Chapter 5

Formulation Development of Nanostructured Lipid Carriers

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FORMULATION DEVELOPMENT OF PACLITAXEL & CYCLOPHOSPHAMIDE LOADED NANOSTRUCTURED LIPID CARRIERS (NLCs)

5.1 METHOD FOR NLCS MANUFACTURING

Lipid phase was prepared by heating solid lipid and liquid lipid in a jacketed vessel preset to 55±2°C followed by addition of Paclitaxel and Acetone (Solvent). After Paclitaxel was dissolved, Cremophor EL was added followed by addition of Cyclophosphamide. Aqueous phase was prepared by dissolving Soluplus in water for injection and further heating the solution to 55±2°C. Aqueous phase was added to lipid phase at elevated temperature under continuous stirring to evaporate the solvent and further cooled at 5 ± 3°C under continuous stirring to form NLCs [1-3]. The schematic representation of the manufacturing process is as presented in Figure 5.1.

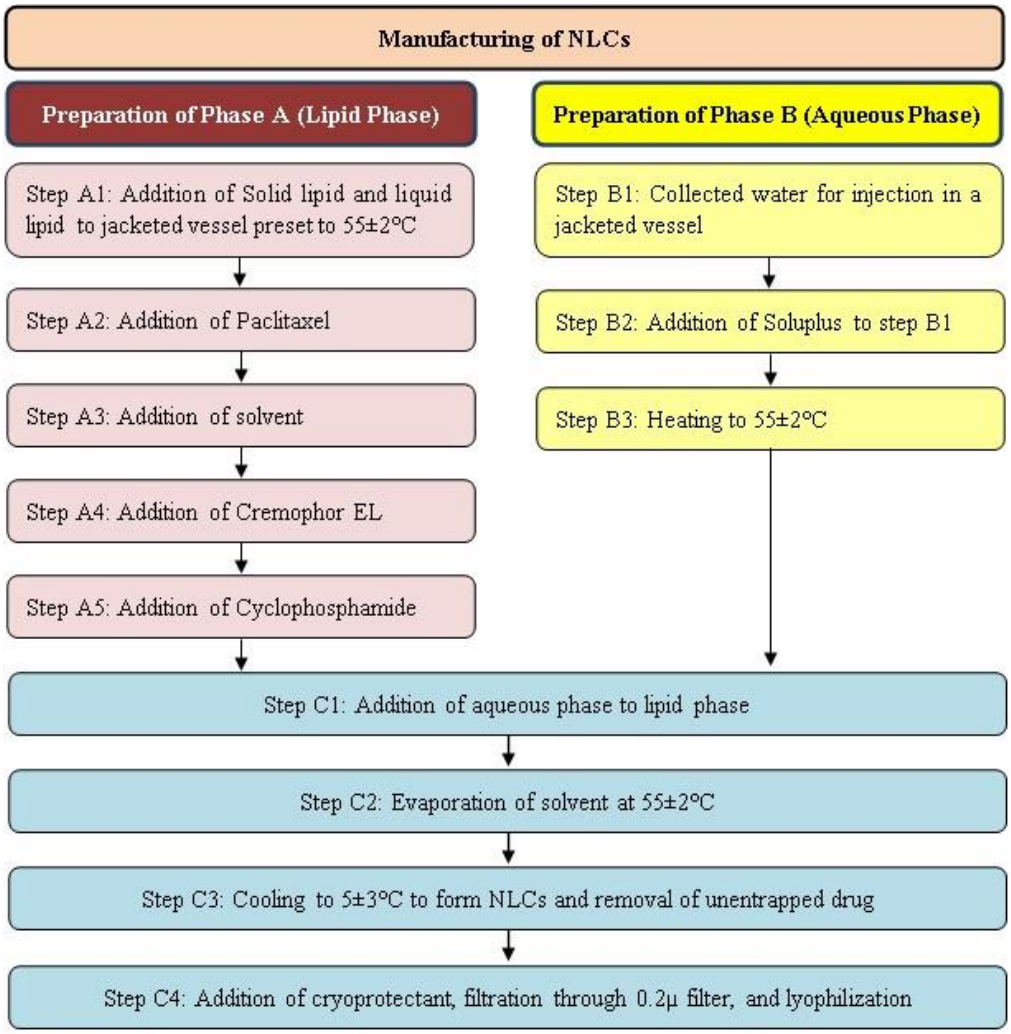


Figure 5.1: Manufacturing process of Paclitaxel and Cyclophosphamide loaded NLCs

As a part of formulation development studies, quality by design (QbD) approach was adopted for the development of Paclitaxel and Cyclophosphamide loaded NLCs. Formulation development contains following elements delineated in ICH Q8 (R2):

[A] Quality Target Product Profile (QTPP)
[B] Critical Quality Attributes (CQAs) of the drug product
[C] Qualitative risk assessment using Ishikawa diagram

The formulation development emphasizes a science and risk based approach to product, process development, and presents findings as a knowledge-based report, where relevant, supporting data have been summarized in appropriate tables or illustrations. Quality by Design is "A systematic approach to development that begins with predefined objectives and emphasizes product & process understanding and process control, based on sound science and quality risk management".

The scientific approach for pharmaceutical development begins with identification of desired dosage form and performance attributes through the QTPP. From this QTPP, a list of potential drug product CQAs was derived. A risk assessment was performed to identify the variables and unit operations which are most likely to impact the CQAs.

[A] Quality Target Product Profile (QTPP)

Quality Target Product Profile (QTPP) forms the basis of design for the development of a drug product. For development of Paclitaxel and Cyclophosphamide loaded NLCs, QTPP was defined based on prior knowledge and literature review as presented in Table 5.1.

Table 5.1: QTPP for development of Paclitaxel and Cyclophosphamide loaded NLCs

Sr. No.	QTPP Element	Target	Justification
1	Dosage Form	Injectable	Pharmaceutical equivalence: Same dosage form as per Innovator TAXOL (Paclitaxel Injection) and CYTOXAN (Cyclophosphamide Injection).
2	Route of administration	Injection, Intravenous	Pharmaceutical equivalence: Same dosage form as per Innovator TAXOL (Paclitaxel Injection) and CYTOXAN (Cyclophosphamide Injection)

Sr. No.	QTPP Element	Target		Justification
3	Stability	At least 6 Months at refrigerated condition (5±3°C)		ICH Q1A (R2) mandates to ensure that product maintains identity, quality, and purity upon storage.
4	Product Description after reconstitution	Clear transparent to translucent liquid with blue tint. Free from any visible particulate matter.		As per ICH Q6A, "A qualitative description of the dosage form should be provided. If any of these characteristics change during manufacture or storage, this change should be investigated and appropriate action taken".
5	Assay of Paclitaxel	90.0 to 110.0 %		According to ICH Q6A, "A specific, stability-indicating assay to determine strength (content) should be included for all new drug products".
6	Assay of Cyclophosphamide	90.0 to 110.0 %		
7	Sterility	Should be sterile		The dosage form is intended to be delivered by intravenous injection. Sterility of injectable is important for patient safety.
8	Particle size distribution	Z-average	< 150nm	Particle size distribution is a critical quality attribute particular to a NLC drug product.
		PDI	< 0.2	
9	Zeta Potential	To be reported		To ensure NLCs stability needed for patient safety and efficacy.
10	Reconstitution time of Lyophilized product	Not more than 1 minute		To ensure complete reconstituted product is clear to translucent liquid free from any visible particulate matter or any undissolved lyophilized mass which is need for patient safety.
11	% water content	Not more than 2.0 %		To yield a stable product by minimizing hydrolytic degradation.
12	Entrapment efficiency	Not less than 90%		Higher entrapment efficiency is needed for patient safety and efficacy.
13	Filterability	Not less than 100 %		Filterability ensures particulate matter free product which is required for patient safety.

[B] Critical Quality Attributes (CQAs) of the drug product

Based on the QTPP, prior knowledge, literature review, and various guidance documents, drug product Critical Quality Attributes (CQAs) were defined. The list of CQAs identified is tabulated in Table 5.2.

Table 5.2: CQAs for development of Paclitaxel and Cyclophosphamide loaded NLCs

Sr. No.	Critical Quality Attributes	Target		Is this a CQA? (Y/N)	Justification
1	Product Description after reconstitution	Clear transparent to translucent liquid with blue tint. Free from any visible particulate matter.		Yes	Description is an indicator of physical stability of dosage form. Hence, it was monitored throughout the drug product development.
2	Assay of Paclitaxel	90.0 to 110.0 %		Yes	Formulation parameters and process parameters may impact the assay of the drug substance. Also, there may be impact of storage conditions on the drug substance assay. Hence, this CQA was assessed throughout the drug product development.
3	Assay of Cyclophosphamide	90.0 to 110.0 %		Yes	
4	Sterility	Should be sterile		Yes*	The dosage form is intended to be delivered by intravenous injection. Sterility of injectable is important for patient safety. However, this CQA was assessed for the final formulation developed at the end of drug product development.
5	Particle size distribution	Z-average	< 150nm	Yes	Formulation parameters and process parameters may impact the particle size distribution. Also, there may be impact of storage conditions on the particle size distribution. Hence, this CQA was assessed throughout the drug product development.
		PDI	< 0.2		
6	Zeta Potential	To be reported		Yes*	This CQA will be assessed for final formulation developed at the end of drug product development. Also, there may be impact of storage conditions on the zeta potential. Hence, this CQA was assessed during the stability study.

Sr. No.	Critical Quality Attributes	Target	Is this a CQA? (Y/N)	Justification
7	Reconstitution time of Lyophilized product	Not more than 1 minute	Yes*	Reconstitution time may get impacted by lyophilization process. Further it will impact safety, quality and ease of administration of drug product. Hence it was evaluated during lyophilization cycle development.
8	% water content	Not more than 2.0 %	Yes	Water in formulation may impact safety of formulation. High Water content may cause hydrolytic degradation of drug product. Hence, This parameter was evaluated during lyophilization cycle development.
9	Entrapment efficiency	Not less than 90%	Yes	Formulation parameters and process parameters may impact the entrapment efficiency. Also, there may be impact of storage conditions on the drug substance assay. Hence, this CQA was assessed throughout the drug product development.
10	Filterability	Not less than 100 %	Yes	Formulation parameters and process parameters may impact the filterability. Hence, this CQA was assessed throughout the drug product development.

* Shall be assessed as and when required during the characterization of optimized product and stability analysis.

[C] Qualitative risk assessment using Ishikawa diagram

All possible variables which were linked with the development of Paclitaxel and Cyclophosphamide loaded NLCs were demonstrated with the help of Ishikawa diagram. These factors were considered as 'low, moderate, and high risk' based on their predicted effect on the Critical Quality Attributes derive from the Quality Target Product Profile. Ishikawa diagram is presented in Figure 5.2.

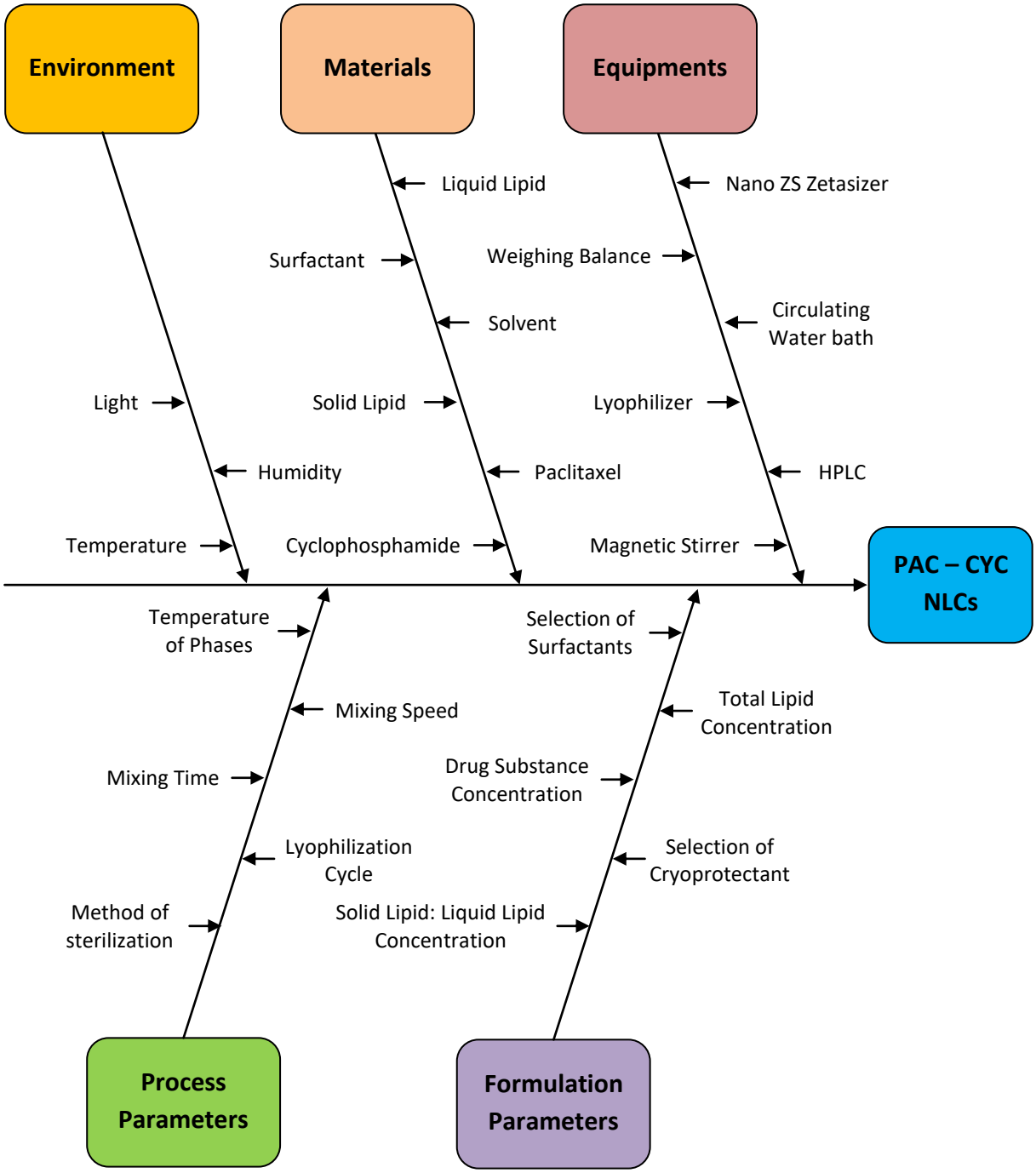


Figure 5.2: Ishikawa diagram of Paclitaxel and Cyclophosphamide loaded NLCs

Based on the above Ishikawa diagram of Paclitaxel and Cyclophosphamide loaded NLCs, the initial qualitative risk assessment is presented in Table 5.4, and justification for initial risk assessment is presented in Table 5.5. Risk assessment was performed to identify and rank parameters with potential to have an impact on the drug product CQAs. The relative risk that each attribute presents was ranked as high, medium, or low. The high risk attributes

warranted further investigation whereas the low risk attributes required no further investigation. The medium risk is considered acceptable based on current knowledge. Further investigation for medium risk may be needed in order to reduce the risk.

