Materials and Methods

METHODOLOGY

4.1. Materials

- Chemicals
- Equipments
- Animals and cell lines

4.2. Preformulation studies

- Physical observation
- FTIR interpretation
- Analytical method development (UV/HPLC)
- Standard calibration curve

4.3. Formulation of polymeric nanoparticles

- ❖ Selection of manufacturing method
- ❖ Polymeric nanoparticles preparation
- PHBV PVA polymeric nanoparticles (PHBV-PVA-DTX)
- PHBV PF127 polymeric nanoparticles (PHBV-PF127-DTX)
- PHBV TPGS polymeric nanoparticles (PHBV-TPGS-DTX)
- PHBV PLH PE-PEG polymeric nanoparticles (PLH-PEG-DTX)
- ❖ Formulation optimization
  - Identification of critical quality attributes (CQA)
  - Ishikawa fishbone diagram
  - Selection of critical material attributes (CMA)
  - Selection of critical process parameters (CPP)

- Control impact matrix (CIM)
- Critical to quality (CTQ) identification
- Failure mode effect & critical analysis (FMECA)
- Impact of CMA and CPP on CQA
- Placket-Burman design (PBD)
- Box-Behnken design (BBD)

4.4. Characterization

- Particle size (Zeta sizer)
- Surface charge (Zeta potential)
- Morphology (SEM, TEM, and AFM)
- Entrapment efficiency (%EE)
- Drug-excipient interaction analysis (FTIR/DSC)
- Analysis of crystallinity (XRD)

4.5. Drug release studies (*In vitro*)

4.6. Cytotoxicity assessment (MCF7 by SRB assay)

4.7. Cellular uptake (confocal microscopy)

4.8. Platelets aggregation study

4.9. Pharmacokinetic studies

4.10. *In silico* and *ivivc* studies

4.11. *In vivo* anticancer activity (C3H/J strain mice)

4.12. Drug-plasma interaction and hemolysis studies

4.13. Stability studies

4.14. Statistical analysis

4.1 Materials

The following are the major chemicals, instruments, animals, softwares used in the various stages of experimental studies.

4.1.1 Chemicals

Sl. No.	Chemicals	Source
1	Docetaxel	Panacea Biotec, Baddi, India
2	PHBV	Sigma-Aldrich, USA
3	Polyvinyl alcohol (PVA)	Sigma-Aldrich, USA
4	PF 127 (Pluronic F-127)	Sigma-Aldrich, USA
5	Poly L-histidine (PLH)	Sigma-Aldrich, USA
6	Tocopherol PEG succinate (TPGS)	Hi Media, Mumbai, India
7	Ketamine	Harman Finochem, Mumbai, India
8	Heparin sodium injection	Biological E. Limited, India
9	Sodium chloride	SD Fine Chemicals, Mumbai, India
10	Calcium chloride	SD Fine Chemicals, Mumbai, India
11	Sodium carbonate	SD Fine Chemicals, Mumbai, India
12	PE block PEG (PE-PEG)	Sigma-Aldrich, USA
13	Acetonitrile	SD Fine Chemicals, Mumbai, India
14	Diethyl ether	SD Fine Chemicals, Mumbai, India
15	Dimethyl sulfoxide (DMSO)	SD Fine Chemicals, Mumbai, India
16	Sodium hydroxide	SD Fine Chemicals, Mumbai, India
17	EDTA	Hi Media, Mumbai, India
18	Ethyl acetate	Hi Media, Mumbai, India
19	Fluorescein iso-thiocyanate (FITC)	Hi Media, Mumbai, India
20	Acetonitrile (ACN)	Merck Ltd., Mumbai, India
21	Ammonium acetate	Hi Media, Mumbai, India
22	Hydrochloric acid	Hi Media, Mumbai, India

23	Sodium bicarbonate ($NaHCO_3$)	Hi Media, Mumbai, India
24	Dichloromethane	SD Fine Chemicals, Mumbai, India
25	Methanol	SD Fine Chemicals, Mumbai, India
26	Ethanol	Merck Ltd., Mumbai, India
27	Formaldehyde	Merck Ltd., Mumbai, India
28	Acetone	Merck Ltd., Mumbai, India
29	Potassium chloride	SD Fine Chemicals, Mumbai, India
30	Potassium dihydrogen phosphate	SD Fine Chemicals, Mumbai, India
31	Membrane filters (0.22 & 0.45 µm)	Hi Media, Mumbai, India
32	Fluorescein sodium	Hi Media, Mumbai, India
33	Amonium acetate	Hi Media, Mumbai, India
34	Glacial acetic acid	Merck Ltd., Mumbai, India
35	Disodium hydrogen phosphate	SD Fine Chemicals, Mumbai, India

4.1.2 Equipments

Sl. No.	**Instruments**	**Source**
1	Digital magnetic stirrer	IKA, Germany
2	Digital electronic balance	Dewinter, India
3	Digital pH meter	IKON Instruments, New Delhi, India
4	Digital microscope	Zeiss, Germany
5	Cooling centrifuge (ELTEK)	RC 4100F, Mumbai, India
6	Particle size analyzer (DELSA)	Beckman Coulter, USA
7	Electric oven	Cintex, Mumbai, India
8	Ultra probe sonicator	UP50H, Hielscher, USA
9	Microplate absorbance reader	Bio-Rad iMark™, USA
10	FTIR 8400S	Shimadzu, Japan
11	CO_2 incubator	Sanyo CO_2 Incubator, Japan
12	Sonicator (Bath type)	WUC, Fisher Scientific, India

13	Stability chamber	NSW-175, New Delhi, India
14	UV-Visible spectrophotometer	Shimadzu 1800, Japan
15	Vortex mixer	Fisher Scientific, India
16	HPLC	Shimadzu, Japan & Agilent, USA
17	Deep freezer	Thermo Scientific, USA
18	Zeta sizer	Beckman Coulter, USA
19	Micro pipettes	Thermo Scientific, USA
20	Differential scanning calorimeter	TA Q1000, USA
21	Powder x-ray diffractometer	Rigaku, Japan
22	Lyophilizer	Labconco, USA
23	FT-IR spectrophotometer	Shimadzu FTIR-8400, Japan
24	Scanning electron microscope	Carl Zeiss, USA
25	Atomic force microscopy (SPM)	NT-MDT, Russia
26	Syringe filter	Millipore, MA, USA
27	Transmission electron microscopy	TECHNAI-20G^2, Czech Republic
28	Dialysis membrane 135	12-14 kDa, Himedia, India
29	Fluorescence microscope	Nikon, Japan
30	NMR Spectrometer	Bruker, USA
31	SDS-PAGE	BioRad, USA
32	Tissue homogenizer	IKA Ultra-Turrax® (T25), Germany

4.1.3 Animals and cell lines

Sl. No.	Animals & Cell lines	Source
1	Charles Foster strain rat	Central Animal House, Banaras Hindu University, India
2	Swiss albino mice	Central Animal House, Banaras Hindu University, India
3	C3H / Jax mammary strain (female mice)	ACTREC, Mumbai, India
4	MCF7 (Breast cancer cell line)	ACTREC, Mumbai, India

4.1.4 Software

Sl. No.	Software used	Source
1	Minitab 17	Coventry CV3 2TE, UK
2	Design Expert 10	MN, USA
3	MODDE 12	Umea, Sweden
4	GastroPlus 9	NY, USA
5	GraphPad Prism 5	CA, USA
6	Origin Pro 8	MA, USA

4.2 Preformulation studies

The preformulation is the fundamental step in the rational development of a dosage form of a drug alone and when combined with excipients. The goal of preformulation includes:

- To establish the physicochemical parameter of new drug substances.
- To establish physical characteristics.
- To establish compatibility with the common excipients.

4.2.1 Physical observation

The morphological identification of drug was done by checking color, odor, taste and physical appearance. It was recorded and documented as evidence for comparing with the other batches during formulation.

Colour - White

Odor - Odourless

Taste - Mildly bitter

Physical appearance - Solid crystalline powder

Inference

The color, odor, taste and powder forms of the drug were physically examined and identified.

4.2.2 FTIR interpretation

The pure drug was combined with IR grade potassium bromide in the proportion of (1:100), and pellets were formed by employing ten metric ton pressure. The pellets were then scanned over a range of 4000-400 cm^{-1} by FTIR instrument (SHIMADZU 8400S, Japan). FTIR spectrum of the drug showed the presence of the peaks which complies with the reference spectra (Figure 4.1). FTIR studies investigate and predict any physicochemical interactions between components in the formulation and can, therefore, be applied to the selection of suitable chemically compatible excipients.

