug Release             | Prolong release(>12 h) | Yes   | Slow and predetermined release to attain prolonged drug absorption          |

Quality Target Product Profile and critical quality attributes

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**Table 3** Ishikawa Fishbone blueprint exhibiting cause-effect relationships among the process and formulation variables for the fabrication of PLGA-based nanoparticles

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**Fig. 2** Ishikawa Fishbone blueprint exhibiting cause-effect relationships among the process and formulation variables for the fabrication of PLGA-based nanoparticles
Establishment of Risk by implementing Fishbone diagram

For the recognition of critical material attributes and critical process parameters for the preparation of PLGA-based nanoparticles and to figure out their influence on critical quality attributes (CQAs), a fishbone diagram was drawn [24] and portrayed in Fig. 2. From the fundamental scientific knowledge gained through the literature survey, particle size, polydispersity index, and entrapment efficiency were assigned as CQAs because these factors have potential influence on the therapeutic efficacy of drug-loaded nanoparticles [14]. Fishbone diagram exhibited twelve manufacturing and process variables which have tendency to affect the properties of the nanoparticles, and hence, these factors are considered for further investigations.

Qualitative assessment of risk

Quantitative assessment of risk

Quantitative assessment of risk was carried out by implementing FMEA (failure mode effective analysis) in order to track likelihood of failure mode native to drug product out [26]. Basing on the impact of each CMA on the drug product, the ranking scores from 1 to 10 were allocated to each CMA for severity, detectability, and risk occurrence, to compute RPN, i.e., risk priority number, which is calculated from the following formula and presented in Table 8.

\[
RPN = \text{Severity}(S) \times \text{Occurrence}(O) \times \text{Detectability}(D)
\]  

The Taguchi design was implemented to the factors native to high RPN for factor screening studies.

Experimental design

Taguchi screening design was adopted for scrutinizing the diagnosing factors affecting QTPP. Taguchi screening

| Table 5 Critical material attributes and risk assessment for nanoformulations |
|-------------------|-----------------|-------------------|
| **Drug product CQAs** | **Critical material attributes** | **Stabilizer concentration (Poloxamer 188)** |
| | Drug | Polymer (PLGA) |
| Particle size | Low | Medium | High |
| PDI | Low | Medium | High |
| Zeta potential | Low | Medium | High |
| Entrapment Efficiency | High | High | Medium |
| Drug Release | Medium | High | Low |

| Table 6 Justification for the initial risk assessment of the material attributes |
|-------------------|-------------------|-------------------|
| **Drug product CQAs** | **Critical material attributes** | **Justifications** |
| | Drug | Polymers | Stabilizer |
| Size/PDI | Drug has no effect on size of particles as it is dispersed in polymeric solutions | The size of particles may vary with concentration of polymers | The size of particles decreases with increase in concentrations of stabilizer as it stabilizes the nanoformulations and prevents aggregations |
| Entrapment efficiency | The physicochemical properties of the drug may influence the entrapment efficiency. The more the lipophilicity of the drug the more is the chances of encapsulation | The optimum level of polymers required for maximizing encapsulation | The stabilizer has little effect on encapsulation |
| Drug release | The properties of drug have medium effect on drug release | The polymers have much influence on drug release as more is the concentration of the polymers less is the drug release | The stabilizer has no effect on drug release |
| Zeta potential | The drug has little effect on zeta potential | The charge of polymers influences zeta potential up to certain extent | The stabilizer has crucial role on zeta potential as it imparts charge |
design was performed with seven factors at two levels and eight runs using design expert 8.0.6 software. The factors selected for Taguchi design were detailed with their levels in Tables 9 and 10. The values of low and high levels were fixed based on preliminary experiment as well as previously reported literatures [27, 28]. Some potential factors such as Eudragit S100 amount, and solvent types were not taken in experimental design in order to avoid unnecessary complications during manufacturing step. The responses evaluated for screening design were particle size, entrapment efficiency, and PDI. The obtained data was analyzed by using ANOVA as well as linear regression to investigate the influence of each independent variable, i.e., CMAs and/ or CPPs on responses.

