Chapter-4

Materials and Methods
4.1. Chemicals

The following chemicals were obtained from the indicated suppliers and used as received.

Table 4.1. List of chemicals used for the study

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine sulphate</td>
<td>Cipla pharmaceuticals, Mumbai, India</td>
</tr>
<tr>
<td>Docetaxel trihydrate</td>
<td>Mac Chem Products (India) Pvt Ltd.</td>
</tr>
<tr>
<td>PLGA (50:50)</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>PVA</td>
<td>Spectrum chemicals, Kochi, India</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>Himedia, Mumbai, India</td>
</tr>
<tr>
<td>Dichloromethane A.R</td>
<td>Merck chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Ethylene Diamine for synthesis</td>
<td>Merck chemicals, Mumbai, India</td>
</tr>
<tr>
<td>N-hydroxy succinimide</td>
<td>Himedia, Mumbai, India</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>Merck chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Coumarin-6</td>
<td>Sigma Aldrich, Bangalore, India</td>
</tr>
<tr>
<td>1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC)</td>
<td>Himedia, Mumbai, India</td>
</tr>
<tr>
<td>DMSO</td>
<td>Merck chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>Spectrum chemicals, Kochi, India</td>
</tr>
<tr>
<td>Methanol</td>
<td>Merck chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Acetonitrile HPLC grade</td>
<td>Merck chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Water HPLC grade</td>
<td>Nice chemicals, Cochin, India</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>Nice chemicals, Cochin, India</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Nice chemicals, Cochin, India</td>
</tr>
<tr>
<td>o-Phosphoric acid</td>
<td>Nice chemicals, Cochin, India</td>
</tr>
</tbody>
</table>
4.2. Equipment

The equipment employed in the formulation and evaluation processes are listed in following table.

*Table 4.2. List of equipment used for the study*

<table>
<thead>
<tr>
<th>Equipments</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasonicator</td>
<td>Sonics vibra cell, USA</td>
</tr>
<tr>
<td>Electronic weighing balance</td>
<td>Libror, Shimadzu, Japan</td>
</tr>
<tr>
<td>Digital pH meter</td>
<td>Systronics, Ahmedabad</td>
</tr>
<tr>
<td>Micropipettes</td>
<td>Borosil, Chennai, India</td>
</tr>
<tr>
<td>UV- visible Double beam Spectrophotometer</td>
<td>UV-1800, Shimadzu, Kyoto, Japan</td>
</tr>
<tr>
<td>FTIR spectrophotometer</td>
<td>Perkin Elmer Spectrum 400, Massachusetts, US</td>
</tr>
<tr>
<td>Differential Scanning Calorimeter</td>
<td>DSC Q20, TA instruments, US</td>
</tr>
<tr>
<td>Vortex mixer (250V, 50 HZ)</td>
<td>Remi, Mumbai, India</td>
</tr>
<tr>
<td>Bath sonicator</td>
<td>Soltech, 220MA, Italy</td>
</tr>
<tr>
<td>Particle size Analyser</td>
<td>Nano-ZS, Malvern Instruments</td>
</tr>
<tr>
<td>Transmission electron microscope</td>
<td>JEOL JEM-2100, Japan</td>
</tr>
<tr>
<td>Scanning electron microscope</td>
<td>JEOL JSM-6390, Japan</td>
</tr>
<tr>
<td>Refrigerated centrifuge</td>
<td>Remi, Mumbai, India.</td>
</tr>
<tr>
<td>X-ray Diffractometer</td>
<td>‘X’ pert PRO, PANalytical instruments, Almelo, The Netherlands</td>
</tr>
<tr>
<td>HPLC</td>
<td>Shimadzu prominence, Kyoto, Japan</td>
</tr>
<tr>
<td>Lyophilizer / Freeze dryer</td>
<td>Subzero lab instruments, Chennai, India</td>
</tr>
<tr>
<td>Nylon 66 filter paper</td>
<td>Himedia, Delhi, India.</td>
</tr>
<tr>
<td>Magnetic Stirrer</td>
<td>Tarson, Mumbai, India.</td>
</tr>
<tr>
<td>Zetasizer nano SZ</td>
<td>Malvern Instruments, MA, USA</td>
</tr>
<tr>
<td>NMR spectrophotometer</td>
<td>Bruker (400 MHz), MA, USA</td>
</tr>
<tr>
<td>Accuri C6 flow cytometer</td>
<td>BD Biosciences, San Jose, CA, USA</td>
</tr>
</tbody>
</table>
4.3. Software

The softwares applied for the optimization and characterization of nanoparticle includes Design Expert, 8.0.7.1 software, (Stat-Ease, Minneapolis, USA).

4.4. Pre formulation Studies

Pre formulation studies are a prerequest for the development of any drug-delivery system for determining the interactions between the Active Pharmaceutical Ingredients, and the excipients incorporated in it. The available data of physico-chemical properties of drug and excipients are useful for the preformulation studies. It also includes various physical and chemical evaluations, analytical method developments and optimization of
the ideal formulation design. The main objective of preformulation is to generate information that is useful to develop most stable and effective formulation.