Table 5.3: Overview of Risk Ranking System

Low	Broadly acceptable risk. No further investigation is needed.
Medium	Risk is acceptable. Further investigation may be needed or can be justified based on the literature review or prior knowledge in order to reduce risk.
High	Risk is unacceptable. Further investigation is needed to reduce the risk.

Table 5.4: Initial Risk Assessment

Drug Product CQAs	**Factor**				
	Environment	**Materials**	**Equipments**	**Process Parameters**	**Formulation Parameters**
Product Description after reconstitution	Low	Low	Low	High	High
Assay of Paclitaxel	Medium	Low	Low	High	High
Assay of Cyclophosphamide	Medium	Low	Low	High	High
Sterility	Low	Low	Low	Medium	Low
Particle size distribution	Low	Low	Low	High	High
Zeta Potential	Low	Low	Low	Medium	Medium
Reconstitution time of Lyophilized product	Low	Low	Low	High	High
% water content	Low	Low	Low	High	High
Entrapment efficiency	Low	Low	Low	High	High
Filterability	Low	Low	Low	High	High

Table 5.5: Justification for Initial Risk Assessment

Drug Product CQAs	Factor	Justification
Product Description after reconstitution	Environment	Environment condition does not impact the product description after reconstitution. Hence, the risk is Low.
	Materials	Materials used do not impact the product description after reconstitution. Hence, the risk is Low.
	Equipments	Equipments used do not impact the product description after reconstitution. Hence, the risk is Low.
	Process Parameters	Process parameters (Lyophilization cycle) and formulation parameters (Selection of cryoprotectant) directly impact the product description after reconstitution. Hence, the risk is High.
	Formulation Parameters	
Assay of Paclitaxel	Environment	Based on literature review, Paclitaxel is sensitive to light conditions. Hence, the risk is medium.
	Materials	Materials used do not impact the assay of Paclitaxel. Hence, the risk is Low.
	Equipments	Equipments used do not impact the assay of Paclitaxel. Hence, the risk is Low.
	Process Parameters	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration directly impact the assay of Paclitaxel. Hence, the risk is High.
	Formulation Parameters	
Assay of Cyclophosphamide	Environment	Based on literature review, Cyclophosphamide is sensitive to light conditions. Hence, the risk is medium.
	Materials	Materials used do not impact the assay of Cyclophosphamide. Hence, the risk is Low.
	Equipments	Equipments used do not impact the assay of Cyclophosphamide. Hence, the risk is Low.
	Process Parameters	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration directly impact the assay of Cyclophosphamide. Hence, the risk is High.
	Formulation Parameters	
Sterility	Environment	Environment condition does not impact the product sterility. Hence, the risk is Low.
	Materials	Materials used do not impact the product sterility. Hence, the risk is Low.
	Equipments	Equipments used do not impact the product sterility. Hence, the risk is Low.
	Process Parameters	Process parameters (selection of method of sterilization) impact the sterility of drug product. Hence, the risk is Medium.
	Formulation Parameters	Formulation parameters do not impact the sterility of product. Hence, the risk is Low.

Drug Product CQAs	Factor	Justification
Particle size distribution	Environment	Environment condition does not impact the particle size distribution. Hence, the risk is Low.
	Materials	Materials used do not impact the particle size distribution. Hence, the risk is Low.
	Equipments	Equipments used do not impact the particle size distribution. Hence, the risk is Low.
	Process Parameters	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration directly impact the particle size distribution. Hence, the risk is High.
	Formulation Parameters	
Zeta Potential	Environment	Environment condition does not impact the zeta potential. Hence, the risk is Low.
	Materials	Materials used do not impact the zeta potential. Hence, the risk is Low.
	Equipments	Equipments used do not impact the zeta potential. Hence, the risk is Low.
	Process Parameters	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration impacts the final formulation which in turn may impact the zeta potential during product storage. Hence, the risk is Medium.
	Formulation Parameters	
Reconstitution time of Lyophilized product	Environment	Environment condition does not impact the reconstitution time of lyophilized product. Hence, the risk is Low.
	Materials	Materials used do not impact the reconstitution time of lyophilized product. Hence, the risk is Low.
	Equipments	Equipments used do not impact the reconstitution time of lyophilized product. Hence, the risk is Low.
	Process Parameters	Process parameters (Lyophilization cycle) and formulation parameters (Selection of Cryoprotectant) directly impacts the reconstitution time of lyophilized product. Hence, the risk is High.
	Formulation Parameters	
% water content	Environment	Environment condition does not impact the water content. Hence, the risk is Low.
	Materials	Materials used do not impact the water content. Hence, the risk is Low.
	Equipments	Equipments used do not impact the water content. Hence, the risk is Low.
	Process Parameters	Process parameters (Lyophilization cycle) and formulation parameters (selection of Cryoprotectant) directly impact the water content of lyophilized product. Hence, the risk is High.
	Formulation Parameters	

Drug Product CQAs	Factor	Justification
Entrapment efficiency	Environment	Environment condition does not impact the entrapment efficiency. Hence, the risk is Low.
	Materials	Materials used do not impact the entrapment efficiency. Hence, the risk is Low.
	Equipments	Equipments used do not impact the particle size distribution. Hence, the risk is Low.
	Process Parameters	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration directly impact the entrapment efficiency. Hence, the risk is High.
	Formulation Parameters	
Filterability	Environment	Environment condition does not impact the filterability. Hence, the risk is Low.
	Materials	Materials used do not impact the filterability. Hence, the risk is Low.
	Equipments	Equipments used do not impact the filterability. Hence, the risk is Low.
	Process Parameters	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration directly impact the filterability. Hence, the risk is High.
	Formulation Parameters	

Based on the above initial risk assessment, process parameters and formulation parameters were observed to have HIGH risk and hence were optimized using OFAT (One Factor At a Time) technique. The parameters studied were:

1. Formulation Parameters
    - [A] Selection of surfactants
    - [B] Total lipid concentration
    - [C] Solid lipid: liquid lipid concentration
    - [D] Drug Substance concentration

2. Process Parameters
    - [A] Mixing time
    - [B] Mixing speed
    - [C] Temperature of phases

The Critical Quality Attributes (Particle size distribution – Z-average and PDI, Entrapment efficiency and filterability) were selected as criteria for optimization of formulation and process parameters.

5.2 OPTIMIZATION OF FORMULATION AND PROCESS PARAMETERS

The optimization of nanostructured lipid carriers (NLCs) heavily relies on the formulation parameters. The stability, drug loading capacity, physicochemical characteristics, and general performance of NLCs are all influenced by these factors.

Selection of Surfactants

The stability of the NLC dispersion is ensured by the surfactants, which form a stabilizing layer around lipid nanoparticles, preventing coalescence and aggregation. Surfactants minimize the interfacial tension between the lipid phase and the aqueous phase, thereby effectively preventing the undesirable aggregation of lipid particles. Surfactants are essential for achieving smaller particle sizes by efficiently lowering surface tension. Surfactants have the ability to enhance the solubility of hydrophobic drugs, thereby increasing their loading capacity within the lipid matrix thereby improving the encapsulation efficiency of drugs within NLCs by stabilizing the lipid matrix.

Various surfactants which can be used for i.v. route viz. Polysorbate 20, Polysorbate 80, Poloxamer 188, Soluplus, and Cremophor EL were tried for preparation of NLCs as per Table 5.6. Five surfactants were screened to select the best surfactant [4-17].

Table 5.6: Surfactants, type, HLB value, and maximum allowable concentration

Sr. No.	Surfactant	Type of Surfactant	HLB Value	Maximum allowable Concentration for i.v. administration
1	Polysorbate 20	Hydrophilic	16.7	1.00 %
2	Polysorbate 80	Hydrophilic	15	69.33 %
3	Poloxamer 188	Hydrophilic	29	2.0 %
4	Soluplus	Amphiphilic	14	75.0 %
5	Cremophor EL	Amphiphilic	13	65.0 %

Based on the literature review, it was concluded that optimization of formulation parameters and process parameters is critical for the development of NLCs. The critical formulation and process parameters evaluated to achieve optimized NLCs along with their levels are presented in Table 5.7.

Table 5.7: Formulation and Process parameters to be optimized

Sr. No.	Parameter			Levels		
				L1	**L2**	**L3**
1	Formulation parameters	Total Lipid Concentration		2%	4%	6%
2		Ratio of solid lipid to liquid lipid		1:1	1:2	1:3
3		Drug substance concentration	Paclitaxel	0.1 %	0.2 %	0.3 %
			Cyclophosphamide	0.875 %	1.75 %	2.625 %
4	Process parameters	Mixing speed (RPM)		250	500	1000
5		Mixing time (Minutes)		5	15	30
6		Temperature of Phases		50±2°C	70±2°C	90±2°C

5.3 LYOPHILIZATION OF NLCs

5.3.1 Selection of Cryoprotectant

Mannitol, Lactose Monohydrate and Dextrose Monohydrate were evaluated as cryoprotectants. The ultimate cryoprotectant was chosen based on a freeze-thaw investigation in which nanostructured lipid carriers (NLCs) containing 4% w/v of each cryoprotectant were frozen at a rate of 0.1°C per minute and subsequently returned to ambient temperature (25°C) at the same heating rate of 0.1°C per minute. After freeze thaw, each sample was analyzed for average particle size distribution, and % entrapment efficiency of Paclitaxel and Cyclophosphamide of the formulated NLCs. Further, the selected cryoprotectant was studied at 3 different concentrations of 2%w/v, 4%w/v, and 6% w/v to optimize its concentration [18-24].

5.3.2 Freeze Drying Microscopy

The freezing temperature and collapse temperature of the formulated NLCs was determined by using LYOSTAT5 Freeze drying Microscope. Briefly, Silicone oil was put on the temperature-controlled element of the freeze-drying Cryo-stage, and a quartz cover slip was placed on it. A drop of the sample was placed in the middle on the quartz cover slip, a metallic spacer was placed around the drop, and a glass cover slip was placed overlapping the sample and spacer. Sample was frozen for up to -45°C at the freezing rate of 2.5°C/minute and held for 5 minutes at -45°C. Further, the sample was dried at the drying rate of 2.5°C/minute and collapse temperature was determined [25, 26].

5.3.3 SMART™ Lyophilization Cycle Development

SMART™ cycle was executed using Lyostar III Lyophilizer of SP Scientific, USA. SMART™ Freeze-Dryer technology eliminates the trial-and-error approach normally involved in developing new lyophilisation cycles. Based on manometric temperature measurement equation and heat and mass transfer theory, pressure rise data is analyzed by an algorithm and converted to a number of critical process parameters. Lyophilization parameters were selected by using SMART™ Programme of Lyostar-3 Lyophilizer (SP Scientific). Parameters required to be fed in the SMART™ program are tabulated in Table 5.8 along with explanation of each parameter [27-33].

Table 5.8: Parameters and Explanation of SMART™ Lyophilization Programme

Sr. No.	Parameter	Explanation	Parameter selected
1	Number of vials loaded in lyophilizer	Total number of vials loaded in the lyophilizer is to be entered.	25 Nos.
2	Vial Inner area (Ap)	The surface area of the inner bottom surface of the vial in square centimetres (cm^2).This information can be obtained from the vial supplier or calculated from vial drawings using the formula $A=\pi*r^2$, where r = the inner vial radius. All the vials used should have the same surface area.	20.9 cm^2
3	Fill volume	The fill volume of the solution in one vial in cubic centimetres. The fill volume should be the same for each vial in the batch.	60 cm^3
4	Fill weight	The net weight in grams of product filled in single vial. The fill weight should be the same for each vial in the batch.	62 gram
5	Tc, Tg' or Teu	The formulation's collapse temperature (Tc), glass transition temperature (T_g'), or eutectic temperature (Teu) should be entered depending on the nature of the product. Where more than one value exists, the lowest value should be used for the safest cycle. Freeze-drying microscopy (FDM) is the best known method of obtaining a product's Tc, Tg' or Teu value.	0.0 °C
6	Concentration of solution	The amount of solute per unit amount of solution (g/g).	0.1 g/g

Sr. No.	Parameter	Explanation	Parameter selected
7	MTM Interval (min)	This value shall determine how often the Isolation Valve is closed during Drying to perform the pressure rise measurements and manometric temperature measurements (MTM).	60 minutes (Default)
8	Nature of Drug Product	For information only, select either protein or small molecule depending on the type of product. This information will be included in the SMART™ data file that is generated during the cycle.	Small molecule
9	Type of Vials	Enter the type of glass vials being used (e.g. Tubing or moulded).Tubing vials are generally recommended for lyophilization applications. This parameter will help determine the heat transfer coefficient used by the program.	Moulded
10	Type of Bulking Agent	Select the appropriate value according to the type of bulking agent being used (e.g. none, unsure, crystalline, or amorphous). This selection will determine the default freezing program, default upper shelf point limit in drying, ramp rates, and shelf set points during the last step(s) of drying.	Crystalline

5.4 CHARACTERIZATION OF PAC-CYC NLCs

5.4.1 Description, Redispersibility and Reconstitution of Lyophilized NLCs

In a glass vial containing 100mg of lyophilized NLCs, 10mL of sterile water for injection and phosphate buffer pH 7.4 respectively and shaken vigorously for 10 times to check the Redispersibility and time required for reconstitution [34-36].