**Optimization of PLGA-based nanoparticles by solvent shifting method using Box-Behnken design**

By using Taguchi design and other qualitative as well as quantitative methods, the three factors were screened and their major impact on the selected responses were observed and subjected to optimization by employing Box-Behnken design at three levels [29]. The three levels (−1, 0, 1) were fixed from the preliminary experiment. Four factors which were evaluated by Taguchi were kept at fixed level, as their influence on CQAs were found to be statistically insignificant considering the ANOVA results and Pareto charts from Taguchi model.

**Enactment of design space and optimization**

The design space was endorsed from the prediction plot obtained from the results of Taguchi design as well as FMEA. The software suggested a batch taking into account desired values of responses. The software suggested formula was fabricated and subjected to evaluation for desired responses. Each predicted response is compared with observed response. The aforesaid process was echoed for BBD[30, 31].

**Design space robustness analysis**

The optimized batch suggested by the software for Taguchi design and BBD were fabricated thrice each for confirmation of reproducibility and lack of variation.

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**Table 7** Justification of the initial risk assessment for manufacturing process

| Critical process attributes | Drug product CQAs | Justification |
|----------------------------|------------------|---------------|
| Probe sonication amplitude | Size/PDI         | Size is influenced by the amplitude of probe sonication |
|                            | Entrapment Efficiency | Size reduction may lead to poor drug encapsulation due to leaching in case of hydrophilic drugs |
|                            | Drug Release      | Size reduction has no influence on drug release |
|                            | Zeta potential    | Zeta potential is seldom affected by size reduction |
| Size reduction time        | Size/PDI         | Sonication time highly influences the particle size |
|                            | Entrapment Efficiency | Entrapment efficiency seldom affected by sonication time |
|                            | Drug Release      | Drug release seldom affected by sonication time |
|                            | Zeta potential    | Zeta potential seldom affected by sonication time |
| Stirring speed             | Size/PDI         | The more is the stirring speed the lesser is the particle size and less stirring speed leads to agglomeration |
|                            | Entrapment Efficiency | Entrapment efficiency is not influenced by the stirring speed |
|                            | Drug Release      | The drug release is not influenced by stirring speed |
|                            | Zeta potential    | The Zeta potential is seldom affected by stirring speed |

**Table 8** Summary of failure mode effective analysis (FMEA) showing RPN scores for various formulation and process parameters influencing the CQAs

| Sl no | Failure mode          | S (severity) | O (occurrence) | D (detection) | RPN (SOD) | Failure effect  |
|-------|-----------------------|--------------|----------------|---------------|-----------|----------------|
| 1     | PLGA conc             | 7            | 6              | 6             | 252       | PS, EE, PDLZP  |
| 2     | PLGA:Eudragit S100   | 5            | 6              | 4             | 120       | PS, EE         |
| 3     | Stirring time         | 7            | 5              | 5             | 175       | EE, PS         |
| 4     | Stirring speed        | 6            | 3              | 4             | 72        | EE, PS         |
| 5     | Sonication time       | 5            | 6              | 3             | 90        | PS, PDI, ZP    |
| 6     | Sonication amplitude  | 8            | 5              | 5             | 200       | PS, PDI        |
| 7     | Conc. of Poloxamer 188| 8            | 6              | 5             | 240       | PS, PDI, EE    |
Characterization of PLGA-based nanoparticles

Measurement of particle dimension (size), polydispersity index, and zeta potential

The nanosuspension was 10 times diluted and subjected to analysis for particle dimension (size), PDI and ZP by the phenomenon of dynamic light scattering (DLS) and electrophoresis light scattering employing Malvern Zetasizer.