4.4.1. Physical appearance

The physical appearance of vincristine sulphate and docetaxel trihydrate was noted and compared with the certificate of analysis supplied by the vendor.

4.4.2. Identification of drug

The drug identification was done by performing UV and FTIR spectral analysis, XRD analysis, and DSC analysis and comparing it with that of the standards in the official books/literature value.

4.4.2.1. Spectrophotometric scanning

1 mg per mL (1000μg/mL) stock solutions of vincristine sulphate and docetaxel trihydrate was prepared by shaking in Remi vortex mixer for 10 minutes. From this solution 1.0mL solution was pipetted out and diluted to obtain 100 μg/mL solutions. The UV spectra of the prepared drug solutions were taken between wavelengths 200-400nm using UV- Vis spectrophotometer (UV 1800, Shimadzu, Japan).

4.4.2.2. Fourier Transform Infrared spectroscopy (FTIR)

FTIR analysis was done to study the chemical structure of vincristine sulphate and docetaxel trihydrate by the functional group analysis of the IR spectra. The samples were analysed by FTIR spectrophotometer Spectrum 400, (Perkin Elmer, Massachusetts, USA) and reported in wave number (cm\(^{-1}\)). The powder sample was added to the FTIR plate, such that the crystal was covered by the sample. Moved the arm on the IR spectrophotometer, and continued the twisting of handle until reaching the sufficient pressure to create the uniform disc of the sample. The scanning range was 400-4000 cm\(^{-1}\).

4.4.2.3. Powder X-ray diffraction (PXRD)

PXRD analysis was performed by X-ray diffractometer (X’ pert PRO, PANalytical, Almelo, The Netherlands) using Cu Ka radiation. The diffraction patterns were recorded from 0\(^{0}\) to 60\(^{0}\) at a diffraction angle of 20.

4.4.2.4. Differential scanning calorimetric analysis (DSC)

DSC was carried out using a DSC Q20 (TA instruments, US) to determine the thermal properties of vincristine sulphate and docetaxel trihydrate. Accurately weighed
samples of about 5 mg sealed in an aluminium pan of 40 µL capacity and equilibrated at 20°C were subjected to DSC run over the temperature range of 25-200°C for heating rate of 5°C/min.

4.4.2.5. NMR

NMR spectroscopic analysis of samples were carried out to identify the molecular structure of the compound as well as to identify the presence of any impurities in it. Samples are dissolved in deuterated DMSO and recorded the NMR spectrum using Bruker 400MHz NMR spectrometer.

4.4.3. Analytical method development

4.4.3.1. UV-Visible spectroscopic analysis of vincristine sulphate (VCS)

1mg/mL solution of vincristine sulphate was prepared by dissolving in water. The working solutions of vincristine ranging from 10 to 60 µg/mL is then prepared using pH 7.4 phosphate buffer by serial dilution of vincristine sulphate stock solution. Absorption at 296nm was recorded using the UV-Vis spectrophotometer (UV1800, Shimadzu, Japan.)

4.4.3.2. HPLC analysis of vincristine sulphate

The HPLC analytical method was developed by slightly modifying the procedure by Jianian Chen et. al (Chen et al., 2011). The chromatographic system used was composed of a Shimadzu LC-20AD UFLC system (Shimadzu, Kyoto, Japan) with a LC-20AD binary pump and a SPD-20A UV–Vis detector. Data processing was performed with LC Solution program. Analysis was carried out on C18 G column (250 mm x 4.6 mm, 5 µm,). The mobile phase 0.02 M sodium dihydrogen phosphate–methanol was taken in the ratio of 70 : 30 v/v with pH 4.7 separately and were filtered through membrane filter (Millipore Nylon disc filter of 0.45 µm) using vacuum filter. This filtered mobile phase was sonicated for 15 min in ultrasonic bath before use for removing the dissolved air. The mobile phase flow rate was 1.0 mL/min, the column temperature was maintained at room temperature, the UV–Vis detection wavelength of vincristine sulphate was set at 276 nm, and the injection volume was 20 µL injected using a Rheodyne injector.

Stock solution of vincristine sulphate (1mg/mL) was prepared in water and stored at -20°C. The working solutions of vincristine with 5, 10, 20, 30, 40, and 50 µg/mL were prepared by serial dilution of vincristine stock solution.
4.4.3.3. **UV-Visible spectroscopic analysis of docetaxel trihydrate (DCT)**

1 mg/ml solution of docetaxel trihydrate was prepared by dissolving in acetonitrile. The working solutions of docetaxel ranging from 10 to 70 μg/mL was prepared using pH 7.4 phosphate buffer by serial dilution of docetaxel trihydrate stock solution. Absorption at 229 nm was recorded using the UV-Vis spectrophotometer (UV1800, Shimadzu, Japan).