5.4.2 Particle size distribution and Zeta Potential

Particle size distribution (Z-Average) and Zeta potential were obtained using Malvern Zetasizer (Nano ZS) for Paclitaxel-Cyclophosphamide loaded NLCs. The temperature for sample testing was selected as 25°C and equilibrium time of 60 seconds was given to each sample. [37-38].

5.4.3 Assay of Paclitaxel

HPLC method from USP 43 NF 38 was adopted for determination of Paclitaxel wherein Shimadzu Chromatograph, Prominence-I LC2030C plus was used. The quantitation of Paclitaxel was done using (250 x 4.6) mm, 5µm column with flow rate of 1.5mL/minute, and Injection volume of 100µL in isocratic mode with column oven temperature of 5°C and detection wavelength of 195nm. Water and Acetonitrile in the ratio of 30:70 was used as mobile phase with run time of 15minutes [39].

5.4.4 Assay of Cyclophosphamide

HPLC method from USP 43 NF 38 was adopted for determination of Cyclophosphamide wherein Shimadzu Chromatograph, Prominence-I LC2030C plus was used. The quantitation of Cyclophosphamide was done using (250 x 4.6) mm, 5µm, L43 column with flow rate of 2.0mL/minute, Injection volume of 100µL in isocratic mode with column oven temperature of 25°C and detection wavelength of 227nm. Water and Acetonitrile in the ratio of 5.5:4.5 was used as mobile phase with run time of 15minutes [40].

5.4.5 Entrapment efficiency

Dilution of NLCs was done with pH 7.4 phosphate buffer (PBS) containing 2 % w/v Tween 80 by adding 1.5mL of Tween 80-buffer system to the NLCs. Tween 80 was added to PBS to avoid the precipitation of drugs in PBS alone. The mixture was then vortexed for 5 minutes. The dispersion was centrifuged for 30 minutes at 15000 RPM (Centrifuge model 2-16P, Sigma, Germany), supernatant was suitably diluted, and the amount of the free PAC and CYC in the dispersion medium was estimated by HPLC. The entrapment efficiency was calculated using following equation:

$$\% \text{ Entrapment efficiency} = \frac{W_{total} - W_{free}}{W_{total}} \times 100$$

Here, the W_{total} is the weight of total drug added and W_{free} is the free drug obtained upon centrifugation [41, 42].

5.4.6 Water content

Water content of lyophilized product was determined using Karl Fisher Coulometer (Metrohm). The instrument was conditioned by setting the temperature of the Thermo prep oven at 100°C, flow set to 80 mL/min. The water content of the product vial was determined against the blank empty vial and reported as percentage [43].

5.4.7 FTIR Spectroscopy

IR-spectrum of Paclitaxel pure drug, Cyclophosphamide pure drug, Lyophilized Placebo NLCs and Lyophilized PAC-CYC NLCs were measured in the solid state by preparing a Potassium Bromide (KBr) pellet. The pure drug was previously ground individually and mixed thoroughly with KBr separately, an infrared transparent matrix at 1:100 (sample KBr) ratio. The pellets were then scanned over a wavelength range of 4000-400 cm-1 and a spectrum was obtained by using a Spectrum –II FTIR spectrometer (PerkinElmer, USA) [44].

5.4.8 Differential Scanning Calorimetry

TA Instruments Discovery DSC Trios V4.2.1.36612 was used for the DSC analysis of Paclitaxel pure drug, Cyclophosphamide pure drug, Lyophilized Placebo NLCs and Lyophilized PAC-CYC NLCs by loading the sample to DSC pan and cycle was run to cooling up to -50°C with ramp 1°C/min followed by heating up to +10°C with ramp 1°C/minutes [45-47].

5.4.9 X-ray Diffraction study

X-ray diffraction (XRD) is a pivotal technique primarily used for the structural analysis of crystalline materials. It plays a crucial role by providing detailed insights into the molecular structure of active pharmaceutical ingredients (APIs) and their polymorphic forms. The technique is also employed in the characterization of drug substances and products, helping to identify and quantify different physical forms and phase transformations during processing and storage. XRD is a powerful non-destructive technique that yields insights into structures, phases, preferred crystal orientations (texture), and various structural parameters, including average grain size, crystallinity, strain, and crystal defects within the material.

XRD peaks arise from the constructive interference of a monochromatic X-ray beam that is scattered at specific angles by each set of lattice planes within a sample. The peak intensities

are dictated by the arrangement of atoms within the lattice. As a result, the XRD pattern serves as a distinctive identifier of the periodic atomic arrangements within a specific material. XRD patterns of Pure Paclitaxel, Pure Cyclophosphamide, and PAC-CYC NLCs were obtained on Nano-viewer (Rigaku, Japan). The instrument utilized Cu-Ka X-rays operated at 45 kV and 60 mA [48, 49].

5.4.10 Morphology by Transmission Electron Microscopy

The PAC-CYC NLCs were analyzed for their shapes prior to lyophilization and following the SMART^{TM} lyophilization process using JEM-2100 TEM. A single drop from each sample was deposited onto a carbon film-coated copper grid (200-mesh). After a span of five minutes, a filter paper positioned at the periphery of the copper grid was employed to eliminate any surplus liquid that remained. Samples were air-dried at room temperature and subsequently examined at 200 kV power. [50].

5.4.11 In-Vitro release studies

The evaluation of the in-vitro release of pure Paclitaxel, pure Cyclophosphamide, and nanocarriers loaded with Paclitaxel & Cyclophosphamide was conducted using the dialysis method. INTAXEL contained Paclitaxel dissolved in a mixture of Ethanol and Cremophor EL (1:1), while ENDOXAN (Cyclophosphamide) was dissolved in Ethanol for the in-vitro release studies. The dialysis membrane underwent a soaking period of 24 hours in the release medium, which consisted of phosphate buffer at pH 7.4, supplemented with Tween-80 to inhibit drug precipitation prior to the experiment. Precisely quantified nanocarriers were positioned within the dialysis membrane, which was meticulously secured to avert any drug leakage. The dialysis membrane was placed in a beaker with a fixed volume of the release medium, which was continuously stirred at 200 RPM, while maintaining the temperature of the release medium at 37.5±2.0°C. At specific time intervals, a consistent volume of release medium samples was extracted and substituted with an equal volume of fresh release media. The samples underwent immediate analysis through the HPLC method to quantify Paclitaxel and Cyclophosphamide [51, 52].

5.4.12 Filterability

% filterability test is to monitor flow decay and gradual pore plugging caused by the product and to determine the maximum volume of product that can be filtered through a 0.2µ filter.

Fixed volume of NLCs/ microemulsion was filtered through 0.2µm PES filter and the % volume filtered was recorded [53].

5.4.13 Sterility

Membrane Filtration Method was used to determine the sterility of optimized NLCs as it is a regulatory method of choice for filterable pharmaceutical products. The reconstituted NLCs were passed through the membrane filter of pore size 0.45µm under aseptic conditions. Once the filtration is complete, filter was added to the culture media and kept for 14 days incubation. Two types of growth media i.e. Fluid Thioglycolate Medium (FTM) and Soybean casein digest medium (SCDM) were used in tests. Sterile water for injection was used as negative controlled whereas growth media exposed to atmospheric conditions was used as positive control [54].

5.5 STABILITY STUDIES

Drug product of Paclitaxel and Cyclophosphamide loaded NLCs were manufactured as per the optimized process and lyophilized vials were loaded in stability for 12 months at 2 to 8°C and for 15 days at 25±2°C/60±5%RH. Batches were evaluated for time required for reconstitution, particle size distribution, PDI, zeta potential, entrapment efficiency, water content, and sterility. Sterility was evaluated only at the end of 12 months stability.

RESULTS AND DISCUSSION

5.6 OPTIMIZATION OF FORMULATION PARAMETERS

5.6.1 Selection of Surfactants

Out of all 5 surfactants, Polysorbate 20 has the lowest allowable concentration of 1 % for intravenous administration. Formulated NLCs shall be administered through intravenous infusion. Elevated concentrations of surfactants may lead to hemolysis, resulting in damage to erythrocytes, as well as other adverse toxicological responses. Consequently, employing them at reduced concentrations mitigates this risk while preserving their efficacy as solubilizers and stabilizers. Surfactants have the potential to influence the functionality of cell membranes and biological barriers, which may result in the disruption of normal physiological processes and the emergence of complications [55-58]. Therefore, all the 5 surfactants were initially evaluated at concentration of 1 %. The results of impact of surfactants are presented in Table 5.9.

Table 5.9: Results of selection of surfactants

Sr. No.	Surfactant	Z-Average (nm)	PDI	% Entrapment efficiency		% Filterability
				PAC	CYC	
1	Polysorbate 80	427.5 ± 66.1	0.400 ± 0.020	76.5 ± 1.2	60.0 ± 2.1	70.0 ± 5.0
2	Polysorbate 20	509.7 ± 19.4	0.511 ± 0.082	72.4 ± 2.0	63.1 ± 1.5	65.0 ± 3.0
3	Poloxamer 188	468.1 ± 25.7	0.445 ± 0.036	49.2 ± 0.6	65.5 ± 0.9	50.0 ± 6.0
4	Soluplus	177.7 ± 36.2	0.187 ± 0.010	97.0 ± 0.2	97.5 ± 0.3	95.0 ± 4.0
5	Cremophor ELP	250.6 ± 11.8	0.200 ± 0.015	95.5 ± 0.3	95.4 ± 0.4	95.0 ± 3.0

The critical micelle concentration (CMC) value of Poloxamer 188 is 0.41mg/mL and therefore higher concentration is required for the stabilization of a formulation. Due to higher CMC value, Poloxamer 188 is not able to stabilize the formulation upon dilution. This was confirmed by Han J. Et Al wherein Paclitaxel emulsion manufactured using Poloxamer 188 as surfactant precipitated upon dilution [59].

Even Polysorbate 20 and Polysorbate 80 were not able to stabilize the NLCs at 1% concentration. Polysorbate 20 and Polysorbate 80 at 1% concentrations were insufficient to

stabilize the NLCs, leading to large particle sizes, poor homogeneity (high PDI), reduced drug encapsulation efficiency, and poor filterability. A higher concentration of these surfactants, likely in the range of 2–15%, could improve the overall stability of the formulation, decrease particle size, narrow the PDI, and increase both the entrapment efficiency and filterability of the NLCs. Polysorbate 80 is typically used in concentrations ranging from 5% to 15% by weight in the formulation of NLCs. This range is effective in stabilizing the lipid nanoparticles and maintaining their structural integrity as researched by Wadsäter et al. [60-63].

Cremophor EL and Soluplus are amphiphilic surfactants that are acceptable for the intravenous route at concentrations as high as 65% and 75% respectively. Z-Average, % Entrapment efficiency, and Filterability were found to be acceptable for both surfactants i.e. Cremophor EL and Soluplus. However, there was a need to increase the filterability to 100% as sterilization by filtration is the only possible approach to achieve sterile NLCs for intravenous infusion and to decrease Z-average with better PDI to achieve better efficacy of the formulated carriers.

This research intends to develop NLCs containing combination of two drugs – Paclitaxel and Cyclophosphamide. Cremophor EL enhances the solubility of poorly water-soluble drugs, which is a critical factor in improving their bioavailability. This is particularly beneficial in NLC formulations, where the solubility of the drug in the lipid matrix is a limiting factor [64].

Soluplus® is an amphiphilic copolymer that forms stable nanomicelles, which significantly enhance the solubility of poorly water-soluble drugs. This property is particularly beneficial in NLC formulations, where solubility enhancement is crucial for drug efficacy [65, 66]. The polymeric nature of Soluplus® allows it to form micelles with a small diameter and narrow size distribution, which are essential for maintaining the physical stability of the drug in aqueous media [65].The critical micelle concentration (CMC) of Soluplus® is low, allowing it to form micelles at relatively low concentrations, which is advantageous for maintaining the stability and bioavailability of the drug within NLCs [67]. The combination of Cremophor EL and Soluplus in NLCs provides a synergistic effect that enhances the overall performance of the drug delivery system. This combination allows for the formulation of NLCs with high

drug loading capacity, improved encapsulation efficiency, and reduced cytotoxicity, as demonstrated in studies involving various drugs by Pittella et al. and Di et al. [68, 69].

Therefore, additional experiments with combination of Soluplus and Cremophor EL were executed. The results are presented in Table 5.10.

Table 5.10: **Results of optimization of concentration of Soluplus and Cremophor EL**

Sr. No.	Surfactant Concentration (%)		Z-Average (nm)	PDI	% Entrapment efficiency		% Filterability
	Soluplus	Cremophor ELP			PAC	CYC	
1	1		177.7 ± 36.2	0.187 ± 0.010	97.0 ± 0.2	97.5 ± 0.3	95.0 ± 4.0
2		1	250.6 ± 11.8	0.200 ± 0.015	95.5 ± 0.3	95.4 ± 0.4	95.0 ± 3.0
3	1	1	158.2 ± 10.1	0.116 ± 0.010	98.9 ± 0.2	96.5 ± 0.3	100.0 ± 0.0
4	1	2	142.7 ± 14.5	0.123 ± 0.010	99.0 ± 0.5	99.6 ± 0.3	100.0 ± 0.0
5	1	4	120.2 ± 4.2	0.138 ± 0.009	100.1 ± 0.7	99.7 ± 0.7	100.0 ± 0.0

There was a significant improvement observed in Z-average, PDI, % entrapment efficiency, and filterability due to combine effect of Soluplus and Cremophor ELP. NLCs containing Soluplus at 1% and Cremophor ELP at 4% showed the smallest particle size with very good PDI value. Lower particle size facilitates the co-delivery of multiple drugs, enhancing synergistic effects in cancer treatment. Smaller NLCs (< 200nm) exhibit improved penetration into tumor tissues, which is essential for effective drug delivery and facilitated enhanced cellular uptake and synergistic anticancer effects in breast cancer cells [70, 71].