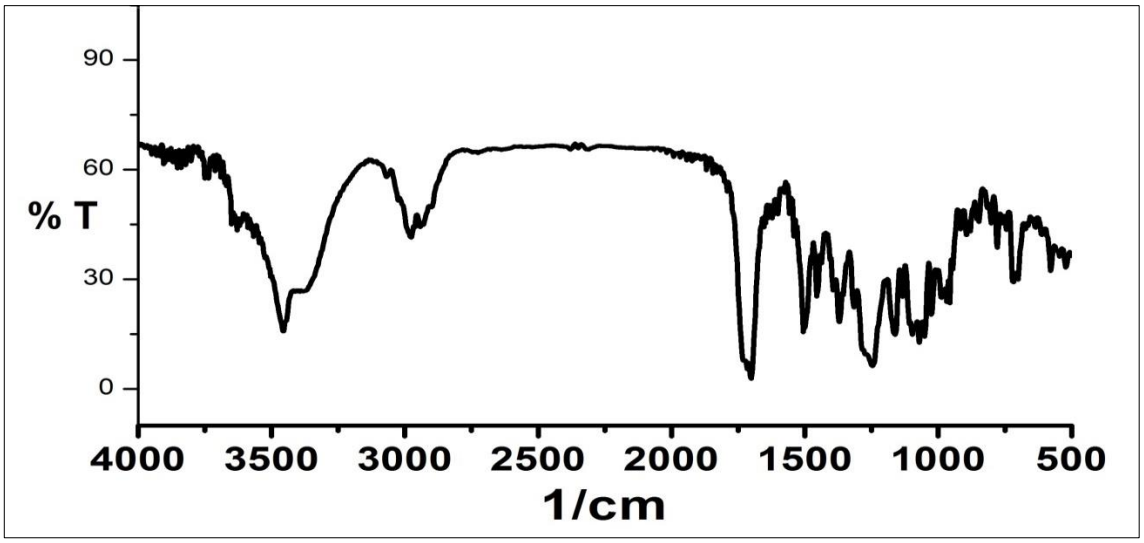

Figure 4.1 FTIR spectrum of docetaxel (sample drug)

Inference

The IR spectrum of drug sample had shown identical peaks as reported in references sample of docetaxel. The FTIR spectra were showing absorption bands as mentioned in Table 4.1.

Table 4.1 FTIR spectra obtained in the spectral region 4,000 to 400 cm^{-1}

Group	DTX (cm^{-1})
O-H stretching	3480
NH stretching	3437
C-H stretching	2979
C=O stretching	1711
C=C stretching	1497
C-O stretching	1109
C-H bending	709

4.2.3 Identification of drug by UV- Visible spectroscopy

4.2.3.1 Preparation of phosphate buffered saline (PBS) pH 7.4

2.38 g of disodium hydrogen phosphate and 0.19 g of potassium dihydrogen phosphate was dissolved in water. 8.0 g sodium chloride was added to above solution, and the solution was diluted with water to produce 1000 mL [Singh et al., 2014].

4.2.3.2 Preparation of stock solution of docetaxel

100 mg of drug was weighed and transferred to 100 mL volumetric flask. Docetaxel was dissolved in 10 mL dichloromethane and shaken manually for 5 min. The volume was made up to the mark with same solvent and the final strength obtained was 1000 µg/mL. Appropriate dilutions of standard drug solution were made with a specific solvent to obtain 5, 10, 15, 20, 25, 35, 40, 45, 50 and 100 µg/mL solutions

and were scanned for spectrum under 200 to 400 nm using double beam 1800S Shimadzu UV spectrophotometer.

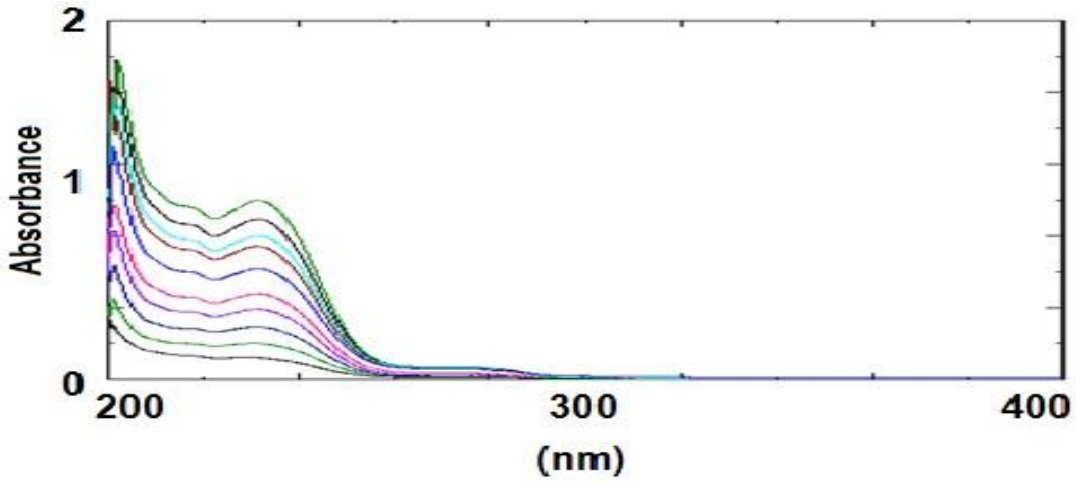

Figure 4.2 UV spectrum of Docetaxel in acetonitrile mixed with pH 7.4 PBS (30:70)

Inference

The solution of docetaxel was found to exhibit maximum UV absorption at 231 nm as illustrated in Figure 4.2. Thus, the procured drug sample of docetaxel complies with the reference spectra showed sharp and clear absorption maxima.

4.2.4 HPLC bio-analytical method development and validation

4.2.4.1 Preparation of sample

10 mg drug was dissolved in 10 mL methanol and shaken manually for 5 min. The volume was adjusted up to the mark with pH 7.4 PBS and the resulting strength obtained was 1000 µg/mL. Further dilution was made with pH 7.4 PBS to make 100 µg/mL solutions (100 ppm) in triplicate.

4.2.4.2 Preparation of plasma sample

Charles Foster healthy rats (mean weight-220 g) were used for the experiment of animal studies (*CAEA no. Dean/2015/CAEC/1416*). The supernatant plasma was collected after 10 min of centrifugation of rat blood mixed wth 0.2% EDTA at 4000 rpm. To the stock solution, 10 mg/mL of docetaxel was added and vortexed (IKA) for 5 min. Further ethyl acetate was added, mixed and centrifuged (Eltek) for 10 min at 5000 rpm to complete extraction procedure. The sample obtained was injected into HPLC system after 0.45 µm Millipore filtration.

4.2.4.3 Preparation of mobile phase

Acetonitrile and phosphate buffer saline (PBS) pH 7.4 was mixed with the ratio (30:70) utilizing Design-Expert software. The mixed solutions were filtered through 0.45 µm membrane filter.

4.2.4.4 QbD approach for method development

The goal of using quality by design (QbD) in HPLC method development is to define a systematic, scientific and proactive approach that begins with predefined objectives along with process control which leads to optimize an analytical method causing reductions in experiment numbers, furthermore improvement of statistical interpretations. Here, a Box-Behnken design was selected to optimize the method development using Design-Expert 10 software for getting the maximum information with the least number of experimental runs [Fan and Stewart, 2002, Kenney et al., 2000, Sowjanya et al., 2013].

4.2.4.5 Experimental design

Three experimental independent variables were chosen based on their significant effect on separation efficiency; namely column temperature (°C), flow rate (mL/min) and organic phase percentage (%). The levels consisting of high (1), middle (0) and low (-1) of all independent variables were selected based on literature survey and historical experimental trials. The number of theoretical plates (Y) which is system suitability parameter was taken as a response. The Box- Behnken design (BBD) was employed consisting of 3 levels, three factors, five center points and one block to make design matrix comprising of 17 runs by utilizing Design-Expert 10 software.

4.2.4.6 Optimization solution for method development

Numerical optimization of Design Expert 10 software was implemented on all the factors by applying constraints on the number of theoretical plates. Based on final optimization data, a fresh new batch was prepared using predicted values of independent variables. The experimental value of the response near to predicted value implies the prediction potential of the consistent design of experiment (DOE).