4.4.3.4. **HPLC Analysis of docetaxel trihydrate (DCT)**

The HPLC analytical method was developed by slightly modifying the procedure by Wei Wu et. al (Wu et al., 2014). Analysis was carried out on a C18 G column (250 mm × 4.6 mm, 5 μm). The mobile phase, acetonitrile and water were taken in the ratio of 55: 45 v/v. The mobile phase was sonicated for 15 min in ultrasonic bath before use for removing the dissolved air. The mobile phase flow rate was 1.0 mL/min, the column temperature was maintained at room temperature, the UV–Vis detection wavelength of docetaxel trihydrate was set at 230 nm, and the injection volume was 20 μL.

Stock solution of docetaxel trihydrate (1 mg/mL) was prepared using acetonitrile and stored at -20°C. The working solutions of docetaxel with 10, 20, 50, 100, 200, and 300 μg/mL were prepared by serial dilution of docetaxel stock solution.

4.4.4. **Drug-Excipient compatibility studies**

Drug excipient compatibility testing is the part of pre formulation study. The stability of a formulation depends on the compatibility of the drug and excipients. Any physical or chemical interaction between drug and excipient can affect bioavailability and stability of drug. To study the physical and chemical compatibility 1:1 mixture of drug and polymer was used. The physical mixture was stored for one week at room temperature before study.

4.4.4.1. **Fourier Transform Infrared Spectrum (FTIR)**

Physical mixtures of the drug with excipients were analysed in Perkin Elmer FTIR spectrum 400 analyser and was compared with the spectrum of the individual components to study any interaction.

4.4.4.2. **Differential Scanning colorimetry (DSC)**

Physical mixtures of drug (vincristine sulphate or docetaxel trihydrate), PLGA, and Folic Acid (1:1:1) was accurately weighed and sealed in an aluminium pan and was equilibrated to room temperature. DSC analysis was carried out using DSC Q20 TA
instrument and run over the temperature range of 25 to 300°C at a heating rate of 5°C/min to obtain the DSC curve and was compared with the DSC curves of the components to detect any interaction.

4.4.4.3. PXRD

Powder X-ray diffraction pattern of physical mixture of PLGA, folic acid and drug (vincristine sulphate or docetaxel trihydrate) was recorded from 0° to 60° at a diffraction angle of 2θ and observed the change in crystallinity of the drug in physical mixture using the X ray diffractometer X’ pert PRO (PANalytical, Almelo, The Netherlands).

4.5. Formulation and optimization of drug loaded nanoparticles using folic acid conjugated PLGA and Transferrin conjugated PLGA

The details of the drugs loaded and polymer-ligand conjugates used in the nanoparticle formulations developed are as in the Table 4.3. The final formulations were of two different conjugates, loaded with two different drugs.

*Table 4.3. Details of the drugs loaded and polymer-ligand conjugates used in the developed nanoparticle formulations*

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Drug loaded</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Vincristine sulphate</td>
<td>PLGA-EDA- Folic acid</td>
</tr>
<tr>
<td>1.2</td>
<td>Vincristine sulphate</td>
<td>PLGA-EDA-Transferrin</td>
</tr>
<tr>
<td>2.1</td>
<td>Docetaxel trihydrate</td>
<td>PLGA-EDA- Folic acid</td>
</tr>
<tr>
<td>2.2</td>
<td>Docetaxel trihydrate</td>
<td>PLGA-EDA- Transferrin</td>
</tr>
</tbody>
</table>

4.5.1. Synthesis of PLGA-EDA- Folic Acid Conjugate

Synthesis is of PLGA- Ethylene diamine- Folic acid conjugate (scheme-1) was optimised in our laboratory by modifying the method developed by Saxena et al (Saxena et al., 2012).
The conjugation of Folic acid with PLGA involves 4 steps:

4.5.1.1. Activation of PLGA

The polymer Poly lactic acid glycolic acid (PLGA 50:50) (0.02 mmol), 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) (0.16 mmol), and N-hydroxy succinimide (NHS) (0.16 mmol) were dissolved in anhydrous Dichloromethane (DCM) in a round-bottom flask, and left to stir overnight. The solution was then filtered (PTFE syringe filter, 0.45 µm) to remove the by-product dicyclohexylurea, and then added drop-wise to cold
anhydrous ether to precipitate the activated PLGA. Ether was decanted and the product was dried under vacuum overnight to obtain the solid activated PLGA.

4.5.1.2. Attachment of Ethylene diamine to activated PLGA

Activated PLGA (0.02 mmol) was dissolved in 8 mL anhydrous DCM in a round bottom flask. Ethylene diamine (0.1 mmol) was dissolved in 2 mL anhydrous DCM and added to the PLGA solution. Ethylene diamine was used in excess to suppress the formation of PLGA–EDA–PLGA triblock copolymers. The mixture was stirred gently at 400 rpm for 6 h under nitrogen atmosphere. The reaction mixture was then added drop-wise to cold anhydrous ether to precipitate the product, which was dried under vacuum. To get rid of the un-reacted EDA, the product was dissolved in Dimethyl sulphoxide (DMSO) and transferred into a dialysis bag (Spectra/Por molecular porous membrane, MWCO 6-8000, Spectrum Laboratories Inc., CA, USA). The contents of the bag were dialyzed against 5 L of de-ionized water for 48 h at 4°C with constant stirring at 600 rpm (with 4 changes). The product was then freeze-dried and stored at −20°C.