Estimation of entrapment efficiency

The nanosuspension was centrifuged at 10,000 rpm for 25 min to sediment the nanoparticles. The clear supernatant was collected and suitably diluted and analyzed for free Cap content by using UV spectroscopy at 273 nm. The % EE was calculated using the following formula

\[
\text{Entrapment efficiency (EE)} = \frac{\text{Total amount of Capecitabine} - \text{Free Capecitabine}}{\text{Total amount of Capectabine}} \times 100
\]

In vitro drug release study

The dialysis membrane with molecular weight layoff 12–14 kDa purchased from HiMedia Laboratories Pvt. Ltd. was kept in phosphate-buffered solution pH 7.4 throughout the night before day of the experiment. Then, the dialysis membrane was opened and 5 mL of nanosuspension was kept inside, and both the ends of the dialysis membrane were closed by dialysis clips and suspended in the dissolution medium. USP II dissolution apparatus was used to carry out the experiment [32]. The same procedure was followed for Cap in pure form by taking 10 mg of the drug in 5 mL water. The study was performed using 500 mL phosphate buffer (pH 7.4) and maintaining temperature at 37 °C ± 0.5 °C and speed of paddle at 50 rpm. The fixed quantity of sample (2 mL) was withdrawn at predestined interval of time and replaced with 2 mL of freshly prepared phosphate-buffered solution pH 7.4 in order to keep the sink condition up. The first 2-h dissolution study was performed using 0.1 N HCl instead of phosphate-buffered solution pH 7.4 as the same way as mentioned in the above process. The samples were subjected to recovery, filtration, suitable dilution, and analysis by high-performance liquid chromatographic technique.

Fourier transform infrared spectroscopy

Translucent KBr (potassium bromide) pellets were prepared by mixing and triturating PLGA-based nanoparticles with KBr and pressing in a mechanical press and analyzed with pure KBr background. The FT-IR spectra were recorded within 400 to 4000 cm⁻¹ having resolution of 4 cm⁻¹.

Table 9 Formulation and process variables along with their high and low levels scrutinized utilizing Taguchi design

| Runs | PLGA conc | PLGA:Eudragit S100 | Stirring time | Stirring speed | Sonication time | Sonication amplitude | Conc. of Poloxamer 188 |
|------|-----------|--------------------|---------------|---------------|-----------------|----------------------|-------------------------|
| 1    | −1        | +1                 | +1            | +1            | +1              | −1                   | −1                      |
| 2    | +1        | +1                 | −1            | −1            | +1              | +1                   | −1                      |
| 3    | +1        | −1                 | +1            | −1            | +1              | −1                   | +1                      |
| 4    | +1        | +1                 | −1            | +1            | −1              | +1                   | +1                      |
| 5    | −1        | −1                 | −1            | −1            | −1              | −1                   | −1                      |
| 6    | −1        | +1                 | +1            | −1            | +1              | +1                   | −1                      |
| 7    | +1        | −1                 | +1            | +1            | +1              | +1                   | +1                      |
| 8    | −1 +1     | −1                 | +1            | +1            | +1              | +1                   | +1                      |

Table 10 Factors under investigation coded value and corresponding actual value

| Factor under investigation | Levels     |
|----------------------------|------------|
| Low (−1)                   | High (1)   |
| PLGA conc                  | 30 mg      | 50 mg      |
| PLGA:Eudragit S100         | 1:1        | 1:5        |
| Stirring speed             | 300 rpm    | 1500 rpm   |
| Stirring time              | 0.5 h      | 2 h        |
| Sonication time            | 2 s        | 5 s        |
| Sonication amplitude       | 30%        | 90%        |
| Conc. of Poloxamer 188     | 0.5%       | 1.5%       |
Fig. 3 Pareto charts for screening factors having major impact on CQAs ((R1) = particle size, (R2) = entrapment efficiency (R3) = PDI) as predicted by Taguchi design.
Differential scanning calorimetry

Approximately 3–5 mg of sample was weighed and analyzed by DSC using DSC-60 (Shimadzu) with TW-60 collection software. The measurements were performed under inert nitrogen atmospheres with a flow rate of 100 mL/min. The samples were heated at 10 °C/min from 30 to 300 °C. An empty aluminum pan was employed as a reference standard.

Transmission electron microscopy of optimized PLGA-based nanoparticles

Transmission electron microscopy of optimized nanoparticles was performed by placing one drop of diluted nanosuspension on copper grid with subsequent staining with 0.1% phosphotungstic acid operating at 90.0 kv. Magnified image was taken using AMT camera system.