5.6.2 Optimization of total lipid concentration

The total lipid concentration is an essential part in optimization in NLCs. The total lipid concentration directly impacts the particle size of NLCs. Lipid concentration higher than the optimized level lead to larger particle size due to the increased amount of lipid available to form the core. However, the lipid matrix needs to solubilise the drug effectively. An optimized lipid concentration ensures sufficient entrapment efficiency, maximizing the amount of drug loaded into the NLCs as it aids in retaining the drug within the carrier,

preventing premature burst or sudden release and degradation. Optimized lipid concentration helps maintain the structural integrity of NLCs, preventing aggregation or fusion of particles and thus plays role in enhancing its stability. The lipid matrix controls the release profile of the encapsulated drug ensuring a sustained and/or controlled release, improving therapeutic outcomes. The total lipid concentration is a pivotal parameter in the formulation of nanostructured lipid carriers. It affects critical attributes such as particle size, drug loading capacity, stability, and release kinetics [72-76]. The results of optimization of lipid concentration are presented in Table 5.11.

Table 5.11: Results of optimization of total lipid concentration

Level	Total Lipid Conc. (%)	Z-Average (nm)	PDI	% Entrapment efficiency		Filterability (%)
				PAC	CYC	
1	2	114.2 ± 3.3	0.147 ± 0.010	99.2 ± 0.6	99.9 ± 0.7	100.00 ± 0.00
2	4	147.7 ± 12.0	0.175 ± 0.013	98.9 ± 0.6	97.9 ± 0.7	83.33 ± 3.06
3	6	267.4 ± 35.2	0.318 ± 0.019	99.1 ± 0.5	98.2 ± 0.6	76.67 ± 6.11

NLCs containing 2% and 4% of total lipid concentration produced NLCs with Z-average below 200nm and very good PDI of below 0.2. At all the 3 levels of total lipid concentration, % entrapment efficiency was observed to be more than 95% which is much desired one, however the filterability was observed to be reduced at 4% and 6%. The filterability of nanostructured lipid carriers (NLCs) is significantly impacted by the total lipid concentration due to the complex interplay between lipid composition, particle size, and the physicochemical properties of the NLCs. The crystallization behaviour of lipids within NLCs is influenced by the total lipid concentration, affecting the morphology and filterability of the particles. Previous studies have demonstrated that higher lipid concentrations can lead to more crystalline structures, which may hinder filterability due to increased rigidity and particle aggregation [77, 78]. The stability of NLCs is closely linked to lipid concentration, with higher lipid content often resulting in increased viscosity. This can negatively impact filterability by creating a more resistant medium for filtration processes [79].

5.6.3 Ratio of Solid lipid to liquid lipid

The ratio of solid to liquid lipids influences the particle size of NLCs. A higher solid lipid content typically results in smaller particles due to the higher melting point and lower fluidity. This ratio affects the morphology of the particles, contributing to the formation of spherical or irregularly shaped NLCs. The ratio determines the ability of NLCs to encapsulate and retain drugs. An optimal balance ensures maximum drug loading by providing an appropriate matrix structure. Solid lipids provide a rigid matrix, while liquid lipids offer flexibility, together creating a stable environment for drug encapsulation.

The solid lipid component helps in forming a stable crystal structure, while the liquid lipid disrupts the perfect crystalline order, preventing premature drug expulsion. A balanced ratio contributes to the long-term stability of NLCs, preventing particle aggregation and ensuring consistent drug release over time. The solid lipid offers a more controlled release due to its rigidity, whereas the liquid lipid can facilitate a faster release. Achieving an optimal balance between solid and liquid lipids is essential for the effective design and application of NLCs in drug delivery systems [72-76]. The results of optimization of ratio of solid lipid to liquid lipid are presented in Table 5.12.

Table 5.12: Results of optimization of solid lipid: liquid lipid ratio

Level	Solid lipid: liquid lipid Ratio	Z-Average (nm)	PDI	% Entrapment efficiency		Filterability (%)
				PAC	CYC	
1	1:1	114.2 ± 3.3	0.147 ± 0.010	99.2 ± 0.6	99.9 ± 0.7	100.00 ± 0.00
2	1:2	164.2 ± 11.9	0.208 ± 0.022	100.1 ± 0.4	99.1 ± 0.7	82.50 ± 1.90
3	1:3	197.8 ± 9.1	0.212 ± 0.057	100.0 ± 0.2	98.5 ± 0.4	60.14 ± 6.65

Increase in Z-average and PDI, and decrease in the filterability of formulated NLCs was observed with increase in the amount of liquid lipid in solid lipid to liquid lipid ratio. When the proportion of liquid lipid is increased, the miscibility between the solid and liquid lipids can lead to a less ordered structure, which results in larger particle sizes. This is because the liquid lipid disrupts the crystalline structure of the solid lipid, leading to a more amorphous and expanded matrix [80, 81]. The presence of liquid lipids creates an imperfect matrix,

which is beneficial for drug loading but can also lead to increased particle size due to the less compact arrangement of lipid molecules. This imperfect matrix is a result of the liquid lipid's ability to reduce the order of the solid lipid structure, thereby increasing the overall volume of the lipid matrix [82, 83].

Studies have shown that as the liquid lipid content increases, the particle size of NLCs tends to increase. This is attributed to the fact that liquid lipids, being less dense than solid lipids, contribute to a larger hydrodynamic diameter of the particles. The increased particle size can hinder the filterability of NLCs, as larger particles are more likely to clog filters and reduce the efficiency of filtration processes [84, 85]. The increase in particle size with higher liquid lipid content is also linked to the reduced stability of the lipid matrix. The less ordered structure can lead to phase separation and aggregation, further contributing to larger particle sizes and reduced filterability [81, 86].

5.6.4 Drug substance concentration

Referring to O Pagani Et Al, the dose of Paclitaxel is 200mg/m^2 and dose of Cyclophosphamide is 1750mg/m^2 for the treatment of breast cancer which conveys that for 1mg of the Paclitaxel, 8.75mg of Cyclophosphamide is dosed. Therefore, ratio of Paclitaxel and Cyclophosphamide in ratio of 1: 8.75 was added during compounding of NLCs [87]. Ratio was increased proportionately for all the 3 levels.

The drug concentration in nanostructured lipid carriers (NLCs) is a critical factor influencing overall effectiveness in drug delivery. The amount of drug substance loaded directly affects the entrapment efficiency. An optimal amount of drug loading ensures maximum encapsulation without exceeding the carrier's capacity. Higher drug loading may result in larger particles size, potentially leading to aggregation and instability. Optimal drug concentration ensures a uniform distribution of the drug substance within the lipid matrix, contributing to consistent particle size and stability preventing issues such as drug crystallization, particle aggregation, or phase separation [72-76]. The results of optimization of drug substance concentration are presented in Table 5.13.

Table 5.13: Results of optimization of drug substance concentration

Level	Drug substance concentration (mg/mL)		Z-Average (nm)	PDI	% Entrapment efficiency		Filterability (%)
	PAC	CYC			PAC	CYC	
1	1	8.75	114.2 ± 3.3	0.147 ± 0.010	99.2 ± 0.6	99.9 ± 0.7	100.00 ± 0.00
2	2	17.5	153.5 ± 8.2	0.179 ± 0.018	88.3 ± 0.6	89.0 ± 1.1	100.00 ± 0.00
3	3	26.25	279.0 ± 25.0	0.328 ± 0.017	67.5 ± 2.3	63.5 ± 2.6	88.67 ± 2.31

Based on above results, there was proportionate increase in Z-average and PDI values, significant decrease in entrapment efficiency with increased drug concentrations. At Level 3, where Paclitaxel was 3mg/mL and Cyclophosphamide at 26.25mg/mL, entrapment efficiency was reduced to almost half and even the filterability was reduced.

As the drug concentration increases, the lipid matrix of the NLCs can become saturated, leading to an increase in particle size. This is because the excess drug may not be efficiently incorporated into the lipid matrix, causing aggregation and growth of the particles [88, 89]. The incorporation of higher drug concentrations can disrupt the crystalline structure of the solid lipids in the NLCs. This disruption can lead to an increase in particle size and PDI as the ordered structure of the lipid matrix is altered [90]. The presence of liquid lipids in the NLCs is intended to reduce crystallinity and improve drug loading. However, at higher drug concentrations, the balance between solid and liquid lipids may be disturbed, leading to less stable NLCs with increased particle size and PDI [90, 91]. The ratio of drug to lipid is a critical parameter. An increase in drug concentration without a corresponding increase in lipid content can lead to a decrease in entrapment efficiency due to insufficient lipid to encapsulate the drug [89, 92].

Based on the above results, total lipid concentration was finalized as 2%, solid lipid: liquid lipid ratio as 1:1, drug concentration for Paclitaxel as 1mg/mL and Cyclophosphamide as 8.75mg/mL.

5.7 OPTIMIZATION OF PROCESS PARAMETERS

5.7.1 Optimization of Mixing Speed

The results of mixing speed are presented in Table 5.14.

Table 5.14: Results of mixing speed

Levels	Mixing Speed (RPM)	Z-Average (nm)	PDI	% Entrapment efficiency		% Filterability
				PAC	CYC	
1	**250**	461.4 ± 17.0	0.243 ± 0.05	85.5 ± 0.2	92.8 ± 0.7	75.00 ± 5.00
2	**500**	152.1 ± 3.8	0.200 ± 0.01	99.6 ± 0.4	100.0 ± 0.2	100.00 ± 0.00
3	**1000**	176.2 ± 11.6	0.194 ± 0.02	94.1 ± 0.4	99.2 ± 0.8	100.00 ± 0.00

At lower mixing speed, the Z-average was observed to be much higher (> 400nm) and % entrapment efficiency was observed to be reduced significantly. Whereas, mixing speed of 500 to 1000 RPM showed Z-average below 200nm with 100% filterability. However, there was insignificant increase in particle size and minor drop in the entrapment efficiency of Paclitaxel at1000 RPM. The relationship between shear intensity and particle size is well-documented, with optimal conditions leading to reduced size and improved uniformity [93].

Lower mixing speeds result in larger particle sizes because the energy input is inadequate to break down lipid aggregates effectively [94]. Entrapment efficiency (EE) is negatively impacted at lower speeds, as inadequate mixing fails to create stable NLCs, leading to drug leakage and lower loading capacity [95]. Filterability is compromised at lower mixing speeds due to larger particle sizes, which can clog filters and hinder the purification process. The Polydispersity index increases, indicating a broader size distribution that complicates filtration [96].

5.7.2 Optimization of mixing time

The results of mixing time are presented in Table 5.15.

Table 5.15: Results of mixing time

Levels	Mixing time (Minutes)	Z-Average (nm)	PDI	% Entrapment efficiency		% Filterability
				PAC	CYC	
1	5	495.2 ± 15.9	0.336 ± 0.05	96.2 ± 0.1	98.1 ± 0.6	60.00 ± 2.50
2	15	152.1 ± 3.8	0.200 ± 0.01	99.6 ± 0.4	100.0 ± 0.2	100.00 ± 0.00
3	30	173.1 ± 7.1	0.204 ± 0.02	98.4 ± 0.6	99.3 ± 0.1	100.00 ± 0.00

At lower mixing time, the Z-average was observed to be much higher (> 450nm) and % filterability was observed to be reduced significantly. Whereas, mixing time of 15 to 30minutes showed Z-average below 200nm with 100% filterability. However, there was slight decrease in the entrapment efficiency of Paclitaxel at mixing time of 5minutes and 30minutes.Mixing intensity and energy are crucial in determining the final particle size distribution. Lower mixing times often correlate with insufficient energy input, which is necessary to achieve the desired particle size reduction and uniformity [97].

The liquid lipids which are a part of NLCs can generate imperfections in the lipid matrix, which may contribute to increased particle size and PDI if not adequately mixed [98]. The decrease in filterability at lower mixing times can be attributed to the larger particle sizes and higher PDI, which result in a more heterogeneous suspension that is difficult to filter [99]. The formation of larger particles and aggregates can lead to clogging of filters, reducing the efficiency of the filtration process [100].

5.7.3 Optimization of temperature of Phases

The results for selection of temperature of phases are presented in Table 5.16.

Table 5.16: Results of temperature of phases

Levels	Phase Temperature	Z-Average (nm)	PDI	% Entrapment efficiency		% Filterability
				PAC	CYC	
1	40 ± 2°C	427.6 ± 22.7	0.390 ± 0.023	62.8 ± 1.3	75.1 ± 1.0	55.0 ± 2.00
2	50 ± 2°C	152.1 ± 3.8	0.200 ± 0.01	99.6 ± 0.4	100.0 ± 0.2	100.00 ± 0.00
3	60 ± 2°C	217.1 ± 11.0	0.232 ± 0.010	100.1 ± 0.5	89.6 ± 1.1	100.00 ± 0.00

At 40±2°C, the Z-average and PDI were observed to be very high whereas entrapment efficiency and filterability were observed to be significantly on lower side. At 50±2°C, Z-average was below 200nm with PDI 0.2 and entrapment efficiency and filterability was observed to 100%.The melting point of the PEG-100 stearate is reported as 47-50°C.

At temperatures below the melting point, lipids are in a solid state, which can lead to increased particle size and heterogeneity. This is because the solid state of lipids can cause aggregation and incomplete crystallization, resulting in a higher Z-average and PDI [101]. The solid state of lipids below their melting point can also hinder the encapsulation process, as the rigidity of the lipid matrix may not allow for efficient entrapment of the active compounds, leading to lower entrapment efficiency [102]. Due to higher Z-value of NLCs, the overall filterability was reduced due to the increased resistance and reduced permeability of the filter [103, 104].

At 60±2°C, the Z-average was observed to be more than 200nm with PDI above 0.2 and there was significant decrease in the entrapment efficiency of Cyclophosphamide. The increase in Z-average size and PDI of NLCs at elevated temperatures can be attributed to the thermal effects on the lipid matrix. Higher temperatures can cause the lipid components to transition from a solid to a more fluid state, leading to aggregation or fusion of particles, thereby increasing the size and heterogeneity of the NLCs [105]. This structural change can also affect the encapsulation efficiency of cyclophosphamide, as the drug may be released or not properly incorporated into the altered lipid matrix. The interaction between cyclophosphamide and the lipid components of NLCs can be influenced by temperature. At higher temperatures, the increased molecular motion can disrupt the interactions that stabilize the drug within the lipid matrix, leading to decreased entrapment efficiency [106].