4.5.1.3. Activation of Folic acid (formation of NHS-folate)

Folic acid (FA) (2 mmol) was first activated with EDC (4 mmol) and NHS (4 mmol) in anhydrous DMSO in presence of 0.1 mL triethylamine as a catalyst, under light protected atmosphere overnight. The solution was filtered to remove the dicyclohexylurea by-product, and then precipitated in cold anhydrous ether. The product was obtained in the dry state after following several steps of ether washing, decantation and vacuum drying.

4.5.1.4. Conjugation of FA to PLGA–EDA

Activated FA, EDC (0.03 mmol) and PLGA–EDA (0.0075 mmol) were co-dissolved in anhydrous DMSO in light protected conditions for 8 h. EDC was added to ensure that all the folic acid is ready to react with PLGA–EDA. Excess amount of activated FA was used to get higher conjugation of FA to PLGA–EDA. The product was filtered, precipitated in methanol, and dried under vacuum. Then, it was dissolved in DCM to precipitate free un-reacted FA, and filtered again. The DCM in the product solution was evaporated under vacuum.

4.5.2. Synthesis of PLGA-EDA-Transferrin Conjugate

Synthesis of PLGA-EDA-Transferrin conjugate was done according to scheme-II.
4.5.2.1. Activation of PLGA

PLGA (0.01 mmol), EDC (0.08 mmol), and NHS (0.08 mmol) were dissolved in anhydrous DCM in a round-bottom flask, and left to stir overnight. The solution was then filtered (PTFE syringe filter, 0.45 μm) to remove the by-product dicyclohexylurea, and then added drop-wise to cold anhydrous ether to precipitate the activated PLGA. Ether was decanted and the product was dried under vacuum overnight to obtain the solid activated PLGA.
4.5.2.2. Attachment of Ethylene diamine to activated PLGA

Activated PLGA (0.01 mmol) was dissolved in 8 mL anhydrous DCM in a round bottom flask. Ethylene diamine (0.05 mmol) was dissolved in 2 mL anhydrous DCM and added to the PLGA solution. Ethylene diamine was used in excess to suppress the formation of PLGA–EDA–PLGA triblock copolymers. The mixture was stirred gently at 400 rpm for 6 h under nitrogen atmosphere. The reaction mixture was then added drop-wise to cold anhydrous ether to precipitate the product, which was dried under vacuum. To get rid of the un-reacted EDA, the product was dissolved in DMSO and transferred into a dialysis bag (Spectra/Por molecular porous membrane, MWCO 6-8000, Spectrum Laboratories Inc., CA, USA). The contents of the bag were dialyzed against 5 L of de-ionized water for 48 h at 4°C with constant stirring at 600 rpm (with 4 changes). The product was then freeze-dried and stored at ~20°C.

4.5.2.3. Activation of Transferrin (formation of NHS-transferrin)

Transferrin (0.00125 mmol) was first activated with EDC (0.0025 mmol) and NHS (0.0025 mmol) in anhydrous DMSO in presence of 0.1 mL triethylamine as a catalyst, under light protected atmosphere overnight. The solution was filtered to remove the dicyclohexylurea by-product, and then precipitated in cold anhydrous ether. The product was obtained in the dry state after following several steps of ether washing, decantation and vacuum drying.

4.5.2.4. Conjugation of Transferrin to PLGA–EDA

Activated Tf, EDC (0.006 mmol) and PLGA–EDA (0.024 mmol) were co-dissolved in anhydrous DMSO in light protected conditions for 8 h. EDC was added to ensure that all the transferrin is ready to react with PLGA–EDA. Excess amount of activated transferrin was used to get higher conjugation of Tf to PLGA–EDA. The product was filtered, precipitated in methanol, and dried under vacuum. Then, it was dissolved in DCM to precipitate free un-reacted Tf, and filtered again. The DCM in the product solution was evaporated under vacuum.

4.5.3. Formulation of drug loaded nanoparticle using polymer conjugates

The ligand conjugated drug loaded polymeric nanoparticles were fabricated by a modified oil-in-water (O/W) single emulsion solvent evaporation/extraction technique (Mattheolabakis et al., 2009). Accurately weighed amounts of ligand conjugated polymer were dissolved in dichloromethane and 5mg drug in 0.1 mL DMSO. Drug solution was
added to polymer solution with gentle stirring to dissolve the contents. The resulting organic phase was added slowly to aqueous phase containing poly vinyl alcohol (PVA) as stabilizer and sonicated using a probe sonicator (Sonics vibracell, USA) at an output of 40W in an ice bath. The formed o/w emulsion was gently stirred at room temperature by a magnetic stirrer (Taron, Mumbai, India) for up to 12 h for complete evaporation of organic solvent. Nanoparticles were separated by centrifuging the resulting suspension at 15,000rpm for 20 minutes at 4°C and washed with distilled water, thrice, to remove the emulsifier and adsorbed drugs. The washed nanoparticles were then freeze-dried for 24 hours using a lyophilizer (Sub zero, Chennai, India) at -85°C and 0.001mbar pressure (Figure 4.5).