Fig. 4 Contour and 3D response surface plots exhibiting the impact of factors, i.e., A amount of PLGA, B Conc. of Poloxamer, and C amplitude of sonication on responses, i.e., R1 = particle size, R2 = % EE, and R3 = PDI of PLGA-based nanoparticles, respectively
In vitro cytotoxicity studies

The cytotoxicity of optimized Cap-loaded PLGA-based nanoparticles was investigated by MTT test to assess the cytotoxicity potential of the formulation [23, 33]. The HT 29 cell lines were seeded in 96-well plates at a density of $1.25 \times 10^4$ cells/well and incubated for 24 h to allow sufficient adhesion. The different concentrations of Cap-loaded PLGA-based nanoparticles and the pure Capecitabine were added to grown cells in 96-well plates in three replicates and incubated for 24 h.

After 24-h incubation, cells were washed with phosphate buffer solutions and 15-µL MTT dye solution was added to each well. The plates were incubated at 37 °C and 5% CO$_2$ for additional 3 h. The medium was discarded, and the formazan crystals were solubilized by adding DMSO to dissolve etrazonium dye. The optical density of each well was measured at 570 nm in a micro plate reader. The absorbance of untreated culture was set at 100%.

In vivo pharmacokinetic studies

The experiment on animal was carried out on male rabbits weighing 1.5 kg and procured by Roland institute of pharmaceutical sciences from Saha enterprises, 386/2 Nilachal, Birati, Kolkata-700051, India. Registration No-1828/PO/Bt/S/15/C PCSEA. The designed scientific animal experimentation procedure was reassessed and sanctioned by Roland institute of pharmaceutical sciences, Berhampur, Institutional Animal Ethical Committee (IAEC), RIPS, Berhampur (IAEC/RIPS/04/2016) on dated 15/07/2016. The all experiments related to animals were conducted as per the Committee for the Purpose of command and surveillance on Experiments on Animals and the animal attention directives of RIPS, Berhampur, IAEC.

Fig. 5 Overlay plot for the impact of various autonomous variables on the dependent variables, i.e., (R1) average particle size, (R3) polydispersity index, and (R2) entrapment efficiency

Fig. 6 Linear correlation plots between the actual and predicted values of various CQAs, (R1) particle size, (R2) % entrapment efficiency, and (R3) polydispersity index (PDI)
Study design and blood sample collections

The rabbits were randomly selected and placed in two groups having 3 rabbits in each group. Both groups were administered orally with Cap suspension containing 10 mg pure drug and Cap nanosuspension equivalent to 10 mg of Cap (optimized nanoformulations) respectively. Blood samples were collected from the marginal ear vein of each rabbit into microcentrifuge tubes and allowed to coagulate followed by centrifugation for 15 min at 2500RPM using cooling centrifuge and the supernatant was collected. The drug was estimated from the collected serum by using HPLC technique using reported method of development [34].

Results

Assessment of risk using fishbone diagram

A fishbone diagram was constructed for identifying most possible risk factors associated with formulation as well as process variables on the critical quality characteristics of Cap-loaded PLGA-based nanoparticles, i.e., average particle size, polydispersity index, and percentage entrapment efficiency. Seven most possible risk factors were identified and subjected to further evaluation using Taguchi design.

Analysis of risk and optimization

Taguchi design with seven factors at two levels was applied to select most important process and formulation factors by taking into account only the main effects. The formulations in Taguchi design with their limits were portrayed in Tables 9 and 10 and the pareto charts for screening factors as portrayed in Fig. 3. The final formulation was optimized by implementing Box-Behnken design (BBD). The leading and dealing effects of tri-independent variants (CMAs and CPPs) on CQAs were evaluated. The effect of independent variables on responses was computed employing polynomial equation from ANOVA analysis as well as three dimensional response surface plot produced by the Design Expert as presented in Fig. 4.