Based on the results of optimization of formulation parameters and process parameters, finalized levels are mentioned in Table 5.17 and finalized formulation composition is mentioned in table 5.18.

Table 5.17: Levels optimized for formulation parameters and process parameters

Sr. No.	Parameters		Level Optimized	
1	Formulation Parameters	Selection of Surfactant	Cremophor ELP	4 %
2			Soluplus	1 %
3		Total lipid concentration	2 %	
4		Ratio of solid lipid to liquid lipid	1:1	
5		Drug Substance concentration	Paclitaxel	1mg/mL
6			Cyclophosphamide	8.75mg/mL
7	Process Parameters	Mixing speed	500 RPM	
8		Mixing time	15minutes	
9		Temperature of Phases	70±2°C	

Table 5.18: Finalized formulation composition for NLCs

Sr. No.	Ingredient Name	Functional role	Quantity
1	Paclitaxel	Active Ingredient	0.1 %
2	Cyclophosphamide	Active Ingredient	0.875 %
3	PEG-100 Stearate	Solid Lipid	1 %
4	PEG-8 Caprylic/Capric Glycerides	Liquid Lipid	1 %
5	Cremophor ELP	Surfactant	4 %
6	Soluplus	Surfactant	1 %
7	Water for Injection	Vehicle	q.s. to 100 %

5.8 LYOPHILIZATION OF NLCs

5.8.1 Selection of Cryoprotectants

Freeze-thaw studies serve as a predictive tool for evaluating the effectiveness of cryoprotectants. They simulate the stress conditions that nanoparticles undergo during freezing and thawing, allowing researchers to assess which cryoprotectants can best preserve the structural integrity and functionality of NLCs [107].These studies help determine the optimal concentration and type of cryoprotectant needed to prevent agglomeration and maintain particle size, polydispersity index, and zeta potential [107, 108].Cryoprotectants like mannitol interact with the lipid matrix of NLCs, preventing ice crystal formation and minimizing mechanical stress during freezing. This interaction is crucial for maintaining the nanoparticles' size and preventing aggregation [108, 109].

The NLCs were formulated with different cryoprotectants as mentioned in Table 5.19 and evaluated for particle size distribution (Average particle size & PDI), and % entrapment efficiency, before and after freeze thaw cycle.

Table 5.19: Compositions containing various cryoprotectants

Sr. No.	Cryoprotectant Name	Batch No.			
		T01	T02	T03	T04
1	Mannitol	Without cryoprotectant	4 %	-	-
2	Dextrose monohydrate		-	4 %	-
3	Lactose monohydrate		-	-	4 %

The results of freeze-thaw studies with different cryoprotectants and without addition of cryoprotectant are presented in Table 5.20.

Table 5.20: Results of before and after freeze thaw with different cryoprotectants

Batch No.		T01		T02		T03		T04	
Cryoprotectant Name		Without cryoprotectant		Mannitol		Dextrose monohydrate		Lactose Monohydrate	
Sr. No.	Test	Results (±S.D.) [n=3]							
		Before Freeze Thaw	After Freeze Thaw	Before Freeze Thaw	After Freeze Thaw	Before Freeze Thaw	After Freeze Thaw	Before Freeze Thaw	After Freeze Thaw
1	Average Particle Size (nm)	82.9 ±3.4	215.2 ±18.6	73.5 ±4.1	80.3 ±6.8	105.9 ±8.3	287.4 ±10.7	95.8 ±7.2	135.1 ±12.5
2	PDI	0.090 ±0.02	0.307 ±0.09	0.120 ±0.04	0.150 ±0.03	0.230 ±0.16	0.415 ±0.10	0.195 ±0.09	0.241 ±0.13
3	% Entrapment efficiency of PAC	100.1 ±0.3	83.5 ±0.8	100.0 ±0.1	99.8± 0.2	100.0 ±0.2	93.8 ±1.0	100.0 ±0.2	95.5 ±0.5
4	% Entrapment efficiency of CYC	99.5 ± 0.3	89.3 ± 0.9	99.8 ± 0.4	99.0 ± 0.5	99.0 ± 0.2	91.4 ± 0.3	99.3 ± 0.5	94.7 ± 0.6

NLCs containing no cryoprotectant showed significant increase in the average particle size and PDI after freeze thaw and significant drop in the entrapment efficiency. NLCs containing Mannitol as cryoprotectant showed minimum change in particle size, PDI, and % entrapment efficiency followed by Lactose monohydrate whereas Dextrose monohydrate containing NLCs shows significant increase in the particle size as well as the PDI and significant decrease in % entrapment efficiency. Mannitol is a readily crystallizing excipient, which facilitates the formation of a homogenous freeze-concentrate. This property is crucial for

maintaining the stability of NLCs during freeze-drying, as it prevents the heterogeneity that can lead to destabilization [110]. The crystallization of mannitol during lyophilization results in a stable lyophilized product with a single glass transition temperature which is beneficial for the structural integrity of NLCs [111].

Mannitol's ability to crystallize effectively during the freeze-drying process also contributes to its role as a bulking agent, providing structural support to the lyophilized cake [112]. The use of mannitol as a cryoprotectant has been associated with stable particle sizes and PDIs even under accelerated stability conditions, indicating its robustness in preserving NLC characteristics over time [109]. Compared to lactose, mannitol provides a more consistent and reliable stabilization effect due to its crystallization properties. Lactose, while increasing the glass transition temperature, does not offer the same level of structural support as mannitol [113]. Dextrose monohydrate, on the other hand, ranks lower in effectiveness as a cryoprotectant for NLCs, as indicated by its inferior performance in maintaining particle size and stability compared to mannitol [109].

Thus, mannitol was found to be suitable cryoprotectant for the formulated NLCs as there was no significant change in the Average particle size, PDI, % entrapment efficiency after freeze thaw. Mannitol was further evaluated at 3 concentrations of 2%w/v, 4%w/v, and 6%w/v by means of freeze-thaw study and the results are presented in Table 5.21.

Table 5.21: Results of Freeze Thaw study with different concentrations of Mannitol

Batch No.		**L01**		**L02**		**L03**	
Mannitol Concentration		**2%w/v**		**4%w/v**		**6%w/v**	
Sr. No.	**Test**	**Results (±S.D.) [n=3]**					
		Before Freeze Thaw	**After Freeze Thaw**	**Before Freeze Thaw**	**After Freeze Thaw**	**Before Freeze Thaw**	**After Freeze Thaw**
1	Average Particle Size (nm)	82.9 ±10.1	100.3 ±9.8	73.5 ±4.1	80.3 ±6.8	125.8 ±7.2	152.5 ±8.3
2	PDI	0.095 ±0.06	0.152 ±0.05	0.120 ±0.04	0.150 ±0.03	0.195 ±0.09	0.141 ±0.087
3	% Entrapment efficiency of PAC	100.0±0.2	96.9±0.5	100.0±0.1	99.8±0.2	100.0±0.2	99.9±0.2
4	% Entrapment efficiency of CYC	99.7±0.5	97.9±0.5	99.8±0.4	99.0±0.5	99.7±0.5	99.3±0.7

Mannitol at 4% concentration has been identified as the optimal cryoprotectant for the stabilization of nanostructured lipid carriers (NLCs) compared to 2% and 6% concentrations. This conclusion is based on its ability to balance the cryoprotective efficacy and the physical stability of the NLCs during freeze-drying. At 4%, mannitol provides an optimal balance between cryoprotection and the prevention of excessive crystallization, which can occur at higher concentrations. This concentration is sufficient to inhibit the crystallization of water, thereby protecting the structural integrity of the NLCs without causing adverse effects associated with higher concentrations [111, 112].

Higher concentrations, such as 6%, may lead to excessive crystallization, which can compromise the stability and functionality of the NLCs. Conversely, lower concentrations, like 2%, may not provide sufficient cryoprotection, leading to increased aggregation and particle size [114]. Studies have demonstrated that mannitol at 4% concentration results in a more stable lyophilized product, with less aggregation and better reconstitution properties compared to lower or higher concentrations [115, 116].

5.8.2 Freeze Drying Microscopy

Freeze drying microscopy (FDM) is a specialized technique used to study the freeze-drying process, particularly focusing on the sublimation kinetics and critical temperature parameters of products. This method is crucial in optimizing the freeze-drying process, which is widely used in the pharmaceutical and food industries to preserve heat-sensitive materials by removing water through sublimation at low temperatures and pressures. FDM allows for the direct visualization of sublimation fronts and helps in identifying critical process parameters, such as the collapse temperature, which is essential for maintaining product quality and stability during freeze-drying. FDM is used to determine the collapse temperature (Tc) of products, which is the temperature just below which the product must be maintained to avoid structural collapse during freeze-drying. This is crucial for ensuring the quality of the freeze-dried cake [117, 118]. By accurately determining critical temperatures and visualizing sublimation, FDM can lead to more efficient lyophilization cycles, reducing drying times and costs [118, 119].

Based on the Freeze Drying Microscopy, PAC-CYC NLCs were observed to be completely frozen at -19.5°C, Onset of collapse was observed at 0.5°C and complete collapse was

observed at 1.5°C. Therefore, 0.5°C at which the collapse initiation was observed in FDM was the collapse temperature (Tc) considered for SMART™ lyophilization cycle. The figure 5.3 depicts the complete freezing, onset of collapse and complete collapse of PAC-CYC NLCs containing 4%w/v mannitol.

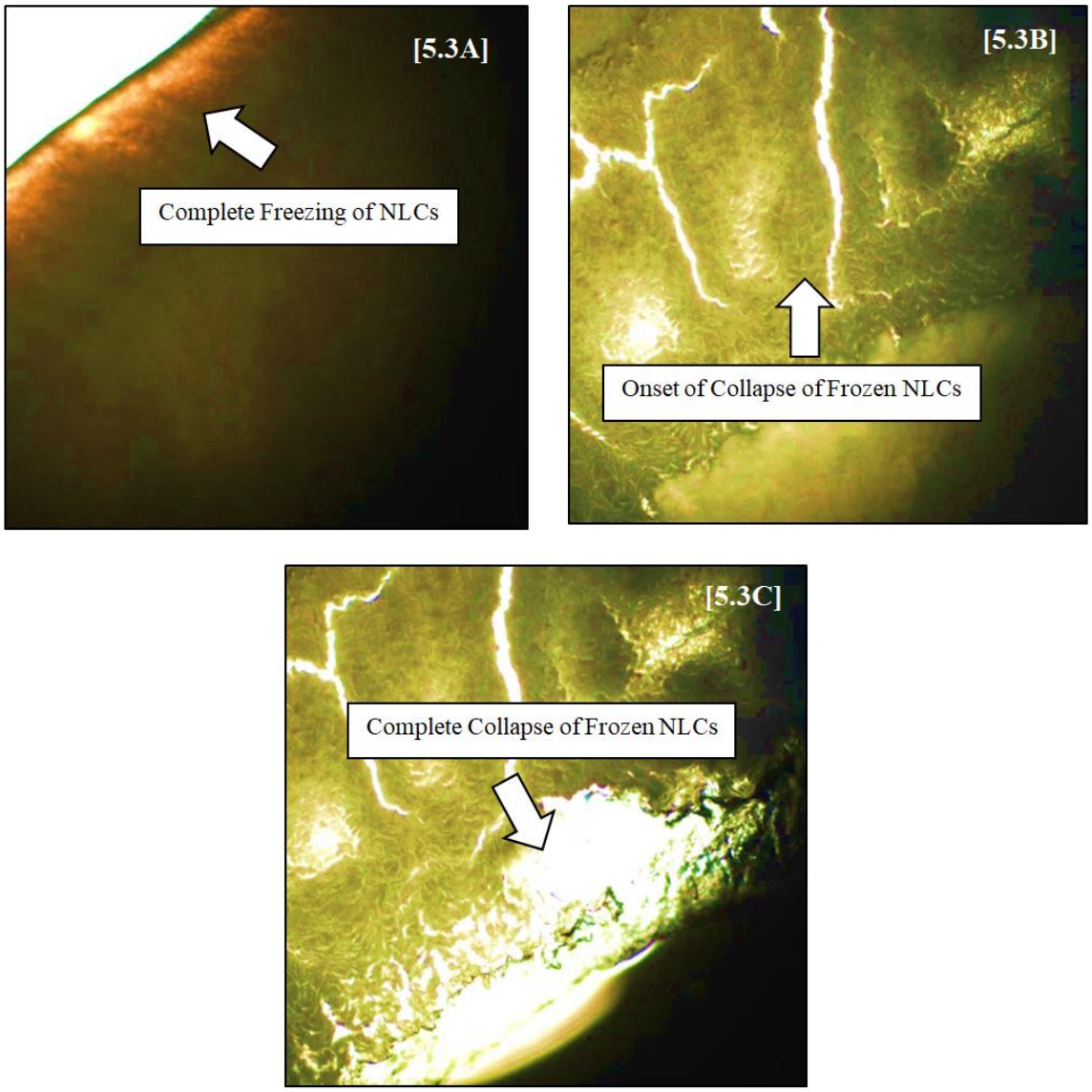


Figure 5.3: Freeze drying microscopy of Optimized NLCs [5.3A] Completion of Freezing at -19.5°C [5.3B] Onset of collapse at 0.5°C [5.3C] Complete collapse at 1.5°C

5.8.3 SMART™ Lyophilization cycle Development

SMART™ Freeze-Dryer technology eliminates the trial-and-error approach normally involved in developing new lyophilisation cycles. Based on manometric temperature measurement equation and heat and mass transfer theory, pressure rise data is analyzed by an algorithm and converted to a number of critical process parameters.

SMART™ is advanced programme in freeze drying technology and it designed the cycle with aid of in-put parameters mentioned in Table 5.22. SMART™ cycle was executed using Lyostar III Lyophilizer.