**Figure 4.5. Schematic representation of formulation of nanoparticles**

### 4.5.4. Optimization of the formulation process

The formulation of drug loaded nanoparticles of desired quality is affected by different factors. Optimizing these factors by trial and error method is tedious and costly. Therefore we used software based Design of Experiments (DoE) for the optimization of the process of nanoparticle formulation. Licenced software of Design Expert ® ver. 8.0.7.1 (Stat- Ease Inc., MN, USA was used for the optimization studies.
4.5.4.1. Pre-optimization

Pre-optimization studies were carried out primarily to choose the independent factors and their levels. The main objective was to obtain nanoparticles with small particle size and high entrapment efficiencies. To study the factors that affect the particle size, and entrapment efficiency, various formulations were prepared with varying polymer and PVA concentrations. The organic to aqueous phase ratio was also optimised. The effect of sonication amplitude and sonication time on the particles size and entrapment efficiency was also studied. The prepared formulations were evaluated by measuring the particle size by dynamic light scattering (DLS) (Zetasizer nano SZ, Malvern Instruments, MA, USA) analysis (Paruchuri et al., 2012).

4.5.4.1.1. Pre-optimization of PLGA conjugate

The ratio of PLGA conjugate was varied at 5, 10, 15 and 20mg (ie, drug : PLGA = 1:1, 1:2, 1:3, 1:4). During the formulation drug content was 5mg and PVA concentration was 1.5%w/v. Sonication amplitude was kept at 40W, phase ratio as 1:3 and sonication time was 6 minutes.

4.5.4.1.2. Pre-optimization of PVA concentration

Surfactant polyvinyl alcohol (PVA) concentration was varied from 1 to 3% to get uniform emulsion with lower particle size and high entrapment efficiency. PLGA concentration was fixed as 10mg (ie, drug to polymer ratio as 1:2) and all other parameters were kept constant.

4.5.4.1.3. Pre-optimization of sonication amplitude

The effect of sonication amplitude (20W to 60W) on particle size and entrapment efficiency of nanoparticles was optimized.

4.5.4.1.4. Pre-optimization of phase ratio

During the pre-optimization studies the organic to aqueous phase ratio was varied from 1:2, 1:3, 1:4 and 1:5 respectively. During the formulation, 10mg of PLGA, 5mg of drug (organic phase) and, 1.5%w/v of PVA were taken and all other parameters were kept constant.

4.5.4.1.5. Optimization of sonication time

Sonication time was varied from 4, 6, 8, 10 and 12 min during the pre-formulation studies. The sonication time was optimized so as to obtain the minimum particle size based
on the DLS analysis. All the other factors were kept constant. Ultra sonicator (Sonics vibra cell, USA) was used for the application of sonication energy and change in the particle size and entrapment efficiency was monitored with change in sonication time.

4.5.4.2. Optimization of nanoparticles by 3² full factorial design

A 2-factor 3-level full factorial design was used for the formulation and optimization of nanoparticles for studying the influence of the two independent variables on the responses (Y₁ and Y₂) particle size and percentage drug entrapment. This design is suitable for exploring quadratic response surface and constructing second order polynomial models. Values of selected variables at different levels, after measuring the responses either simple linear (Y = X₀ + X₁A + X₂B) or interactive (Y = X₀ + X₁A + X₂B + X₅AB) or quadratic (Y = X₀ + X₁A + X₂B + X₃A² + X₄B² + X₅AB + E) models can be evolved by carrying out multiple regression analysis of the data and F statistics to identify statistically significant terms. The reduced equation, an equation containing only statistically significant terms is then used for drawing contour plots to see the influence of selected variables when changing from low to high level. The non-linear quadratic model generated by the design is in the form,

\[ Y = X₀ + X₁A + X₂B + X₃A² + X₄B² + X₅AB + E \]

where, Y is the measured response associated with each factor level combination: X₀ is an intercept; X₁ - X₅ are the regression coefficient; A, B are the factor studied and E is the associated error term.