4.2.4.7 Risk assessment studies with evaluation of robustness and ruggedness

As the selected experimental method was consistent and will remain all over the lifetime of the product, the evaluation of robustness and ruggedness become necessary for method verification. A risk-based approach according to QbD ideology set out in ICH Q8, and Q9 was applied to recognize the potential risk. An analytical procedure like robustness is a measure of its capacity which remains

unaffected by small variations in method parameters. The ruggedness of analytical method explains as the degree of reproducibility in the results of the same samples under a variety of conditions [Fan and Stewart, 2002, Patel et al., 2007, Sahoo et al., 2009, Singh, 2015].

4.2.4.8 Method validation approach

All the new methods developed are to be validated. A typical analytical character that has to be considered according to ICH guidelines are as follows [Baranda et al., 2005, Heinisch and Rocca, 2004, Sabitha et al., 2009, Singh, 2015].

4.2.4.8.1 Precision

The precision of an analytical procedure is the closeness of the agreement between a series of measurements obtained from multiple sampling. The precision is expressed in the form of standard deviation and relative standard deviation or coefficient of variation. It is mainly classified into three levels: repeatability, intermediate precision, and reproducibility. To be acceptable, the values of precision should be <15% at all concentrations.

4.2.4.8.2 Linearity and range

The linearity is the ability to obtain test results within a set of given range which is directly proportional to the concentration of the analyte in the sample. Calibration curve of docetaxel in plasma was established over the concentration ranges from 5-100 µg/mL. For construction of the calibration curve, five different calibration standards were prepared for the range 5-100 µg/mL and processed as discussed in

Sub-section 4.2.4.2. Drug concentrations versus corresponding peak area of the drug were plotted. The calibration curve was done in triplicate.

4.2.4.8.3 Limit of detection and limit of quantitation

Limit of detection is the lowest amount of analyte in a given sample which can be detected resulting in a peak area of 3 times the baseline noise. The limit quantitation procedure is the lowest amount of analyte in a given sample which can be quantitated as accuracy (±15% bias), and precision (<15 RSD) provided a peak area with a signal-to-noise ratio higher than 10. Both were estimated from signal-to-noise ratios in accordance with ICH Q2B guideline.

4.2.4.9 Stability indicating analytical method

Stability testing of a drug substance provides information about degradation mechanism, pathway, and interaction between the drug and excipients. It is the drug capacity to maintain its quality, purity, and strength throughout the retest period. Stability indicating assay can detect and quantify the degradation products and impurities, for this, a stress testing of the drug substances in the form of forced degradation is required. Stress testing was carried out at 10°C increments above accelerated stability studies with 75% relative humidity or greater [Heinisch and Rocca, 2004, Sabitha et al., 2009, Singh, 2015].

4.2.4.9.1 Degradation studies of drug in acidic condition

Docetaxel at a concentration of 10 mg/mL was subjected to forced degradation in 2 mL of acidic medium (0.1N HCl) and was refluxed for 72 h. Further, it was

neutralized with 0.1N sodium hydroxide, and appropriate dilutions were made with mobile phase. 10 µL of this degraded solutions were injected into chromatographic condition. From the peak areas in the chromatogram, the percentage of the non-degraded drug was determined.

4.2.4.9.2 Degradation studies of drug in alkaline condition

10 mg/mL docetaxel was subjected to forced degradation in 2 mL of 0.1N sodium hydroxide for 72 h. Further, it was neutralized with 0.1N HCl, and appropriate dilutions were injected into the chromatographic column.

4.2.4.9.3 Degradation studies of drug in oxidation condition

3% v/v hydrogen peroxide was used as an oxidizing medium for forced degradation of docetaxel. 10 mg/mL docetaxel with 2 mL 3% v/v hydrogen peroxide was subjected to a stress condition, and this degraded solution was injected into the column to get chromatogram.

4.2.4.9.4 Degradation studies of drug in thermal condition

Thermal degradation studies were carried out in a dry, hot air oven at 50°C for the period of 72 h with exposed 10 mg/mL of the standard drug in a Petri dish. Finally, degraded solutions were injected into the chromatographic condition, and percentage content of non-degraded drug was determined.

4.2.4.9.5 Degradation studies of drug in photo (UV light) condition

A medium pressure mercury lamp (emits UV light in the range of 200-400 nm) was used for degradation study of standard drug docetaxel. Accurately 10 mg of docetaxel was weighed and transferred into 10 mL volumetric flasks and made up to the volume with mobile phase. From the volumetric flask, the solution was transferred into Petri dish and was exposed to UV light for 72 h. The percentage content of non-degraded drug was determined.

4.3 Preparation of PHBV PVA polymeric nanoparticles and characterization

4.3.1 Formulation of docetaxel-loaded nanoparticle

Docetaxel-loaded PHBV polymeric nanoparticles were prepared by modified emulsification solvent evaporation technique [Sahana et al., 2010, Wang et al., 2011]. Briefly, 1 mg docetaxel and 15.29 mg PHBV were dissolved in dichloromethane. The first emulsion was prepared by dispersing 3.96 mg/mL PVA aqueous solution in organic phase utilizing IKA high shear homogenizer at 10,718 rpm for 5 min. Afterward, the formed primary emulsion was mixed with external aqueous phase containing 0.4% w/v PVA under stirring and then the whole mixture was sonicated using ultra probe sonicator for 10 min. Final w/o/w emulsion was stirred overnight on the magnetic stirrer (IKA RH digital) to evaporate organic solvent. The resultant dispersion was centrifuged, washed, lyophilized (Labconco) and stored in a desiccator until further use.

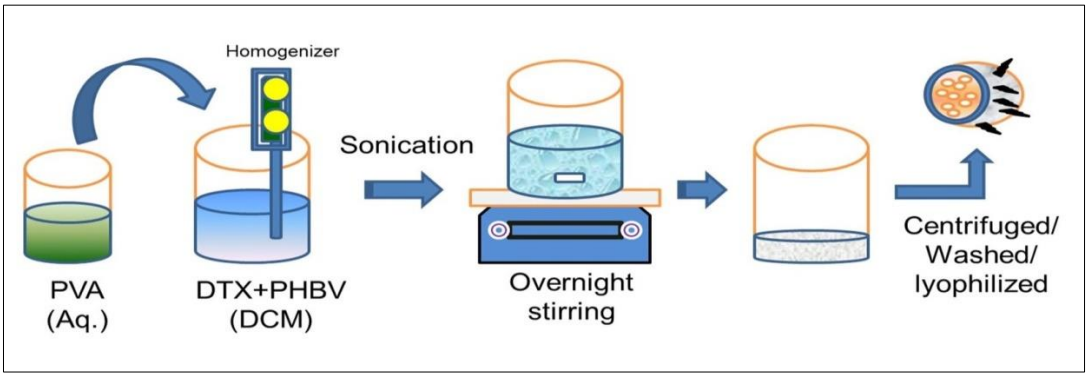

Figure 4.3 Preparation of PHBV PVA polymeric nanoparticles

4.3.2 Risk assessment studies

The development of dosage form with the concept of QbD requires in-depth knowledge of risk assessment control strategy. These are QTPP (quality target product profile), categorizing CQAs (critical quality attributes), identifying CMA (critical material attributes) and CPP (critical process parameter) [Singare et al., 2010, Singh et al., 2005a, Wang et al., 2014]. This information is further used to develop an optimized and robust manufacturing method that can produce a consistent product over time. Risk assessment studies help to identify the material attributes and process parameters which play a significant role on the product CQA (Fig. 4.3). Furthermore, failure mode effect analysis (FMEA) provides an evaluation of rank modes of relative effectiveness to priorities critical to quality (CTQ) variables as high, medium and low. Table 4.2 shows prioritization levels of the independent variable before drafting DOE to optimize the NPs manufacturing process.