Table 5.22: Parameters for SMART™ Cycle

Sr. No.	Parameter	Input value	
1	Number of Vials	25	Nos.
2	Vial Inner area (Ap)	20.9	cm^2
3	Fill Volume	60	cm^3
4	Fill Weight	62	g
5	Tc, T_g, Teu	0.0	°C
6	Concentration of solution	0.1	g/g
7	Nature of Drug Product	Small Molecule	
8	Type of Vials	Moulded	
9	Type of bulking agent	Amorphous	
10	MTM Interval	60 minutes (Default)	

Based on above mentioned input parameters, SMART™ programme developed Lyophilization cycle. The screenshot of the representative SMART™ programme is presented in Figure 5.4. The final optimized SMART™ Lyophilization cycle is presented in Table 5.23.

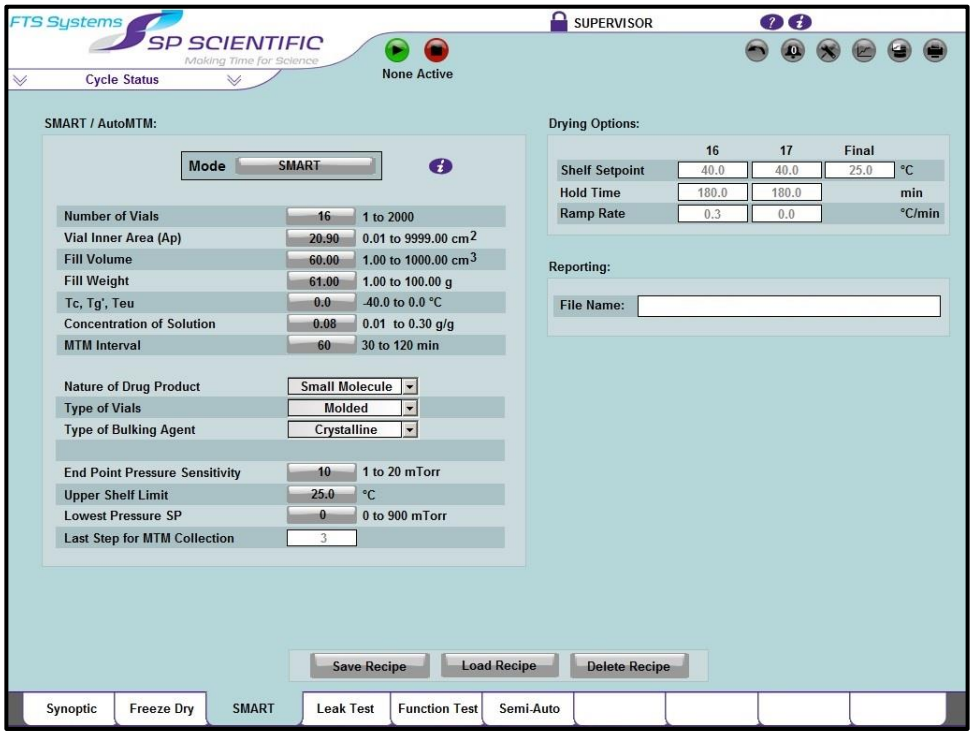


Figure 5.4: Screenshot of Representative SMART™ programme

Table 5.23: SMART™ Lyophilization Cycle (Final Optimized Cycle)

Cycle Step	Step	Temperature(°C)	Vacuum (mTorr)	Step time (minutes)
Freezing	Ramp	5	Not Applicable	22
	Hold	5		30
	Ramp	-5		10
	Hold	-5		30
	Ramp	-40		35
	Hold	-40		120
	Ramp	-22		18
	Hold	-22		180
	Ramp	-40		18
	Hold	-40		120
Total freezing time				**583 minutes**
Primary Drying	Ramp	-15	150	50
	Hold	-15	150	31
	Ramp	25	150	80
	Hold	25	150	3488
Total primary drying time				**3649 minutes**
Secondary Drying	Ramp	40	150	50
	Hold	40	150	360
Total secondary drying time				**410 minutes**
Total Cycle time				**4642 minutes (77.36 hours)**

Lyophilization was performed for NLCs with and without Mannitol using SMART™ lyophilization cycle and the results were compared with the NLCs with and without Mannitol before lyophilization. The results for NLCs lyophilized without cryoprotectant was evaluated against NLCs lyophilized with Mannitol. The critical quality attributes viz. product description, reconstitution time, particle size (average), PDI, Zeta potential, % water content, % entrapment efficiency, were studied and results are tabulated in Table 5.24.

Table 5.24: Results of the before vs. after lyophilization and with Mannitol vs. without Mannitol

Critical Quality Attribute	Before Lyophilization		After Lyophilization	
	NLCs w/o Mannitol	NLCs with Mannitol	NLCs w/o Mannitol	NLCs with Mannitol
Product Description after reconstitution	Clear transparent with blue tint	Clear transparent with blue tint	Milky with blue tint	Clear transparent liquid with blue tint
Reconstitution time	Not Applicable	Not Applicable	118 seconds	45 seconds
Particle size (Average)	69.6±8.1nm	58.6±4.2nm	389.2±51.7nm	59.5±3.6nm
PDI	0.09±0.02	0.08±0.01	0.38±0.10	0.08±0.06
Zeta Potential (mV)	-1.4±0.3	-3.4±0.3	Cannot be measured	-3.0±0.3
% water content	Not Applicable	Not Applicable	1.7±0.3	0.8±0.4
% Entrapment efficiency of PAC	100.0±0.5	100.2±0.3	85.2±2.7	100.0±0.4
% Entrapment efficiency of CYC	99.3±0.2	99.5±0.5	88.4±1.0	99.0±0.8

NLCs lyophilized without any cryoprotectant exhibited milky appearance after reconstitution with water for injection whereas NLCs lyophilized with Mannitol produced clear transparent liquid with blue tint. Without cryoprotectants, NLCs tend to form a milky dispersion due to particle aggregation and scattering of light. In contrast, the presence of Mannitol as a cryoprotectant resulted in a clear liquid with a blue tint, indicating better particle dispersion and stability [115, 120]. This difference is primarily due to the role of cryoprotectants in preventing aggregation during the freeze-drying and reconstitution processes. Without cryoprotectants, NLCs are prone to aggregation due to the formation of ice crystals during freeze-drying, leading to a milky appearance upon reconstitution [115]. The lack of a stabilizing agent like Mannitol results in increased particle size and polydispersity, which contributes to the scattering of light and the milky appearance [120].

Based on the above results, no significant change in particle size, PDI and zeta potential of mannitol containing NLCs was observed before and after lyophilization whereas there was

significant increase in particle size and PDI for the reconstituted NLCs in absence of a cryoprotectant and also it was not possible to measure the zeta potential for these NLCs. Mannitol, in particular, has been shown to be effective in maintaining small particle sizes and low PDI values, as it provides a stabilizing effect that prevents the collapse of the nanoparticle structure during the removal of water[109, 121]. Without cryoprotectants, NLCs experienced significant aggregation, resulting in larger particle sizes (>400 nm) and higher PDI (>0.3), indicating a broad size distribution [122, 123].

The presence of Mannitol resulted in no significant change in particle size and PDI, reflecting a more uniform and stable nanoparticle population [109, 115]. Cryoprotectants like Mannitol work by replacing water molecules and forming hydrogen bonds with the lipid components of NLCs, thus preserving their structure during freeze-drying [115,121]. Zeta-potential measurementwithout cryoprotectant after lyophilization was challenging due to the destabilization and aggregation of particles. This aggregation led to unmeasurable zeta potential values, as the particles no longer maintained their original dispersion state. The destabilization of the electric double layer due to aggregation results in a loss of the distinct charge separation necessary for accurate zeta potential determination [124].

The drop in entrapment efficiency for lyophilized nanostructured lipid carriers (NLCs) without cryoprotectant, compared to those containing mannitol, can be attributed to the protective role cryoprotectants play during the freeze-drying process. The absence of a protective matrix results in structural changes that can compromise the stability and functionality of the nanoparticles [125]. Cryoprotectants like mannitol help maintain the structural integrity of nanoparticles by preventing aggregation and preserving particle size, which are crucial for maintaining entrapment efficiency. Without cryoprotectants, nanoparticles are prone to aggregation and increased particle size during lyophilization, leading to a significant drop in entrapment efficiency [126]. Studies have shown that formulations without cryoprotectants exhibit increased particle size and reduced stability, which negatively impacts entrapment efficiency [126].

5.9 CHARACTERIZATION OF LYOPHILIZED NLCs

5.9.1 Description, Redispersibility and Reconstitution of Lyophilized NLCs

The lyophilized NLCs were found to be readily redispersible in sterile water for injection and phosphate buffer pH 7.4 as dispersion was observed within 2 times of shaking. The time required to reconstitute in sterile water for injection was 69 seconds and for phosphate buffer pH 7.4 was 74 seconds [127-129]. The product description after reconstitution was observed to be clear translucent liquid with blue tint and free from any visible particulate matter.

Mannitol as cryoprotectant prevented aggregation of NLCs during freeze-drying, maintaining their stability and size, which is crucial for rapid redispersion [115, 121]. It acts as a bulking agent in the final lyophilized product, ensuring that the nanoparticles remain evenly distributed, and easily rehydrated [111]. Mannitol's ability to inhibit crystallization in frozen systems contributes to the preservation of the nanoparticle structure, facilitating quick reconstitution [111]. The preserved particle size and structure due to mannitol's cryoprotective properties allow for rapid reconstitution in aqueous solutions [121]. The reconstitution time of not more than 120 seconds is achieved due to the efficient dispersion of nanoparticles facilitated by mannitol [115]. The lyophilized product's ability to dissolve quickly and readily is crucial for its use in injectable formulations [115].

5.9.2 Particle size distribution and zeta potential

The particle size distribution of the optimized NLCs revealed that the average Z-average was 53.9 ± 2.0nm with PDI of 0.125 ± 0.018. The graph of particle size distribution is presented in Figure 5.5. The zeta potential of the NLCs was observed to be -5.3 ± 2.7 mV. The graph of zeta potential is presented in Figure 5.6.

The particle size achieved was below 100nm with PDI less than 0.1 confirms that optimized process and product parameters along with optimized lyophilization process were suitable for the development of Paclitaxel and Cyclophosphamide loaded NLCs. Particle size below 100 nm is crucial for nanostructured lipid carriers (NLCs) containing anticancer drugs due to its significant impact on drug delivery efficiency and therapeutic efficacy [130]. Smaller particles enhance cellular uptake, improve tumor penetration, and facilitate sustained drug release, which are essential for effective cancer treatment [130-132].

The enhanced permeability and retention (EPR) effect, a phenomenon where nanoparticles preferentially accumulate in tumor tissue, is more pronounced with sub-100 nm particles, leading to improved drug delivery to cancer cells [131, 133]. Sub-100 nm particles are more readily internalized by cancer cells, leading to higher intracellular concentrations of the drug. This is due to their ability to penetrate cellular membranes more efficiently than larger particles [130, 131]. This size-dependent accumulation leads to higher drug concentrations at the tumor site, enhancing the overall antitumor efficacy [130-133]. A PDI below 0.1 indicates that the particles are nearly monodisperse, meaning they have a uniform size. This uniformity is crucial for ensuring that the NLCs behave predictably in biological systems, which is essential for effective drug delivery [134]. NLCs with a low PDI are more stable over time, reducing the risk of aggregation or separation, which can compromise the efficacy of the drug delivery system [134].

The graph of Zeta potential is show in Figure 5.5 revealed that there is no significant charge on the manufactured NLCs. Paclitaxel and Cyclophosphamide are neutral compounds in its pure form, meaning they does not possess an inherent charge. The charge properties of Paclitaxel and Cyclophosphamide are primarily influenced by the formulation and delivery systems used, rather than the active pharmaceutical ingredient (API) itself [135-139]. Cremophor EL is a polyethoxylated castor oil, which is a non-ionic surfactant, meaning it does not carry a net charge [140, 141].

The non-ionic nature is due to the ethoxylation process, where ethylene oxide is reacted with castor oil, resulting in a compound that is neutral in charge [141]. Solid lipid and liquid lipid used in formulating NLCs does not have any charge on them. PEG-100 stearate specifically has been shown to reduce zeta potential from more negative values to near zero [142]. However, Soluplus solutions possess charge which is typically neutral to slightly negative [143]. Thus, no other components possess charge by themselves and the observed zeta potential can be attributed to the use of Soluplus as surfactant in formulation of NLCs.

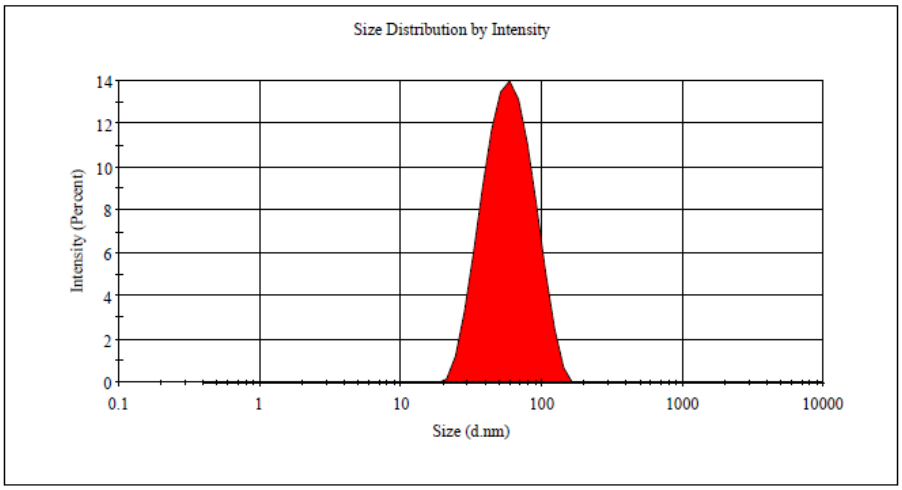


Figure 5.5: Graph of Particle size distribution of NLCs

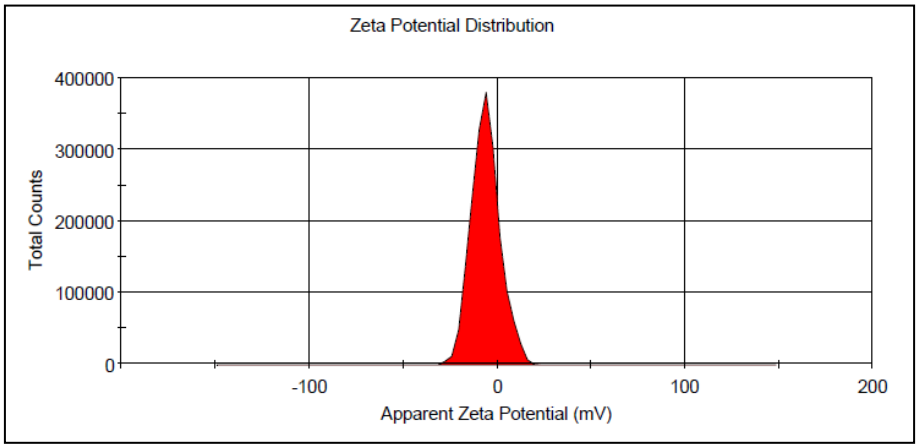


Figure 5.6: Graph of Zeta Potential of NLCs

5.9.3 Entrapment efficiency, Assay of Paclitaxel, and Assay of Cyclophosphamide

The entrapment efficiency of Paclitaxel was obtained as 100.0±0.4 % whereas for Cyclophosphamide it was obtained as 99.0±0.8 %. Assay of Paclitaxel in NLCs was determined as 99.5 ± 0.6 % and assay of Cyclophosphamide in NLCs was reported as 100.2 ± 0.1 %. As per the USP monograph of Paclitaxel Injection and Cyclophosphamide Injection, the acceptable specification for assay is 90.0 to 110.0 % and reported results adhere to the specification limit.