The polynomial equations for particle size (R1), EE (R2), and PDI (R3) were presented as follows.

\[
\text{PS(R1)} = +221.76 + 12.03 \times A + 0.23 \times B - 1.41 \times C - 0.89 \\
+ A \times B - 2.65 \times A \times C - 0.028 \times B \times C - 9.68 \\
+ A^2 - 3.79 \times B^2 + 0.32 \times C^2
\]  

\[
\text{EE(R2)} = +62.62118 + 6.25250 \times A - 1.97375 \times B - 0.11125 \times C
\]  

\[
\text{PDI(R3)} = +0.53 - 0.26 \times A - 0.047 \times B + 0.077 \times C
\]  

(4)

where A, B, and C are concentration of PLGA, concentration of Poloxamer 188, and amplitude of sonication, respectively.

![Fig. 7 Linear correlation plots between the normal and residual values of various CQAs, (R1) particle size, (R2) % entrapment efficiency, and (R3) polydispersity index (PDI)](image)
Selection of model for particle size

The quadratic model for particle size was found to be significant having $F$ value 6.07 and $p$ value 0.0134. The coefficient of regression with a $p < 0.05$ implies that the model terms are significant, whereas the values $> 0.1$ are insignificant. The predicted $R^2$ value of 0.06942 is very close with the adjusted $R^2$ value of 0.7403 which again indicates the model is significant.

Selection of model for entrapment efficiency

The linear model for EE was found to be significant having $F$ value 9.21 and $p$ value 0.0016. The coefficient of regression with a $p < 0.05$ implies that the model terms are significant, whereas the values $> 0.1$ are insignificant. The predicted $R^2$ value of 0.5232 is very close with the adjusted $R^2$ value of 0.6061 which again indicates the model is significant.

Selection of model for polydispersity index

The quadratic model for particle size was found to be significant having $F$ value 6.11 and $p$ value 0.0080. The coefficient of regression with a $p < 0.05$ implies that the model terms are significant, whereas the values $> 0.1$ are insignificant. The predicted $R^2$ value of 0.2328 is not close with the adjusted $R^2$ value of 0.4895 which may be attributed due to large block effect.

Optimization of critical material attributes along with critical process parameters with respect to critical quality attributes

The software was fed with the targeted criteria to accomplish the software suggested architecture. One of the software suggested solutions was selected based on the desirability value as a design space and was practically applied for its authentication as portrayed in Fig. 5 and the linear correlation plots between (actual versus predicted) and normal versus residuals were presented in Figs. 6 and 7, respectively. The software predicted desirability value was found to be 0.98 which assures 98% predictability to work out the destination with revamped CMAs and CPPs. The more is the value of desirability, the more is the chance to obtain the goal. A formulation was fabricated by taking optimized quantities of CMAs and CPPs, and its CQAs were scrutinized. The particle size (Z-average), % EE, and PDI value of optimized formulation were found to be 195 nm, 75%, and 0.214, respectively. As the particle size is under 200 nm, the cellular uptake will be enhanced because the cellular uptake depends on the particle size. The entrapment efficiency is less which may be attributed due to hydrophobic nature of PLGA. The PDI value $< 0.25$ signifies that the particles are of uniform size.

![Image of average particle size, PDI, and zeta potential of optimized PLGA-based nanoparticles](image)

![Image of cumulative percent drug release versus time in h of cap and optimized nanoformulations](image)
Measurement of zeta potential

The zeta potential of the optimized formulation was found to be $-6.65$ as portrayed in Fig. 8. As the zeta potential value was found to be very less, the formulation may be less stable and may not in favor of biological uptake but it is forecasted to be secure as negatively charged particles decline the likelihood of cytotoxicity. Due to the presence of negative charge on the surface of the nanoparticles, they may undergo nonspecific adsorption to the cellular membrane.

Estimation of entrapment efficiency

The %EE of the optimized formulation was observed to be 72%. From the equation $\text{EE}(R^2) = +62.62118 + 6.25250 \times A - 1.97375 \times B - 0.11125 \times C$, it indicates that as the coefficients of $B$ and $C$ are negative, increasing the concentration of poloxamer and amplitude of sonication decreases the value of EE, whereas as the coefficient of $A$ is positive, increasing the conc. of PLGA increases the value of EE.