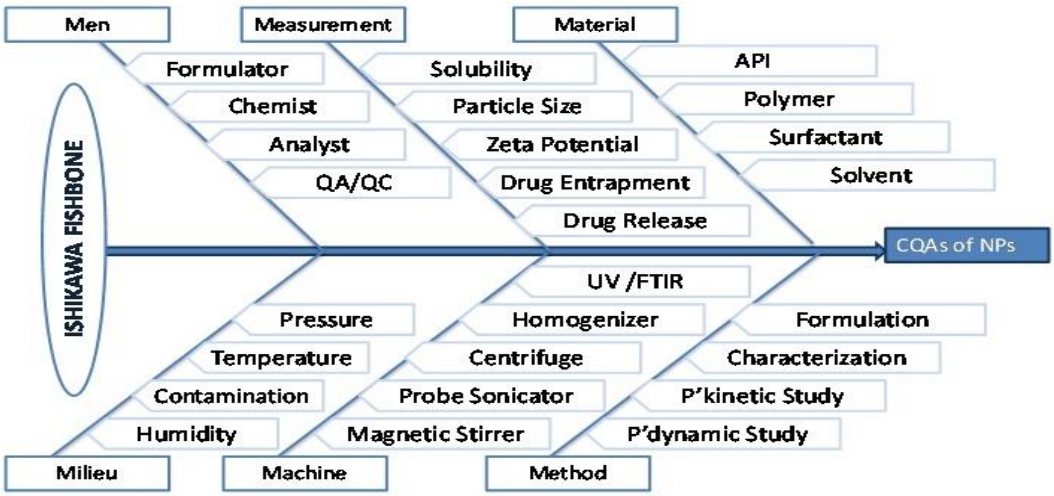

Figure 4.4 Ishikawa fishbone diagram is showing risk assessment studies

Table 4.2 Prioritized Independent variables based on FMEA study

CTQ (Independent Variables)	Responses (Dependent Variables)			
	Particle Size	**Zeta Potential**	**Polydispersity Index**	**Entrapment Efficiency**
Polymer type	Low	Low	Low	Low
Polymer concentration	High	Low	Medium	High
Surfactant type	Low	Low	Low	Low
Surfactant concentration	High	High	High	Medium
Solvent type	Low	Low	Low	Low
Solvent ratio	Medium	Low	Low	High
Temperature	Low	Low	Low	Low
Homogenizer speed (H. Speed)	Medium	Low	Medium	Medium
Homogenization time (H. Time)	High	Low	Low	High
Ultra sonication time (US. Time)	High	Low	Medium	Low
Stirring speed (S. Speed)	Medium	Medium	Low	Medium
Stirring time (S. Time)	Medium	Medium	Low	Low
Centrifugation speed (C. Speed)	Low	Low	Low	Medium
Centrifugation time (C. Time)	Low	Low	Low	Medium

4.3.3 Factor screening design studies

The DOE approach was performed systematically by first employing preliminary screening design and then response surface methodology (RSM). Plackett-Burman design (PBD) is the best-suited design for screening the factors which reduce the number of experiments. It was used to estimate the correlations between factors and responses through Minitab 17 software. PBD employed Pareto chart to select the important factors responsible for the significant effects.

4.3.4 Optimization and statistical analysis

The Box-Behnken design was selected to optimize docetaxel-loaded PHBV nanoparticles using Design-Expert 10 software. BBD has the benefits of exploration of complete design space with reduced experimental runs, without aliasing interaction factors [Singh et al., 2005a, Singh et al., 2005b]. The factors and their levels were chosen on the basis of primary screening and data mining. The independent variables (A) polymer concentration, (B) surfactant concentration, (C) homogenizer speed, (D) homogenization time and (E) ultrasonication time were selected with their levels of constraint for optimization. A suitable model was chosen based on statistical data like *p*-value, r-square and lack of fit. Based on the model, a quadratic equation was generated to describe the effect of independent variables on the response. After defining and selecting proper constraint, the predicted optimization solution was generated which was used further for *in vitro* characterization.

4.3.5 Determination of particle shape and size

The nanoparticle can be morphologically visualized by SEM, TEM, and AFM which determines particle shape and size. They also help in identification of nanocapsule, nanosphere, and other nanoformulation. Particle size is mainly determined by measuring changes in the intensity of light scattered by suspended particles using particle size analyzer and photon correlation spectroscopy. As larger particles have large cores, they allow more entrapment of drug and slow diffusion; whereas smaller particles have a large surface area which shows fast diffusion but leads to aggregation of particles during storage. NPs less than 10 nm results in rapid clearance by the kidney and more than 100 nm have higher tendency to be cleared by cells of mononuclear phagocytes (opsonins) which is reticuloendothelial (RES) or mononuclear phagocyte (MPS) systems of the body. For passive targeted chemotherapy, the size of NPs should be between 200-400 nm; so as to achieve EPR effect, NPs has to extravasate (migration, internalization, and accumulation) into broken blood vessels to achieve desired cell death.

4.3.6 Determination of surface charge, density, and properties

Measurement of surface charge and properties of the nanoparticle was done by using Desla Nano C, Beckman Coulter, USA instrument. Nature and intensity of surface charge determine NPs interaction with the biological environment and compounds. Zeta potential is analyzed to study the electrophoretic mobility (viscosity and electric field strength), colloidal property and physical stability of the formulation during storage. NPs with zeta potential above ± 30 mV have been shown to be stable in suspension, as high surface charge prevents aggregation of the particles. Neutrally

and negatively charged particles have very low opsonization rate as compared to positively charged particles. NPs surface hydrophobicity determines the absorption of opsonins (a blood protein) and their binding acts as a bridge between particulate matter and phagocytes. Thus, to minimize opsonization and prolong NPs circulation, coating with a biodegradable hydrophilic polymer is necessary.

4.3.7 Determination of entrapment efficiency

Successful NPs should have high drug loading capacity as it reduces the quantity of matrix materials for administration. Drug loading can be done by drug incorporation method (during NPs production) and adsorption or absorption method (after NPs production). Entrapment efficiency is determined by separation of the supernatant free drug content using centrifugation method [Chalikwar et al., 2012, Fazil et al., 2012, Sahana et al., 2010]. The entrapment efficiency is expressed as:

$$\% EE = \frac{Total\ drug\ added - Supernatant\ free\ drug}{Total\ drug\ added} \times 100$$

After sonication, NPs (SUVs) was taken in a centrifuge tube and was centrifuged at 15,000 rpm (1,37,088 G) for 20 min at 4 ^{0}C in an ultra-cold centrifuge (ELTEK RC 4100F) to get a white pellet. Supernatant layer from centrifuge tube was separated as it contains free drug by micropipette. To the remaining pellet in the centrifuge tube, PBS pH 7.4 was added and was then vortexed thoroughly for 3 min. The resultant white suspension was lyophilized and stored until further use.

4.3.8 In vitro drug release studies

In vitro drug release study was performed in determining the permeation rate of NPs which depends on; (a) NPs matrix erosion or degradation, (b) Drug diffusion through NPs matrix, (c) Desorption of adsorbed drug, and (d) Solubility of the drug. Pre-treated dialysis membrane-135, molecular weight cut off between 12-14 kDa was immersed in 100 mL of pH 7.4 phosphate buffer saline and stirred magnetically at 100 rpm at 37 ^{o}C [Fazil et al., 2012, Masood et al., 2013, Zhang et al., 2010, Zhang and Feng, 2006]. Aliquots of 5 mL were withdrawn from release medium and replaced with fresh phosphate saline buffer at regular time interval. The solution was filtered, and respective concentrations were measured by HPLC method. Finally, the drug release kinetics were evaluated considering four models including Zero order, First order, Higuchi's equation and Korsmeyer-Peppas model. Based on the relevant correlation coefficients comparison and linearity test, the mechanism of drug release was selected. The regression coefficient R^2 value closer to 1 indicates the best model fitting of the release mechanism.

4.4 Preparation of PHBV PF127 polymeric nanoparticles and characterization

4.4.1 Formulation of docetaxel-loaded nanoparticle

Docetaxel-loaded PHBV nanoparticles were fabricated by modified emulsification-solvent evaporation technique [Zakeri-Milani et al., 2013]. Briefly, 18.38 mg PHBV along with docetaxel were dissolved in small volume of water immiscible volatile solvent dichloromethane. The first emulsion was prepared by dispersing 4 mg/mL

PF-127 aqueous solution in organic phase using IKA high shear homogenizer (10,000 rpm for 5 min). The resulting primary emulsion was further mixed into the aqueous phase containing 0.4% w/v stabilizer under stirring, and the mixture was sonicated by ultra-probe sonicator for 9.40 min. After emulsification, the emulsion was magnetically stirred overnight on IKA RH digital stirrer to evaporate organic solvent. The nanoparticles formed were then centrifuged (RC 4100F, Eltek) and lyophilized for storage.