NLCs are made from a combination of solid and liquid lipids, which creates an imperfect matrix that enhances drug loading capacity and stability [144, 145]. The presence of liquid lipids reduces the ordered structure of solid lipids, increasing the entrapment efficiency of

drugs within the NLCs [145]. The stability of NLCs is enhanced by their ability to maintain particle size and drug content over time, which is crucial for achieving very good entrapment efficiency [146].

5.9.4 Water content

The water content of lyophilized NLCs was observed to be 0.5± 0.1 %. The water content of lyophilized Nanostructured Lipid Carriers (NLCs) below 1% indicates a high level of stability and quality in the lyophilized product. Low moisture content is crucial for maintaining the structural integrity and prolonging the shelf life of lyophilized products [147-149]. It prevents issues such as cake collapse, product degradation, and reduced shelf life, which are common problems associated with higher moisture levels in lyophilized formulations [147, 148]. Low water content is a marker of successful lyophilization, indicating that the process was conducted under optimal conditions to remove moisture effectively [147-149].

5.9.5 FTIR Spectroscopy

The Paclitaxel and Cyclophosphamide loaded NLCs were characterized by FTIR spectra. FTIR spectra of Paclitaxel, Cyclophosphamide, and Paclitaxel & Cyclophosphamide loaded NLCs are presented in Figure 5.7. The peaks characteristic to Paclitaxel and Cyclophosphamide are presented in Table 5.25.

Table 5.25: Characteristic peaks of Paclitaxel and Cyclophosphamide

Drug Name	Characteristic peak	Peak observed in pure drug		Peak observed in PAC-CYC NLCs	Peak observed in Placebo NLCs
Paclitaxel	Carbonyl (C–O) stretching	1733.96		1735.73	No peak
	Stretching of ester C–O at 1244 cm^{-1}	1244.39		1249.26	No peak
Paclitaxel and Cyclophosphamide	C-O stretching of Amide group	PAC	1646.90	1631.42	No peak
		CYC	1651.58		
Cyclophosphamide	C-Cl stretching	844.64		839.02	No peak

The shift in the amide bond peak from 1646.90 cm^{-1} in pure Paclitaxel and 1651.58 cm^{-1} in Cyclophosphamide to 1631.42 cm^{-1} in Paclitaxel-Cyclophosphamide loaded NLCs and

Stretching of ester C–O from 1733.96 $cm^{-1}$and 1244.39 cm^{-1} in pure Paclitaxel to 1735.73 cm^{-1} and 1249.26 cm^{-1}in Paclitaxel-Cyclophosphamide loaded NLCs can be attributed to the interaction between Paclitaxel and the lipid matrix of the NLCs. This shift is a common occurrence when drugs are encapsulated in nanocarriers, as the drug's environment changes, affecting its vibrational frequencies [150, 151]. The encapsulation in NLCs involves interactions with lipids, which can alter the chemical environment around the drug molecules, leading to shifts in FTIR peaks. The shift in the amide bond peak suggests a change in the chemical environment due to encapsulation [151]. Such shifts are typical when drugs are encapsulated in lipid-based carriers, as the lipid matrix can influence the drug's vibrational properties [152]. The shift does not indicate a change in the chemical structure of Paclitaxel but rather a change in its interaction environment [151].

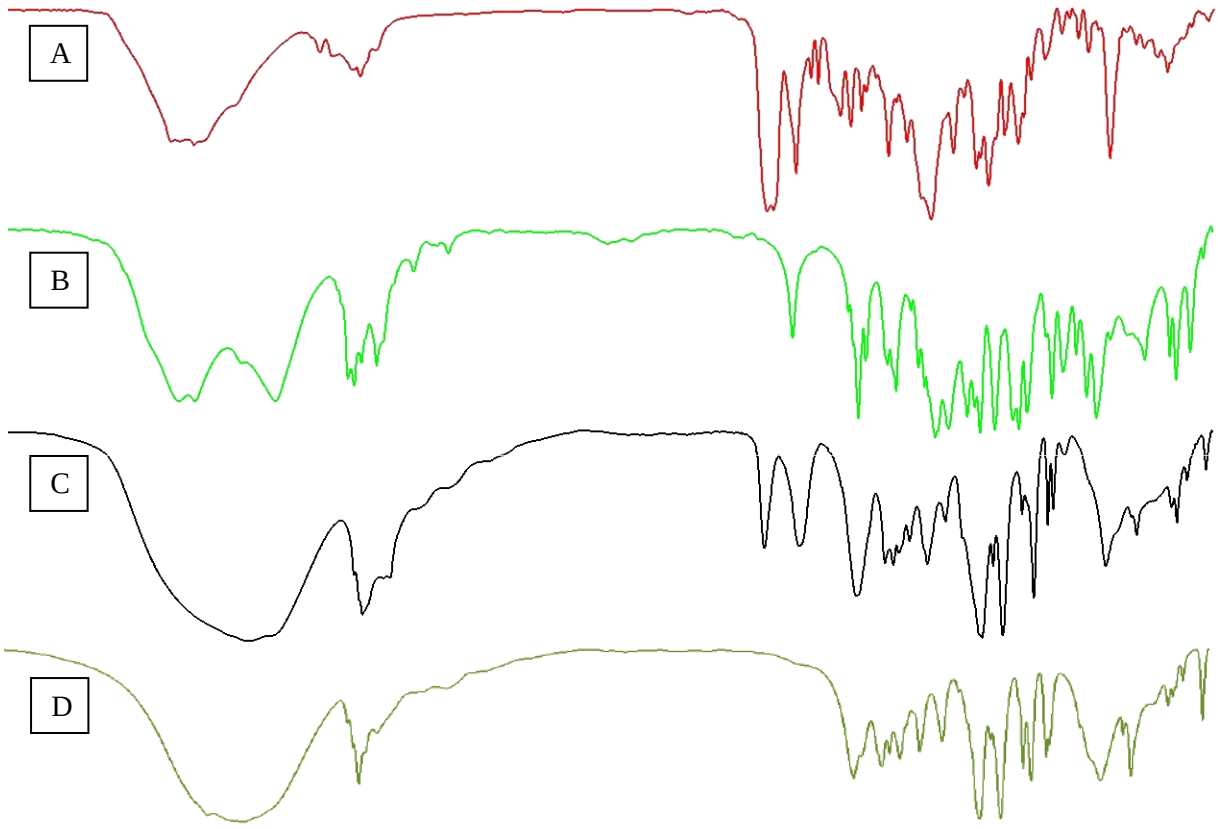


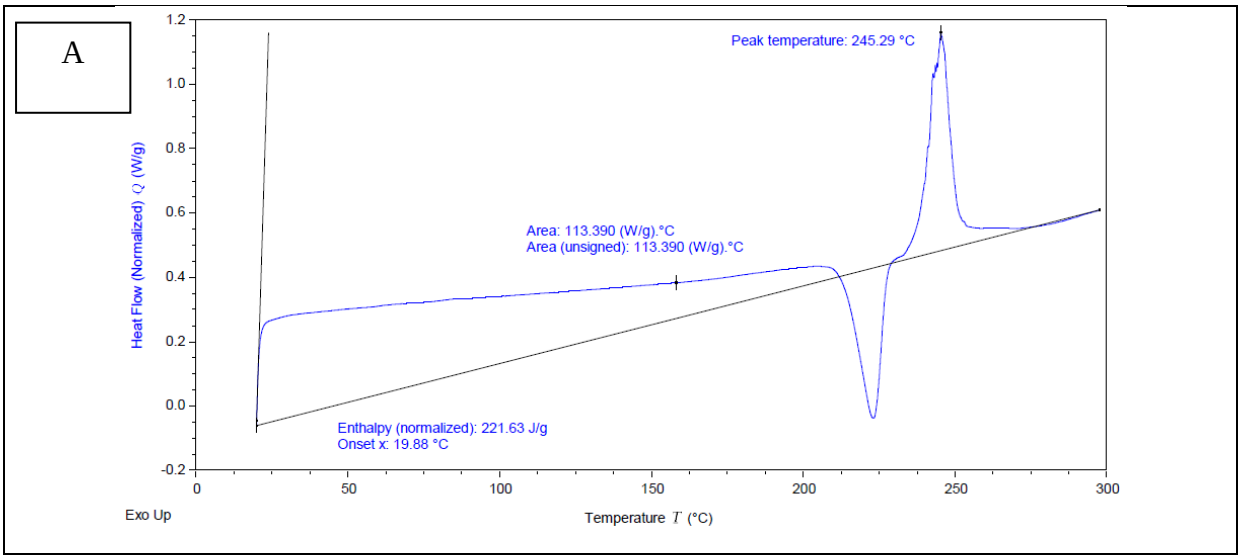
Figure 5.7: FTIR Spectra of [A] Paclitaxel pure drug [B] Cyclophosphamide pure drug [C] Paclitaxel and Cyclophosphamide loaded NLCs [D] Placebo NLCs

The slight shift in the C-Cl stretching from 844.64.09cm^{-1} in pure Cyclophosphamide to 839.02cm^{-1} in Paclitaxel-Cyclophosphamide loaded NLCs can be attributed to the interaction between the drug and the lipid matrix of the NLCs. The lipid matrix in NLCs can interact

with Cyclophosphamide, altering its molecular environment and affecting vibrational frequencies [153, 154]. The presence of solid and liquid lipids in NLCs can lead to changes in the drug's conformation, impacting the C-Cl vibrational mode [155, 156]. Cyclophosphamide is molecularly dispersed within the NLCs, which can lead to changes in its vibrational spectrum due to altered intermolecular interactions [157]. The stability of the drug within the NLCs can also contribute to the shift in vibrational frequencies, as the lipid matrix provides a stable environment that can influence the drug's molecular vibrations [158].

5.9.6 Differential scanning calorimetry

Paclitaxel, Cyclophosphamide, Paclitaxel-Cyclophosphamide loaded NLCs, and Placebo NLCs were analyzed by DSC (Refer Figure 5.8). Thermogram of NLCs did not show the melting peak of Paclitaxel and Cyclophosphamide suggesting that Paclitaxel and Cyclophosphamide were present in the NLCs either in amorphous form or molecularly dispersed.



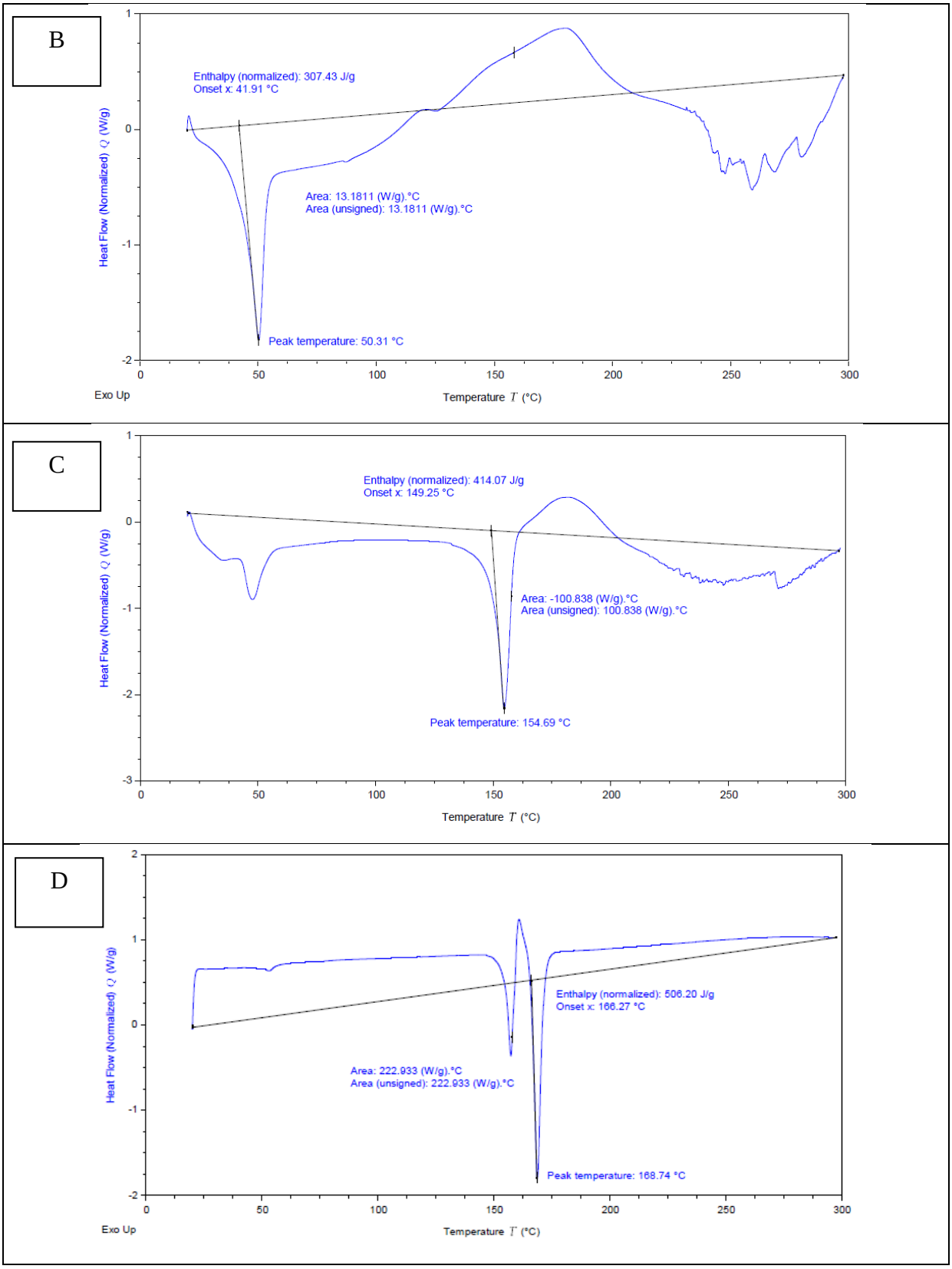


Figure 5.8: DSC Thermograms [A] Pure Paclitaxel [B] Pure Cyclophosphamide [C] Paclitaxel & Cyclophosphamide loaded NLCs [D] Placebo NLCs