Based on the pre-optimization studies three square designs was used for optimization. The phase ratio, and sonication time were found to have greater effect on particle size and entrapment efficiency. Based on the data from the pre-optimization, three levels were chosen and the design matrix was fixed. The particle size and percentage drug entrapment were chosen as the responses on which the success of the formulation depends. The three square design matrix and the factors with their levels are shown in Table 4.4, 4.5 respectively.
Table 4.4. Factors and levels used in the design

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Phase ratio</td>
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</tr>
<tr>
<td>Sonication time (min)</td>
<td>6</td>
</tr>
</tbody>
</table>

| Dependent variables   | Y₁ - Particle size |
|                       | Y₂ - Percentage drug entrapment |

Table 4.5. 3² full factorial design matrix

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Factor 1 Phase ratio</th>
<th>Factor 2 Sonication time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1:3</td>
<td>6</td>
</tr>
<tr>
<td>F2</td>
<td>1:4</td>
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</tr>
<tr>
<td>F13</td>
<td>1:4</td>
<td>8</td>
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</tbody>
</table>

4.5.5. Particle size distribution

The mean particles size and zeta potential were determined by laser diffraction particle size analyzer (Zetasizer Nano ZS- Malvern instruments, MA, USA). Prepared nano particulate dispersions were diluted with triple distilled water and stirred using a
mechanical stirrer at 500 rpm for 5 min in order to reduce aggregation. From the diluted sample 2 mL was pipetted and analyzed for particles size distribution.

4.5.6. Determination of encapsulation efficiency and drug loading

The amount of encapsulated vincristine sulphate and docetaxel trihydrate in the nanoparticles and the drug loading efficiency were evaluated by a direct method. 5mg of the freeze dried nanoparticles were vortexed with 2mL of DCM for 1h and was filtered through 0.22 µm membrane filter. Then the drug content in the filtrate was analyzed by HPLC. The percentage encapsulation, a measure of encapsulation efficiency, was calculated as the ratio of the drug content in the freeze dried powder to the initial drug amount added (Arya et al., 2011). The drug loading was the ratio of the drug content to the freeze dried powder.

\[
\text{Encapsulation efficiency} = \frac{\text{entrapped drug}}{\text{total drug added}} \times 100
\]

4.6. Characterization of ligand conjugated drug loaded polymeric nanoparticles

The freeze dried folic acid conjugated and transferrin conjugated PLGA nanoparticles bearing vincristine sulphate and docetaxel trihydrate were characterized using different techniques as follows.

4.6.1. FT-IR

FTIR analysis was done to study the chemical properties of vincristine sulphate, docetaxel trihydrate, and drug loaded nanoparticles by functional group analysis. The samples were analysed by FTIR Spectrum 400 (Perkin Elmer, Massachusetts, USA) analyser and reported in wave number (cm\(^{-1}\)). The scanning range was 400-4000 cm\(^{-1}\).

4.6.2. DSC

DSC thermograms of the physical mixture, folic acid conjugated drug loaded (vincristine sulphate, docetaxel trihydrate) PLGA nanoparticles, and transferrin conjugated drug loaded (vincristine sulphate, docetaxel trihydrate) PLGA nanoparticles were recorded and compared.
4.6.3. PXRD

Powder X ray diffraction pattern of physical mixture of drugs, polymer, & ligands, and ligand conjugated drug loaded nanoparticles were compared to study the change in crystal structure using X ray diffractometer X’ pert PRO, (PANalytical, Almelo, The Netherlands).

4.6.4 Shape and surface morphology by Transmission Electron Microscopy (TEM)

Transmission Electron microscopy offers images with more resolution and magnification over Scanning Electron Microscope. TEM can be utilized for obtaining more detailed surface images of nanoparticles. Transmitted electrons (those that do not scatter) are used to produce the two dimensional image of the particle by TEM.

The nanoparticles were suspended in Milli Q water. A tiny drop of sample was pipetted on the parafilm using a micropipette. Placed the shiny side of the TEM grid on the drop and left it for 20 minutes for the particles to adsorb on to the grid. Removed the grid and placed it on a tissue paper (Kimwipe without fibers) with shining side up for 1 hour for drying. Image scanning of the sample were performed under different magnifications.

4.6.5. Particle size distribution and zeta potential

The particle size distribution and zeta potential (ZP) of surface modified optimized formulation of nanoparticles were analyzed by dynamic light scattering technique using Zetasizer Nano ZS, nanoseries, Malvern Instruments, MA, USA. 0.1% by weight of the sample was prepared in phosphate buffered solution (PBS) of pH 7.4 for zeta potential measurement.

4.6.6. In-vitro drug release studies

Drug release from the nanoparticles was studied using a dialysis technique. Freeze dried nanoparticles equivalent to 5 mg drug were weighed and dispersed in 1mL of phosphate buffer solution at pH 7.4 and placed in a dialysis bag (Spectra/Por®, molecular weight cut off 12000 Da) sealed at both ends. The dialysis bag was soaked in 100 ml of phosphate buffer solution (pH 7.4) and maintained at 37 ±0.5°C and at 100 ±5 rpm in a shaker. At predetermined time intervals, individual samples were taken and was replaced with fresh phosphate buffer solution to maintain the sink condition. The amount of drugs (vincristine sulphate and docetaxel trihydrate) released into the medium was quantified by HPLC and compared with a standard calibration curve generated using known
concentrations of drugs (Saxena et al., 2012). The experiment was triplicated and calculated the standard deviation.