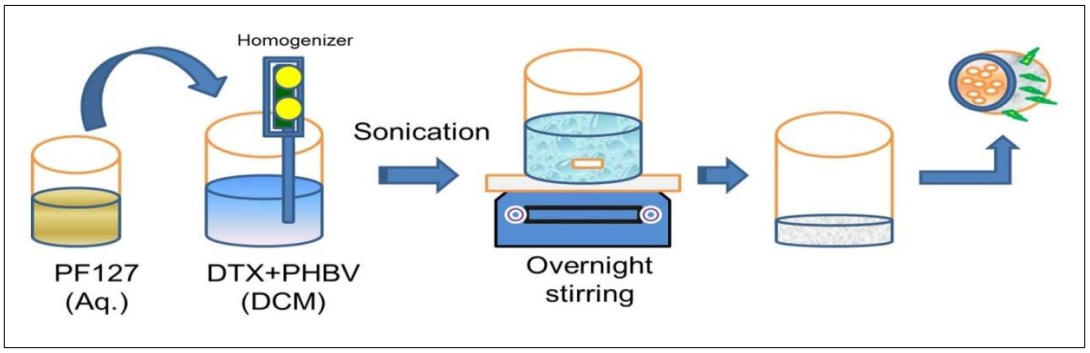

Figure 4.5 Preparation of PHBV PF127 polymeric nanoparticles

4.4.2 Risk assessment studies

Risk assessment study is necessary to enhance knowledge of various material attributes and process parameters employed in the product manufacturing. It starts with the collection and identification of all possible root cause in the manufacturing processes influencing product quality (Figure 4.4). Risk ranking and filtering (RRF) was done to categorize the critical quality attributes (CQA) by ranking variables based on probability and severity. The independent variables having risk score above five were considered to be high-risk factors (Table 4.2). After finding the risk score, appropriate filtering action was taken, and selected CQAs were further

subjected to screening studies. The Plackett-Burman design quickly screens the potential risk factors among several input variables obtained by RRF.

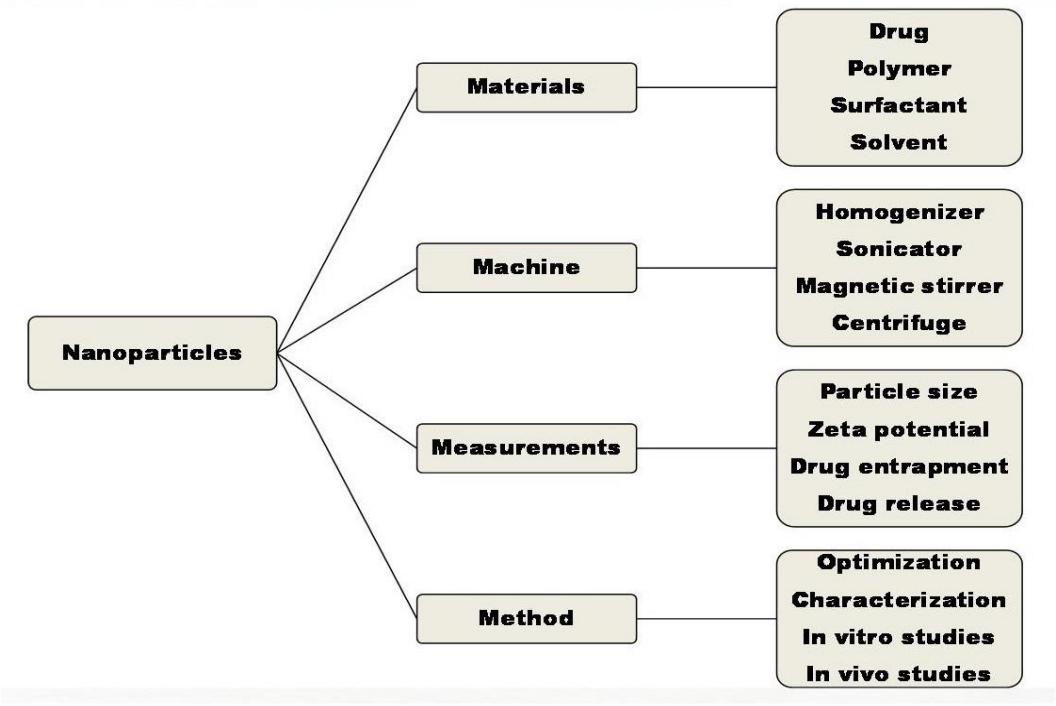

Figure 4.6 Risk management flow chart containing material attributes and process parameters

4.4.3 Optimization and statistical analysis

Box-Behnken design, among another response surface methodology, is the most cost effective, as it requires lesser experimental runs for optimization [Singh et al., 2005b, Singh et al., 2010]. Based on Plackett-Burman factor screening design, five independent variables out of ten factors were selected for response surface methodological analysis. The Box-Behnken design was employed to generate a polynomial statistical equation by using Design Expert 10 software for optimization of formulations.

Table 4.3 Risk ranking and filtering analysis based risk score of probability and severity

(Independent Variables)	Risk Ranking & Filtering			
	Probability (P)	Severity (S)	Risk Score	Risk Action
Polymer type	1	4	4	Non CQA
Polymer concentration	2	8	16	CQA
Surfactant type	1	3	3	Non CQA
Surfactant concentration	2	7	14	CQA
Solvent type	1	4	4	Non CQA
Solvent ratio	2	5	10	CQA
Homogenizer speed	1	7	7	CQA
Homogenization time	2	4	8	CQA
Sonication time	2	7	14	CQA
Stirring speed	1	6	6	CQA
Stirring time	2	4	8	CQA
Centrifugation speed	1	6	6	CQA
Centrifugation time	1	5	5	CQA

The non-linear quadratic equation produced by BBD is;

$$\mathbf{Y}=X_0+X_1A+X_2B+X_3C+X_4D+X_5E+X_6AB+X_7AC+X_8AD+X_9AE+X_{10}BC+X_{11}BD+X_{12}BE+X_{13}CD+X_{14}CE+X_{15}DE+X_{16}A^2+X_{17}B^2+X_{18}C^2+X_{19}D^2+X_{20}E^2.$$

Where Y is the response, X_0 is intercept, X_1 to X_{20} represents regression coefficient, and A to E are factors (independent variables). BBD consist, a set of runs which includes replicated center points and factorial points which were used to evaluate the main effects. It utilizes only three levels for each factor, i.e., -1, 0 and 1 to design a set of runs. All runs of the experiments were conducted in triplicate and randomized order to maintain precision and error minimization. ANOVA analysis was carried out for deciding the significance and influence of each independent variable on responses. The response surface plots like contour and 3-D plots were generated to identify the interaction between factors and responses. Based on statistical data, multiple linear regression analysis, quadratic equation, response surface

methodology and constraint, an optimized batch was generated for further characterization processes [Gannu et al., 2009, Solanki et al., 2007, Vardhan et al., 2017a].

4.4.4 Morphological studies

The morphology and shape of the PHBV nanoparticles were investigated by atomic force microscopy, scanning electron microscopy and transmission electron microscopy. SEM observation was performed using Carl Zeiss Inc., USA at 10 kV of accelerating voltage. Sample preparation was done by dropping the formulation suspension on a small glass slide, air dried and sputtered with gold under vacuum. TEM micrograph was performed with TECHNAI-20G^2, Czech Republic at 15 kV of accelerating voltage. A drop of nanoformulation was placed on a copper grid coated with carbon film. Scanning probe microscope NT-MDT, Russia was used for AFM study of surface morphology and particle distribution. The shape of images was observed with a scanning rate of 0.5 Hz [Budhian et al., 2005, Hao et al., 2012, Mahdavi et al., 2010].

4.4.5 Particle size and surface charge

Particle size determination helps in determining release profile, tissue penetration and cellular uptake of nanoparticles. It is strongly affected by molar mass and polymeric concentration. The size analysis of PHBV nanoparticles was performed by a dynamic light scattering analyzer (Desla Nano C, Beckman Coulter, USA) by placing the formulation in a cuvette made of polystyrene. This yields the mean particle diameter and polydispersity index for particle size distribution in the suspension. The nanoparticles were analyzed three times, and before introduction

into poly-propylene cuvettes, the formulation was sonicated in milli-Q water to minimize the interparticle interactions [Budhian et al., 2007, Mainardes and Evangelista, 2005]. The zeta potential was calculated by measuring electrophoretic light scattering and surface charge of nanoparticles using Desla Nano C, Beckman Coulter. Three measurements were made for each formulation. Nanoparticles having the zeta potential above ±30 mV were considered to be stable in suspension, and the negative charge value protects them from opsonization [Sharma et al., 2014, Yousefi et al., 2009].