Estimation of dimensional analysis of nanoparticles and PDI

The average particle size and PDI value of the optimized nanoparticles were found to be 195 nm and 0.214, respectively, as portrayed in Fig. 8. From Eq. 2, it was observed that the conc. of PLGA has positive impact on particle size, but conc. of Poloxamer 188 and amplitude of sonication have negative impact on it, whereas from Eq. 4, it was observed that both the conc. of PLGA and Poloxamer 188 have negative impact and amplitude of sonication has positive impact on PDI.

In vitro drug diffusion study

USP Type II dissolution apparatus was used to carry out in vitro drug release studies. In order to maintain the

![Fig. 10 FTIR spectra of A pure drug, B physical mixture, and C PLGA](image)
desired temperature and simulate biological conditions throughout the experiment, this apparatus was preferred which is not possible in case of drug diffusion studies. It was observed that initially up to 2 h in 0.1 N HCl negligible quantity of drug released due to presence of Eudragit S100 as pH sensitive polymer followed by very slow rate of drug release up to 4 h in phosphate-buffered solution pH 7.4 which may be attributed due to slow diffusion of drug from the stagnant layer formed by the outermost layer PLGA. This observation revealed that drug was entrapped completely in the core nanoparticles. After 10 h, the rate of drug release was little bit faster and within 24 h up to 80% drug released which may be due to lack of combination of polymers. The cumulative percent release of drug versus time in h was presented in Fig. 9.

FT-IR studies

The drug-excipient compatibility studies as well as identification of drug were performed by means of FT-IR spectroscopy. The FTIR spectra of Cap, PLGA, physical mixture of Cap and PLGA(1:1), and Cap-loaded nanoparticles were portrayed in. The Capecitabine exhibited characteristic bands of different functional groups. The peaks at 3520 cm\(^{-1}\) and 3254 cm\(^{-1}\) corresponding to O–H and N–H vibrations, 1647 cm\(^{-1}\), 1713 cm\(^{-1}\), 1043.3, and 1248.6 cm\(^{-1}\), indicate pyrimidine carbonyl stretching vibrations and urethane carbonyl stretching vibrations, C-F stretching vibrations, and the presence of tetrahydrofuran ring, respectively. The major peaks found above confirms the structure of Cap which provides its identity. The FTIR spectra of optimized Cap-loaded nanoparticles revealed

Fig. 11  DSC Thermograms of A pure drug, B physical mixture, and C PLGA
the absence of major peaks of drug which confirms the better encapsulation of drug inside the nanoparticles, but it retains maximum peaks of PLGA and Poloxamer 188. Moreover, the presence of all major peaks of the components confirmed the drug and excipients compatibility as portrayed in Fig. 10.

**Differential scanning calorimetry**

The thermo grams of Cap exhibited an endothermic peak at 128 °C corresponding to its melting point (121 °C) and the thermo grams of Optimized nanoformulations exhibited peak at 127.95 °C. In the themograms of optimized lyophilized nanoformulation, there was no peak was observed at 128 °C which indicates there was no crystalline drug material in it which may be attributed due to complete entrapment of drug in the core of the nanoparticles. As the sharp endothermic peaks were observed in the pure drug as well as in the physical mixture of drug, PLGA and Poloxamer 188 at 128 °C which indicates drug and excipients are quite compatible with each other as presented in Fig. 11.

**Transmission electron microscopy**

The TEM study of the optimized nanoformulation revealed that all most all nanoparticles were of spherical in shape as presented in Fig. 12. Neither irregular-shaped nor rod-shaped particles were observed from TEM images which may be accounted for removing un-entrapped or
surface bound drug particles from nanoparticles by efficient washing.

**In vitro cytotoxicity studies**

The main reason of selection of HT29 cell lines to perform cytotoxicity studies was to simulate the colorectal cancer cells. From the cytotoxicity studies, it was found that the optimized formulation of Cap-loaded PLGA nanoparticles were more cytotoxic than the pure drug of Cap as portrayed in Fig. 13 and Table 11 which indicates that the optimized nanoparticles has more potential to kill the colorectal tumor cells.