4.6.7. Kinetic assessment of drug release from ligand conjugated nanoparticles

The data obtained from in-vitro release studies of optimised formulations of vincristine sulphate and docetaxel trihydrate loaded PLGA-ligand conjugated nanoparticles were fitted to various models such as zero order, first order, Higuchi and Korosmayer Peppa’s to ascertain the kinetic modelling of drug release (Joshi, S. A., Chavhan, S. S. & Sawant 2010).

To study the release kinetics, the data obtained from in vitro drug release studies were plotted in various kinetic models.

2. First order rate kinetics: Log cumulative percentage of drug release Vs time
3. Higuchi model square: Cumulative percentage of drug release Vs square root of time
4. Korsmeyer peppas model: Log cumulative percentage of drug release Vs log time

The model with the highest correlation coefficient ($r^2$ values approaches unity) was chosen as the best fit model.

4.6.7.1. Zero order kinetics

A zero order release can be predicted using the equation

$$Q_t = Q_0 - K_0 t$$

where,
$Q_0 =$ Initial amount of drug present in solution (most cases $Q_0 =$0)
$Q_t =$ The amount of drug release at time t
$K_0 =$ Zero order release rate constant

A graph of cumulative percentage of drug released Vs time would yield a straight line with a slope equal to $K_0$.

4.6.7.2. First order kinetics

The first order describes the release from system where the release rate is concentration dependent. It can be described by following equation:
Where,
\[ K_1 = \text{First order release rate constant} \]

### 4.6.7.3. Higuchi model kinetics

The drug release can be predicted by the following equation:

\[ Q = K t^{1/2} \]

Where, \( K \) is Higuchi dissolution constant, \( t \) is the time in hours.

The model predicts that the drug release from the dosage form is directly proportional to the square root of time.

### 4.6.7.4. Korsmeyer Peppa’s model

To evaluate the mechanism of drug release, the in vitro release data was plotted in Korsmeyer equation as log cumulative percentage of drug release Vs log time and the exponent \( n \) was calculated through the slope of straight line (Pagar and Vavia, 2013).

Korsmeyer equation is as follows

\[ \frac{M_t}{M_\alpha} = K t^n \]

where,
\( M_t/M_\alpha \) is the fractional solute release
\( t \) is the release time.
\( K \) is the kinetic constant

### Table 4.6. The release exponent (n) values and mechanism of drug transport

<table>
<thead>
<tr>
<th>Release exponent (n)</th>
<th>Drug transport mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;0.45</td>
<td>Fickian diffusion</td>
</tr>
<tr>
<td>0.45 &lt; n = 0.89</td>
<td>Non-Fickian transport</td>
</tr>
<tr>
<td>0.89</td>
<td>Case II transport</td>
</tr>
<tr>
<td>Higher than 0.89</td>
<td>Super case II transport</td>
</tr>
</tbody>
</table>
4.7. In-vitro bioactivity studies

**Cell line: MCF-7 (human breast cancer cell line), from NCCS Pune, India.**

**Materials used:**

DMEM (Dulbecco’s modified eagles medium), fetal bovine serum (FBS) and MTT reagent from sigma Aldrich, USA.

Tissue culture flasks, micro centrifuge tubes, 96 well microculture plates from Tarson and Nunc, USA.

Gentamycin, Streptomycin, and penicillin procured from KMC hospital, Manipal.

**Instruments:**

1. Biosafety class II cabinet
2. Inverted Microscope (Olympus)
3. Microplate reader (ELISA Reader, Bio-Tek)
4. BD Accuri C6 flow cytometer

**Methods:**

i. Maintenance of cell lines

MCF-7 (human breast cancer cell line) procured from NCCS Pune were grown in 25 cm² tissue culture flasks containing Dulbecco’s modified eagles medium (DMEM) supplemented with 10% FBS, 1% L-glutamine and 50μg/ml gentamycin sulphate at 37°C in CO₂ incubator in an atmosphere of humidified 5% CO₂ and 95% air. The cells were maintained by routine sub culturing in 75cm² tissue culture flasks.

ii. Sub culturing process of cell lines

The culture media from the flasks containing monolayer culture was aspirated and washed with sterile phosphate buffered saline (PBS). To the flasks, 3 mL of 0.1% trypsin-EDTA solution was added and after few seconds it was aspirated and flask was kept in incubator 2-3 min. for detachment. The flasks were removed from the incubator and gently tapped to detach all the adhering cells. The cell detachment was confirmed by observing under an inverted microscope (Nikon Eclipse TE 2000-5, Japan). Once the cells were completely detached from the flasks, 2-3 mL of DMEM media containing 10% FBS was added and mixed well.
Cell viability was checked with a small sample of the suspension by trypan blue dye exclusion test. From the stock cell suspension, 1 x 10^5 viable cells/ml suspended in media were seeded in 25cm^2 tissue culture flask containing about 4mL of fresh media and incubated until the flasks attained 60-70% confluence.