4.4.6 Determination of entrapment efficiency

The entrapment efficiencies of docetaxel were calculated by separation of the supernatant free drug content using centrifugation method [Lee et al., 2008, Singh et al., 2010]. The NPs were centrifuged at 4^0C using ultracold centrifuge for 20 min. Supernatant layer from centrifuge tube was separated, and to the remaining residue, PBS pH 7.4 was added and vortexed thoroughly. The entrapment efficiency is expressed as [Kheradmandnia et al., 2010]:

$$\% EE = \frac{Drug\ added - Free\ drug}{Drug\ added} \times 100$$

4.4.7 In vitro drug release studies

Phosphate buffer saline (PBS) with pH 7.4 was used as a receptor media in the study of *in vitro* drug release. A dialysis membrane-135, having a molecular weight cut off between 12-14 kDa was immersed in 100 mL of phosphate buffer saline maintained at 37±0.5^{0}C. The release medium was stirred magnetically at 100 rpm, and aliquots of 5 mL samples were withdrawn maintaining a sink condition by replacing with

fresh prewarmed (37±0.5^{0}C) PBS media, which was analyzed by HPLC method for quantification. Based on regression coefficient R^2, the mechanism of drug release kinetics was evaluated considering four models including Zero order, First order, Higuchi's and Korsmeyer-Peppas model [Bernabeu et al., 2014, Costa and Lobo, 2001, Seju et al., 2011].

4.5 Preparation of PHBV TPGS polymeric nanoparticles and characterization

4.5.1 Formulation of docetaxel-loaded nanoparticle

Docetaxel-loaded PHBV polymeric nanoparticles varying the proportion of TPGS were prepared by modified emulsification solvent evaporation technique [Kumari et al., 2010, Vardhan et al., 2017a]. Briefly, 1 mg docetaxel and 10.83 mg PHBV were dissolved in dichloromethane. The first emulsion was prepared by dispersing 4 mg/mL TPGS solution in organic phase utilizing IKA high shear homogenizer at the speed of 4,962 rpm. Afterward, the formed primary emulsion was mixed with external aqueous phase containing 0.4% w/v TPGS under stirring, and then the whole mixture was sonicated for 8.39 min using ultra probe sonicator (UP50H, Hielscher). Final w/o/w emulsion was stirred overnight on a magnetic stirrer to evaporate organic solvent. The resultant dispersion was centrifuged, washed, lyophilized and stored in a desiccator until further use.

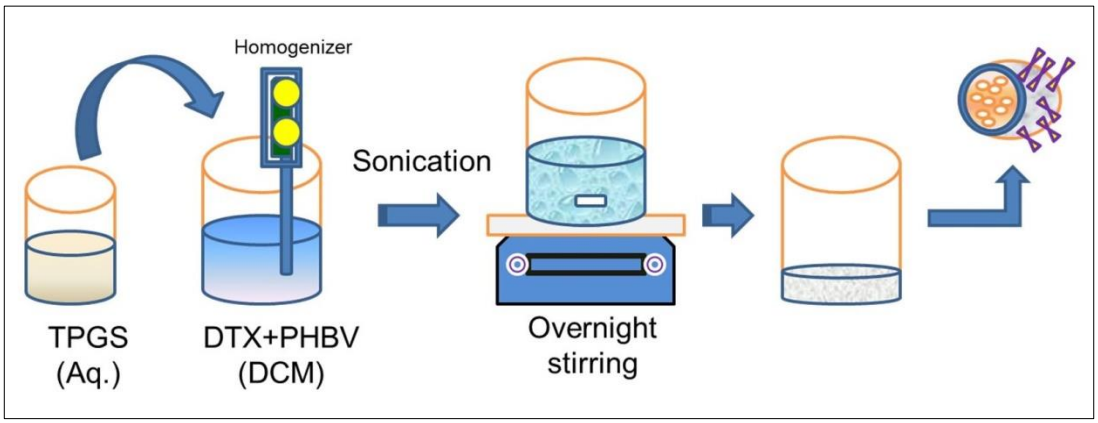

Figure 4.7 Preparation of PHBV TPGS polymeric nanoparticles

4.5.2 Formulation by design approach

The implement of QbD approach in product development is mandatory demands of regulatory agencies to obtain a robust product [Kourti and Davis, 2012]. Here, statistical analysis, quadratic equations and graphical outcomes were used to find the impact of independent variables influencing responses of the formulation. The optimization study starts with the identification of critical quality attributes (CQA) and selection of CQA variables concerning materials and processes influencing the development of a nanoparticulate system [Vardhan et al., 2017a]. Further, basic fundamental independent variables were screened out by pre-optimization studies utilizing Placket-Burman design (PBD) [Dhat et al., 2017]. The selected factors and responses include the concentration of polymer, homogenizer speed, ultrasonication time, particle size and entrapment efficiency. The selected independent variables or factors were subjected to response surface methodology (RSM) towards response variables which directly represents the product quality. The experimental region in RSM is defined by lower and upper level of each factor, and it is depicted

graphically as 2-D and 3-D plots. The effects of factors were estimated by the generation of a mathematical model and statistical evaluation [Hao et al., 2011, Zidan et al., 2007]. Lastly, the desirability function was calculated to predict optimum levels of independent variables for model adequacy. For this purpose, a numerical optimization technique was employed wherein the responses were assigned with a goal to minimize, maximize, in range or target under a set of constraints.

4.5.3 Morphological studies

The geometry, shape, and size of nanoparticles surface were investigated by sophisticated instruments like the SEM, AFM, and TEM [Gaumet et al., 2008]. The surface morphology of drug loaded nanoparticles was examined using SEM. The prepared sample was mounted on a brass stub, and vacuum coating was done with gold. The sample was imaged at a different resolution to have closure look of the surface morphology. For TEM micrograph, a drop of suspension was placed over 300 mesh TEM grid coated with carbon film and 1% phosphotungstic acid solution for negatively stained. The copper grid was air dried at room temperature before loading in the microscope TECHNAI-20G^2, Czech Republic. For AFM study a Scanning probe microscope (NT-MDT, Russia) was used, and formulation was analyzed for their surface and particle size distribution with scanning rate 0.5 Hz.

4.5.4 Particle size and surface charge

The size of nanoparticle considered being a decisive factor in the determination of rate and extent of drug release and absorption. Dynamic light scattering technique was used for determining the size of prepared nanoparticles [He et al., 2010]. The sample was illuminated with a laser beam, and the intensity of scattered light by particle based on the brownian motion was measured by Desla Nano-C, Beckman Coulter, USA. All the analysis were carried out in triplicate. Surface charge and zeta potential of prepared nanoparticles were measured similarly as particle size determination by zeta sizer instrument [Vardhan et al., 2017a]. The absence of aggregation, precipitation, any sign of coalescence of nanoparticles, affirmed by zeta potential closure to ±30 mV indicated stability. All measurements were done in triplicate.

4.5.5 Determination of entrapment efficiency

Weighed known quantities of optimized nanoparticles were dissolved in 10 mL distilled methanol to evaluate loading efficiency. Samples were first vortexed and then centrifuged for 20 min at 4°C in ELTEK RC 4100F. The supernatant free drug was collected, suitably diluted and analyzed at 231 nm using UV-Visible spectrophotometer (Shimadzu 1800).

The entrapment efficiency is expressed as [Vardhan et al., 2017a]:

$$\%\, EE \; = \frac{Total\ drug\ present - Supernatant\ free\ drug}{Total\ drug\ present} \times 100$$

4.5.6 In vitro drug release studies

In vitro drug release testing of prepared nanoparticles plays a significant role in establishing therapeutic performance and ensuring drug release behavior [Adjei et al., 2016]. This study was carried out on a beaker where optimized nano formulation equivalent to 25 mg of drug suspension was filled in a dialysis bag (135 membrane and molecular weight cut off between 12-14 kDa of HiMedia) and immersed in 100 mL of pH 7.4 saline buffer maintained at 37±0.5°C. The release medium was stirred magnetically at 100 rpm. Aliquots of 2 mL were withdrawn at predetermined time intervals and to maintain sink condition replenished with an equal volume of fresh media. The released drug was analyzed by reverse-phase HPLC method in triplicate. The drug release kinetics was evaluated based on regression coefficient R^2 considering four models including Zero order, First order, Higuchi's and Korsmeyer-Peppas.