**Pharmacokinetic studies**

Figure 14 illustrates the serum pharmacokinetic profile of Cap and the optimized nanoformulation of Cap. The pharmacokinetic parameters were computed by employing Microsoft excel sheet and presented in Table 12. The comparative pharmacokinetic study was carried out between the Cap dispersion (Pure drug) and the optimized nanoformulation loaded with Cap. The Cmax, Tmax, AUC, Cl, and MRT of Cap nanoformulation were found to be increased as compared to the pure drug of Capecitabine, which pointed out quick onset of action as well as long absorption phase of Cap from nanoformulation as compared to the pure drug whereas the values of t1/2, Vd, Ke, and Cl and MRT found to be decreased with Cap nanoformulation. The decrease in Vd reveals the enhancement of solubility of the drug after fabrication into nanoformulation. The increase in Cmax and AUC in case of nanoformulation suggests that the dose of Cap can be reduced in the form of nanoformulation.

**Discussion**

The PLGA was selected in our research as a sustained release polymer due to its biodegradability and biocompatibility nature [35]. Solvent shifting method employed in our research is based on the interfacial deposition of a polymer following shifting of a semi-polar solvent (acetone in our research) miscible with water from a lipophilic solution. The solvent shifting method was adopted as it is simple, involves few steps, and reproducible in nature. P.H. Rajasree et al.[4] prepared PLGA nanoparticle surface modified with chitosan by double emulsion solvent evaporation technique and encapsulated with Eudragit S100 to target colon. Fishbone diagram followed by Taguchi screening design was used to minimize variables and to screen potentially influencing QTPP factors. Box-Behnken design was adopted for optimization of PLGA-based nanoparticulate formulations. From the FTIR studies, it was found that there is no significant shift of peaks or additional peaks found in case of optimized formulation from that of pure drug. From the DSC studies, the endothermic peak of both the drug as well as the

![Fig. 13 Cytotoxicity studies of pure drug and optimized PLGA-based nanoparticles as % inhibition vs concentration curve](image-url)

![Table 11 In vitro cytotoxicity assay of Capecitabine and Capecitabine-loaded PLGA-based nanoparticles against HT29 cell lines](table-url)
optimized formulation was found to be approximately same. Hence, from the FTIR studies as well as from DSC studies, it was confirmed that there is no interaction between drug and other excipients. The TEM study of the optimized nanof ormulation revealed spherical shape of all most all nanoparticles. Neither irregular-shaped nor rod-shaped particles were observed from TEM images which may be accounted for removing un-entrapped or surface bound drug particles from nanoparticles by efficient washing. The free Cap as well as Cap-loaded PLGA nanoparticles at different concentrations significantly reduced HT 29 cell lines in a dose-dependent manner, but the drug-loaded nanoparticles killed HT 29 cell lines more significantly as compared to free drug. The Cap-loaded nanoformulations were evaluated for other cell lines vis-à-vis MCF7 and PANC1. R. Nazari-Vanani et al. [36] studied cytotoxicity effect of CAP and CAP-loaded nanoniosomes using MCF7 and PANC1 cell lines and reported the toxicity of drug in the nanoniosomes to be higher than the free drug. The half maximal inhibitory concentration (IC50) of CAP-Span 20 against MCF7 and PANC1 cell line was obtained as 69.11 µg/mL and 128.90 µg/mL, respectively. Surface modified PLGA nanoparticles loaded with cap for effective delivery of the drug to the prostate were reported by Shu Ben Sun et al. [37]. Rajiv Kumar et al. [38] synthesized method for PEGylated PLGA nanoparticles encapsulating anti-cancer drug doxorubicin for cancer imaging and therapy. And they provided simple and robust PLGA-based platform for efficient drug delivery and imaging of cancer cells in vitro and in vivo. Kuldeep Nigam et al. [39] developed lamotrigine (LTG)-loaded PLGA nanoparticles and analyzed in vitro cytotoxicity using MTT assay and reported dose-dependent cytotoxicity for developed LTG-PLGA-NPs.