**iii. Trypsinization**

To obtain a single cell suspension from a monolayer culture, cells were dislodged from the culture flasks by trypsinization. From a 60-70% confluent flask, the culture media was aspirated out using a micropipette. Cells were washed with 3 mL of PBS to remove trace amount of media.

To each culture flask 3 mL of trypsin-EDTA was added and after few seconds it was aspirated and the flask was kept in the incubator for 3-4 min for cell detachment. Culture flasks were observed under an inverted microscope (Nikon Eclipse, Japan) to ensure that cells were completely dislodged. Trypsin activity was stopped by adding 2-3mL media containing 10% FBS.

**4.7.1. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay:**

**4.7.1.1. Principle**

MTT is taken up by the viable cells and reduced to formazan by the “Succinate-tetrazolium reductase” system that belongs to the mitochondrial respiratory chain functioning in metabolically active cells. Formazan formed, is a purple colored water-insoluble product that is largely impermeable to cell membranes, thus resulting in its accumulation within the healthy cells which is solubilized by adding Dimethyl sulphoxide (DMSO). The optical density (OD) of purple colored solution developed was read using a conventional ELISA plate reader at 540nm (maximum absorbance). The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which, in turn, may be interpreted as a measure of viability and/or cell number.

**4.7.1.2. Procedure**

Exponentially growing cell lines were harvested from 25cm^2 tissue culture flask and a stock cell suspension (5X10^6 cell/mL) was prepared. A 96-well flat bottom tissue culture plate was seeded with 5 x 10^3 cells in 0.1 mL of DMEM medium supplemented with 10% FBS and allowed to attach for 24h. Test compounds were prepared just prior to the experiment and serially diluted with medium to get the desired working solutions. After 24
h of incubation, cells were treated with 100 µL of test solutions were incubated for 24 h. Each treatment was performed in triplicates. After the treatment, drug containing media was removed and washed with 200µL of PBS. To each well of the 96 well plate, 100µL of MTT reagent (Stock: 1mg/ml in serum free medium) was added and incubated for 4h at 37°C. After 4h of incubation the plate was blotted on tissue paper to remove the MTT reagent. To solubilize formazan crystals in the wells, 100µL of 100% DMSO was added to each well. The optical density (O.D) was measured by an Enzyme Linked immunosorbent Assay (ELISA) plate reader at 540 nm. O.D of each well was read and expressed as percentage cell survival: (absorbance of treated wells / absorbance of control wells x 100).

4.7.2. Estimation of Coumarin 6 tagged nano formulation uptake by flow cytometry

4.7.2.1. Procedure

MCF 7 (1 x 10⁶) cells were seeded in 96-well plates and incubated for 24 h at 37°C temperature in humidified CO₂ incubator. After 24 h of incubation, medium was aspirated from plates and cells were washed with DMEM medium. 10µL of nano formulations (at their respective IC₅₀ concentrations) were incubated at 37°C temperature in CO₂ incubator (for 2 h and 24 h). Then formulation in medium was aspirated and cells were washed three times with PBS and scraped in 1mL of fresh PBS using cell scraper. The cells were analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) excitation at 488 nm and emission at 533/30. A minimum of 10,000 events was acquired and analysis of flow cytometric data was performed using BD software. The first gating was done for single cell population selection on FSS vs SSC dot plot. The second dot plot was FL-1-A vs FL-4-A (533/30 vs 675/25) for selection of coumarin 6 fluorescence in FL-1 channel. The internal fluorescence or auto-fluorescence was normalized using plain normal cells. The shift in mean fluorescence in FL1-A vs count plot was calculated (Ge et al., 2009).

4.7.3. Cell cycle analysis

4.7.3.1. Principle

The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye (e.g. propidium iodide) that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height
(amplitude) proportional to the total fluorescence emission from the cell. Four distinct phases could be recognized in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). However, G2- and M-phase, which both have an identical DNA content, could not be discriminated based on their differences in DNA content.

### 4.7.3.2. Procedure

MCF-7 (1× 10^6 cells) was seeded in 25 cm² flasks and after overnight adherence, incubated with test compounds (at their respective IC₅₀ concentrations) for 24h. Cells were detached by trypsinization and mixed with floating cells, centrifuged and washed with PBS. The cell pellets were fixed in 70% ice-cold methanol at -20°C for 24 h. Cell pellets were washed with PBS and isotonic PI solution [25 µg/ml propidium iodide, 0.03% NP-40 and 40 µg/ml RNase A] was added. The stained cells were analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using excitation at 488 nm and emission at 575/40 nm. A minimum of 10,000 events were acquired for each sample and data analysis was done by using BD Accuri™ C6 software (Pozarowski and Darzynkiewicz, 2004; Reddy et al., 2015).

### 4.8. Stability studies

The stability studies were carried out according to ICH Q1A guidelines with optimized nanoparticles (ICH, 2003). According to these guidelines the developed nanoparticles were stored at 5° ±3°C in a refrigerator for a period one year and samples were withdrawn in every 3 months. Three batches of each nanoparticle formulations were kept in the refrigerator and analysed for the changes in particle size and percentage drug content.