4.6 Preparation of PHBV PLH PE-PEG polymeric nanoparticles and characterization

4.6.1 Formulation of docetaxel-loaded nanoparticle

Docetaxel-loaded PHBV PLH PE-PEG polymeric nanoparticles were formed by modified emulsification solvent evaporation technique following three steps [Kumari et al., 2010, Vardhan et al., 2017a]. Firstly, 1 mg of docetaxel and 13.0711 mg of PHBV were dissolved in dichloromethane in a beaker under gentle mixing with a magnetic stirrer. The first emulsion was prepared by dispersing 4 mg/mL of TPGS solution in organic phase utilizing high shear homogenizer (IKA) at the speed of 8,476 rpm. Afterward, the formed primary emulsion was mixed with external

aqueous phase containing 0.4% PVA w/v under stirring, and then the whole mixture was sonicated for 7.5 min using ultra probe sonicator. Final emulsion was stirred overnight on a magnetic stirrer to evaporate organic solvent. The resultant NPs were centrifuged and washed. In the second step, 4% v/v PLH was dissolved in 10 mL of ultrapure Milli-Q water, and HCl (0.1N) was added dropwise to get pH 5. Now the prepared NPs were suspended and stirred in developed acidic solution of PLH. Further, NaOH (0.1N) was added dropwise to increase the pH towards basic up to 8. The PLH coated PHBV NPs were collected by the filtration process. In third and last step, PLH coated NPs were stirred in 20 mL cold water (4^0C) containing 4% w/v PE-PEG for 10 min. The pegylated pH-responsive particles were collected by centrifugation at 10,000 rpm for 5 min. Finally, the formed NPs were lyophilized and stored until further use.

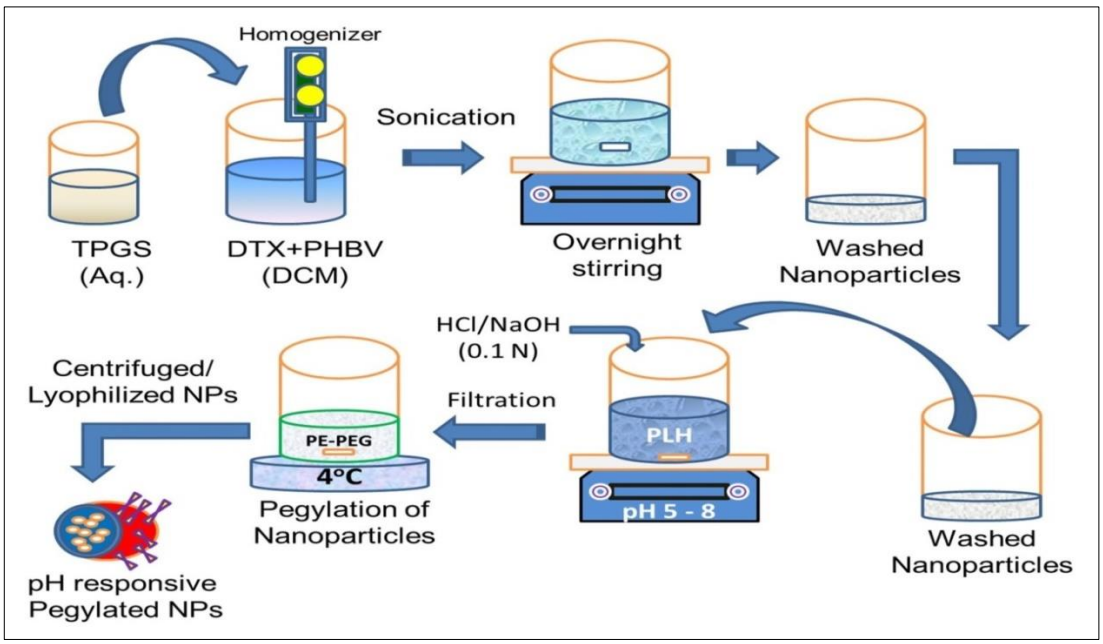

Figure 4.8 Preparation of PHBV PLH PE-PEG polymeric nanoparticles

4.6.2 Formulation by design approach

Docetaxel-loaded PHBV PLH PE-PEG polymeric nanoparticles were optimized using three factors, three levels Box-Behnken design (BBD). Box- Behnken designs (BBD) are response surface method used to examine the relationship between a set of independent variables and one or more responses. In BBD all design points fall within the safe operating zone and consist of only three levels which make it more convenient and less expensive to run the experiments [Singh et al., 2005b, Singh et al., 2010]. Polymeric concentration, homogenizer speed and ultra-sonication time were selected as factors over responses i.e. particle size and percentage entrapment efficiency. The formed design generated 15 trial runs including 3 center points and experimentations were performed to get an optimized formulation.

4.6.3 Morphological studies

The morphological geometry of formed pegylated nanoparticles was observed using SEM, AFM and TEM [Vardhan et al., 2017a]. For SEM imaging, a drop of prepared samples was mounted on aluminum stub by double-sides sticky discs of conductive carbon, gold coated by the sputtering method and observed at a different resolution under scanning electron microscope. The photomicrographs were taken at the same magnifications for all the samples to facilitate comparison between them [Gaumet et al., 2008]. AFM was employed to study the shape and size distribution of prepared nanoformulations. The nanoparticles were diluted with buffer, and few drops were placed on a microscopic slide. The sample was air-dried and analyzed under microscopic scanner. The TEM micrographs were collected using a copper grid with

300 meshes. Here, a drop of nanoformulation was placed on TEM grid, air dried and analyzed for surface characteristics under transmission electron microscope.

4.6.4 Particle size and surface charge

The particle size and polydispersity index of prepared nanoparticles were determined by using Desla Nano-C, Backman Coulter, USA. This technique is based on photon correlation spectroscopy by measuring the rate of fluctuations in dynamic light scattering by particles [He et al., 2010]. Nanoformulation was diluted with ultrapure Milli-Q water and analyzed for the measurement of the size and polydispersity index. PDI indicates the width of the monomodal decay rate distribution with values between zero to one. The zeta potential of nanoparticles was also determined by the same Desla Nano-C instrument using the same procedure as particle size. Here, nanoparticles were placed in zeta cell, and the movement within the electric field determines their electric charge [Vardhan et al., 2017a]. All the measurements were done in triplicate, and zata potential closure to ±30 mV indicated colloidal stability.

4.6.5 Proton (^{1}H) NMR spectroscopy

An NMR technique characterizes the surface composition, chemical properties, and structure of functionalized nanoparticles [Zhang et al., 2007]. ^{1}H NMR spectrum was obtained using Bruker DRX-500 FT-NMR, USA. 3 mg of prepared nanoparticles and pegylated nanoformulations were dissolved in 0.5 mL of deuterated dimethyl sulfoxide (DMSO-d6) solvent. NMR spectroscopy was carried out in the pulsed FT

mode at a 500 MHz resonance frequency for protons using tetramethylsilane (TMS) as the internal standard.

4.6.6 Determination of entrapment efficiency

Briefly, known quantity of optimized nanoparticles was vortexed and centrifuged at 10,000 rpm for 20 min at 4°C using a cooling centrifuge (ELTEK RC 4100F) to separate unentrapped drug. The supernatant free drug was collected, diluted and subsequently analyzed at 231 nm UV-Visible spectrophotometer (Shimadzu 1800) [Vardhan et al., 2017a]. The percentage entrapment efficiency was calculated according to the following equations:

$$\%\, EE\ = \frac{Total\ drug\ - Supernatant\ free\ drug}{Total\ drug} \times 100$$

4.6.7 In vitro drug release studies

In vitro, drug release studies were performed to ensure the drug release behavior and release kinetic of prepared nanoformulation [Adjei et al., 2016]. In brief, nanoparticles equivalent to 25 mg drug was placed in a dialysis bag having molecular weight cut-off between 12-14 kDa (HiMedia, India) [Vardhan et al., 2017b]. The dialysis bag was then suspended into a beaker containing 100 mL PBS of pH 7.4 and stirred magnetically at 100 rpm. The temperature of release medium was maintained at 37±0.5°C throughout the study. Aliquot of 2 mL of samples were withdrawn at predetermined time points and replaced with the same volume of fresh PBS to maintain sink condition. The cumulative drug release was determined using the RP-HPLC method. Further, drug release kinetics was evaluated considering four

models including Zero order, First order, Higuchi's and Korsmeyer-Peppas based on their regression coefficient (R^2).