In our research, we have implemented QbD approach for developing PLGA and Eudragit S100-based nanoparticles by solvent shifting method for colon targeting for effective management of colorectal cancer which is supported by in vitro cytotoxicity studies using HT 29 cell lines for the first time which is not reported earlier. In our research, we

**Table 12** Pharmacokinetic parameters of Capecitabine and Capecitabine nanosuspension after oral administration

| Pharmacokinetic parameters | Pure drug (Capecitabine) | Capecitabine loaded nanosuspension |
|----------------------------|---------------------------|-----------------------------------|
| \( C_{max} \) (mcg/mL)     | 0.0583 ± 0.004            | 0.0898 ± 0.005                    |
| \( T_{max} \) (h)          | 6 ± 1.59                  | 4.5 ± 1.41                        |
| \( AUC_{0-\infty} \) (mcg h/mL) | 0.4247 ± 0.06           | 1.1367 ± 0.08                     |
| \( MRT_{0-\infty} \) (h)   | 10.39 ± 1.75              | 11.27 ± 1.76                      |
|\( Ke \) (1/h)              | 3.336817 ± 0.12           | 6.457382 ± 0.22                   |
|\( T1/2 \) (h)              | 0.207683 ± 0.03           | 0.107319 ± 0.04                   |
|\( Vd \) (mL)               | 398.1988 ± 10.55          | 29.10717 ± 1.95                   |
|\( Cl \) (mL/h)             | 97.12529 ± 2.56           | 187.9561 ± 4.59                   |

Data conferred as mean ± SEM, n = 3

\( C_{max} \) peak plasma concentration, \( T_{max} \) time to achieve peak plasma concentration, \( Ke \) elimination rate constant, \( Vd \) volume of distribution, \( AUC_{0-\infty} \) area under the curve from time of administration to infinite time, \( Cl \) clearance, \( Vd \) volume of distribution, \( T1/2 \) elimination half life

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Fig. 14 Mean serum concentration vs. time profiles obtained after oral administration of Capecitabine and optimized PLGA-based nanosuspension to rabbit respectively.
used solvent shifting method for preparing NPs which is simple, a single-step manufacturing technique, easily scalable, and cost-effective as compared to other techniques reported before. We employed HT 29 cell lines for in vitro cytotoxicity studies as these cell lines are human colorectal adenocarcinoma cell line, better and reliable result can be obtained. In the cytotoxicity studies by MTT assay, % inhibition of Cap-loaded Nps was found to be 73.16% at 10 µg/mL in our research where as previously reported by Shu-Ben Sun et al.[37] was 75.42 ± 4.02% at 1000 µg/mL. Hence, our nanoformulations are better optimized using QbD approach as compared to previously reported optimizations by other authors.

### Conclusion

The investigation made by us successfully testify the application of logical QbD approach in the systematized improvement of optimized nanoformulations by solvent shifting method exploiting elementary, adequate and cost-effective PLGA-based nanoparticles for promising delivery of Capecitabine for effective treatment of colorectal cancer. Exertion of QbD-assisted approach guided in screening relevant formulation and process parameters for fabrication of nanoparticles and consequently attaining optimum drug delivery with required amount of drug at colonic site. To such a degree the in vitro drug release study, the in vitro cytotoxicity studies using HT 29 cell lines as well as in vivo pharmacokinetic studies endorse the controlled release and cytotoxic potential to tumor cells for once a day administration bringing about efficient, secure, and patient centric and patient compliant outcomes. The future in vivo cytotoxicity studies using suitable animal model will be undertaken in order to investigate the anti tumor potential of the optimized Capecitabine-loaded PLGA-based nanoparticles.

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### Author contribution
All authors contributed to conduct the experiment, testing, and the writing of this manuscript.

### Availability of data and materials
Not applicable.

### Declarations

#### Ethics approval and consent to participate
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### Competing interests
The authors declare no competing interests.

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