4.7 Cytotoxicity studies

The cellular toxicity of docetaxel-loaded PHBV nanoformulations were determined by sulforhodamine B (SRB) assay [Liu et al., 2008]. For this purpose, human breast cancer cells (MCF-7) were obtained from the ACTREC, Tata Memorial Centre, Mumbai and maintained using Dulbecco's modified eagle medium (DMEM) in 5% CO_2 humidified atmosphere at 37°C. It contains 10% fetal bovine serum (FBS), 100 µg/mL penicillin, and 100 µg/mL streptomycin. Further, the MCF-7 suspension was seeded in 96 well culture plates and incubated for 24 h. The treatments of cells were done with pure drug, optimized formulations, positive control (standard) and untreated cell as a negative control. All the treated cells were incubated for 24 h. 100 µL of trichloroacetic acid was added, and after 4 h of incubation, cells were stained with 100 µL of SRB solution. After washing the unbound dye with 1% acetic acid, remaining protein-bound dye was extracted with 10 mM unbuffered tris hydroxyl methylamino methane solution. The formed plates were agitated, and absorbance was measured at 570 nm using a multimode reader. The cytotoxicity and cell viability of the nanoparticle formulations were investigated concerning GI_{50} reported fifty percent cancer cell growth inhibition [Leroux et al., 2006, Nemati et al., 1994, Vichai and Kirtikara, 2006, Zhang and Feng, 2006]. Further, the cellular uptakes of nanoparticles mounted on microscopic slides were observed by confocal laser scanning microscope.

4.8 Cell uptake studies

For cell uptake, MCF7 breast cancer cells were cultured and seeded at a density of $2x10^5$ cells/well into 24-multiwell plates (Tarsons, India) and grown until they reached 80-90% confluence [Vardhan et al., 2017b]. The optimized NPs containing fluorescent agent (FITC) were incubated in 1 mL of medium, and loaded cells for overnight at 37°C supplied with 5% CO_2. Then, the medium was removed, washed thrice with cold phosphate buffer saline (PBS) and the fluorescence intensity of the cells was evaluated using triton x-100 (0.1% solution) as lysis agent. The obtained confocal microscopic images mounted on microscopic slides fixed with 8% v/v formaldehyde solution confirms intracellular uptake of the optimized formulations and their interaction with macrophage cells.

4.9 Pharmacokinetic study

An *in vivo* study was done to estimate the bioavailability and pharmacokinetics of the drug and formulations [Wang et al., 2016]. Charles Foster rats *(150-200g body weight)* of either sex used for *in-vivo* studies were obtained from Central Animal House, Banaras Hindu University. Proper diet with standard conditions was provided to animals for five days before the study. The experimental protocol was approved by the Central Animal Ethical Committee (no. Dean/2015/CAEC/1416) of the University. Rats were housed in polypropylene cages and fasted overnight before the experiment. Rats were housed under standard conditions as per guidelines for the care and use of laboratory animals (CPCSEA, GOI). The animals utilized in this study were not subjected to any form of treatment or medication. The animals were kept under anesthesia using diethyl ether whenever necessary to prevent any pain

and discomfort [Bayne et al., 2015]. They were divided randomly into six groups' i.e. pure drug, control and formulations (F1, F2, F3, & F4) each group consisting of six rats. Drug and NPs (12.6 mg/Kg) in water for injection were administered via tail vein (*i.v.* route) using tuberculin syringe with a 27G needle. Blood samples were serially collected from the retro-orbital sinus at time points of pre-dose, 6, 12, 24, 48, 72, 96, 120 and 144 hours after drug administration. Blood samples were collected in K3-EDTA tubes and centrifuged at 4000 rpm for 10 min. Further, the supernatant plasma sample was extracted with ethyl acetate on vortex mixer followed by centrifugation as mentioned in ***Sub-section 4.2.4.2*** [Lu et al., 2006]. The rat plasma samples were analyzed by HPLC using reverse phase C_{18} column for quantification of the drug. The plasma pharmacokinetic parameters were calculated by PKPlus™ software using intravascular non-compartmental analysis model. Peak plasma concentration *(C_{max}),* time to reach C_{max} *(T_{max})* and area under the curve (AUC_{0-t}) were obtained from plasma concentration-time curves. The elimination half-life ($t_{1/2}$) was calculated by $0.693/K_{el}$. Total plasma clearance (*CL*) calculated by *dose/AUC*. The mean residence time was calculated by the ratio of *AUMC* and *AUC*. The pharmacokinetic parameters were compared for statistical significance by student's t-test and those having probability value ≤0.05 were considered to be significant.

4.10 In vivo-in silico and IVIVC studies

In silico pharmacokinetic prediction and computer simulations study was done to predict *in vivo* absorption profile of optimized nanoparticles with the help of GastroPlus™ software [Parrott and Lavé, 2002]. The physicochemical parameters

were entered into the compound tab of software. In the pharmacokinetics tab, the parameters obtained from PKPlus™ module were imported. The simulated model was considered to be of high prediction accuracy as per calculated fold error.

$$fold\ error = 10\ [\log(predicted/observed)]$$

The *in vitro-in vivo* correlation (IVIVC) was used to visualize the correlations between *in vitro* release and *in vivo* availability of prepared optimized formulations [Somayaji et al., 2016]. The IVIVC was achieved by various models and regression correlation analysis and was done to select the best-fit model. The Single Weibull method of Mechanistic absorption model was used to form a correlation. This was done by IVIVCPlus™ module using mechanistic deconvolution followed by convolution to predict and reconstruct the plasma concentration-time profile of developed formulations.

4.11 In vivo anticancer activity

The anticancer activity of optimized formulations were tested using murine breast tumor-bearing model [Walia et al., 2016]. C3H/J mammary strain of female mice was used for the evaluation process. The mice were further divided into seven groups of six mice each. The groups were named as control, formulation (F1, F2, F3, & F4), pure drug and positive control (standard). All the possible efforts were made to minimize animal suffering. The tumor cells were subcutaneously implanted on each mice and were anesthetized during implantation. The group of mice received an appropriate amount of dose through i.v. route (tail vein) except for control, which receives same saline solution [Vardhan et al., 2017b]. The relative tumor volume

was calculated for each group by measuring tumor size with a digital caliper for tumor volume calculation. GraphPad Prism was used for statistical analysis. The animals were checked for survival data, weight loss data and relative tumor volume for 30 days. Photographs of mice were taken on the first and last day of treatment with the optimized formulations.

4.12 Drug-Plasma interaction and hemolysis studies

In parenteral drug delivery especially via intravenous, there will be chances of a critical interaction of administered NPs with plasma and blood components. That can modify the pharmacological effects and biodistribution of the dosage form [Aggarwal et al., 2009]. The reticuloendothelial system (RES) uptake of any particulate materials is preceded by opsonization process, the absorption of serum protein bound to optimized NPs was studied. Evaluation of plasma protein on different formulation surface was performed by using SDS-PAGE gel electrophoresis [Magdeldin et al., 2014]. Here, the aqueous phase of each sample was loaded onto vertical slab gel consisting of stacking gel (10%) and resolving gel (12%). The gels were run at 100V for 90 min in SDS Tris buffer. The experimentation was done on plain rat plasma, drug treated plasma, drug alone, and optimized formulations treated with plasma.

The evaluation of hemolysis is a essential need for every intravenous administration to prove hemocompatibility [Fornaguera et al., 2015]. The heparinized blood sample of the rat was centrifuged at 4000 rpm for 10 min, and plasma layer was removed. To the erythrocyte pellet, the equal volume of PBS solution was added again subjected to centrifugation at 4000 rpm for 10 min. Optimized formulations of 25

μg/mL were mixed with 2 mL erythrocyte suspension and were incubated for 45 min at 37°C. The samples were diluted, dried and stained with leishman stain and peripheral blood smears were prepared on glass slides and analyzed with an optical microscope (Dewinter).

4.13 Stability study

The stability testing study is done to provide evidence on how the drug quality and drug product varies with time under the influence of ambient conditions such as temperature, humidity, and light. Freshly prepared freeze dried powder sample of different formulations were packed in amber coloured glass vials, sealed and placed in stability chamber. Accelerated stability studies were carried out over a period of 6 months at 40±2^{o}C, 75±5% RH as per ICH guideline and drug loaded lyophilized different PHBV nanoparticles were redispersed in PBS and analyzed for particle size, zeta potential, and % entrapment efficiency [Blessy et al., 2014].

4.14 Statistical analysis

All the experimental data are expressed as mean ± standard error of the mean (SEM). Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by Bonferroni post hoc test and Student-Newman-Keuls multiple comparisons tests wherever necessary. GraphPad Prism™ -5.01 (GraphPad Software Inc., San Diego, CA, USA) software was used for statistical analysis. Origin Pro™ -8 software (Origin Lab Corporation, MA, USA) was used for graph representation. A p-value < 0.05 was considered as statistically significant. In the case of formulation optimization, Minitab™ 17 (UK) was used for primary factor

screening; Design Expert™ -10 (MN, USA) and MODDE Pro™ -12 (Sweden) were employed for response surface methodology. GastroPlus™ 9 (NY, USA) utilized for pharmacokinetics, *in silico*, and *in vitro-in vivo* correlation studies.