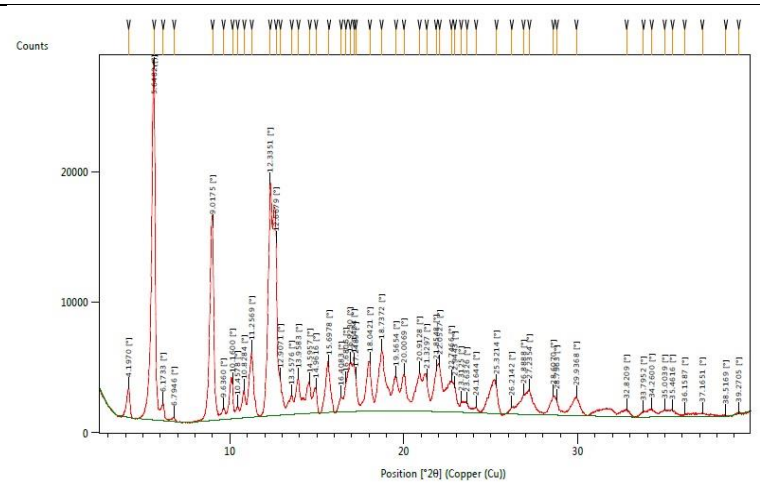
Pure Paclitaxel showed sharp exothermic peak at 245.29°C and pure Cyclophosphamide showed sharp endothermic peak at 50.31°C, whereas Paclitaxel-Cyclophosphamide loaded NLCs containing Mannitol as cryoprotectant showed a small endothermic peak at 50°C and sharp endothermic peak at 154.69°C. No sharp exothermic peak was observed at 245.29°C and no sharp endothermic peak at 50.31°C was observed. The observed thermal behaviour of Paclitaxel-Cyclophosphamide loaded NLCs with Mannitol as a cryoprotectant can be attributed to the interactions and modifications in the physical state of the drugs when incorporated into NLCs. The absence of the sharp exothermic peak at 245.29°C for Paclitaxel and the endothermic peak at 50.31°C for Cyclophosphamide indicates a change in the crystalline structure of these drugs when encapsulated in NLCs. This encapsulation leads to improved solubility and stability, which is a key advantage of using NLCs for drug delivery [152, 159]. The encapsulation of drugs in NLCs can lead to the formation of amorphous or less crystalline forms, which do not exhibit the same thermal transitions as the pure drugs [160]. The use of Mannitol as a cryoprotectant helped stabilize the NLCs during freeze-drying, which can further influence the thermal behaviour of the encapsulated drugs [161]. The absence of the original thermal peaks suggests that the drugs are in a more stable form within the NLCs, possibly due to the formation of a solid solution or a different crystalline structure [162].

A sharp endothermic peak at 154.69°C in Paclitaxel-Cyclophosphamide loaded NLCs is attributed to different polymorphic forms of mannitol [163]. The presence of an endothermic peak around 50°C is associated with the transformation of mannitol's supercooled liquid (SCL) to a low-energy amorphous phase, known as Phase X, which is facilitated by stronger hydrogen bonds [164, 165]. Two melting peaks have been observed in Placebo NLCs, at 155°C and 166°C, which are also attributed to different polymorphic forms of mannitol [163].

5.9.7 X-Ray Diffraction study

Paclitaxel, Cyclophosphamide, Paclitaxel & Cyclophosphamide loaded NLCs and Placebo NLCs were analyzed by XRD, and XRD graphs are provided in Figure 5.9. XRD is a crucial analytical technique for characterizing lyophilized NLCs due to its ability to provide detailed insights into the crystallinity and structural properties of these systems.

A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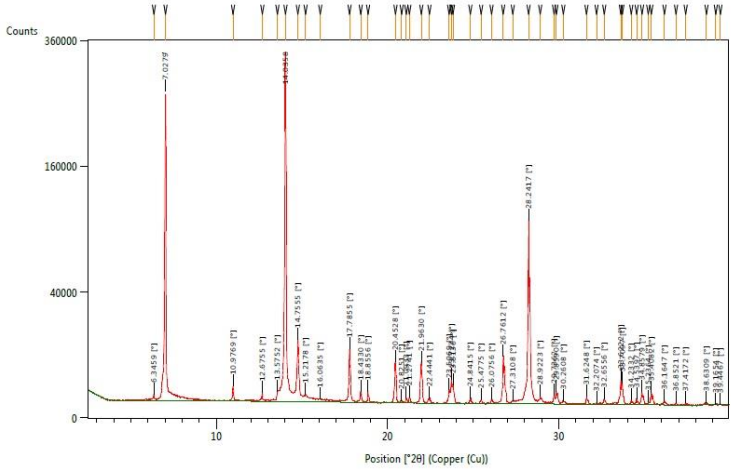
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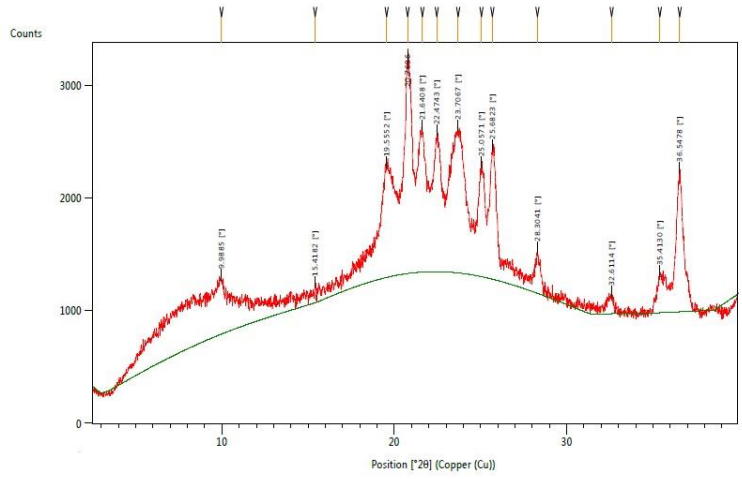
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Position [°2θ] (Copper (Cu))

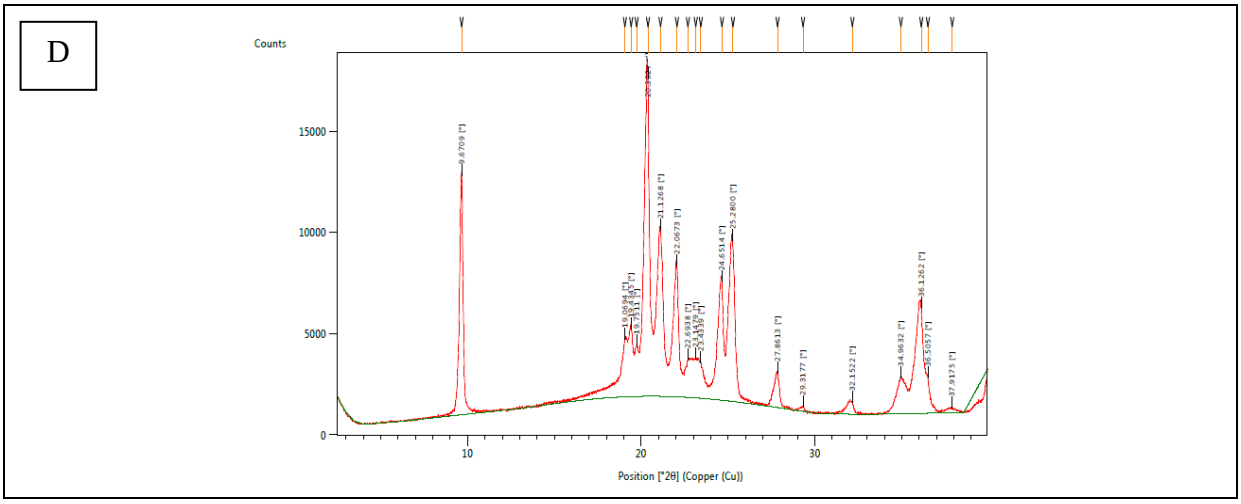

Figure 5.9: XRD graph [A] Pure Paclitaxel [B] Pure Cyclophosphamide [C] Paclitaxel and Cyclophosphamide loaded NLCs [D] Placebo NLCs

XRD spectrum of pure Paclitaxel exhibited crystalline behaviour with several peaks at 2Θ values of 5.64°, 9.02°, and 12.34°. Also, XRD spectrum of pure Cyclophosphamide exhibited crystalline behaviour with several peaks at 2Θ values of 7.03°, 14.04°, and 28.24°. However, the XRD spectrum of PAC-CYC NLCs exhibited amorphous behaviour with no significant peaks at any of the above mentioned 2Θ values suggesting that Paclitaxel and Cyclophosphamide were present in the NLCs either in amorphous form or molecularly dispersed [166, 167].

The amorphous behaviour of PAC-CYC NLCs in XRD spectra, as opposed to the crystalline nature of pure Paclitaxel and Cyclophosphamide, can be attributed to the molecular dispersion or amorphous form of these drugs within the nanostructured lipid carriers (NLCs). This transformation is often a result of the encapsulation process, which alters the molecular arrangement and prevents the formation of a crystalline lattice. The absence of distinct peaks in the XRD spectrum indicates a lack of long-range order, which is characteristic of amorphous materials [166, 167].

5.9.8 Morphology by Transmission Electron Microscopy

The images obtained by TEM (shown in Figure 5.10) revealed that the PAC-CYC NLCs were discrete spherical lipid carriers. Based on the images obtained from TEM, it is clearly understood that there is no significant difference observed between the formulated NLCs before and after lyophilization. The particle size based on TEM was observed as 45.95 ± 7.98nm.

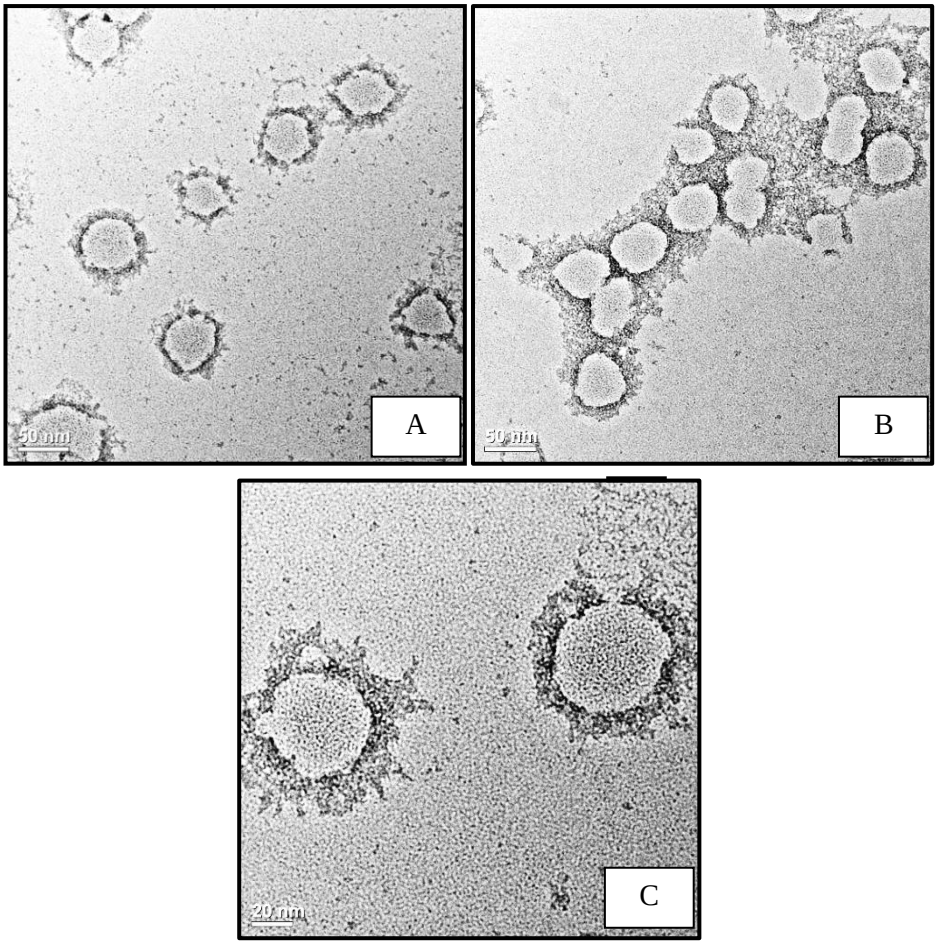

Figure 5.10: TEM Images of PAC-CYC loaded NLCs [A]: Before lyophilization [B & C]: After SMART™ lyophilization

5.9.9 In-vitro release study

The graph of % cumulative release versus time in hours is presented in Figure 5.11.

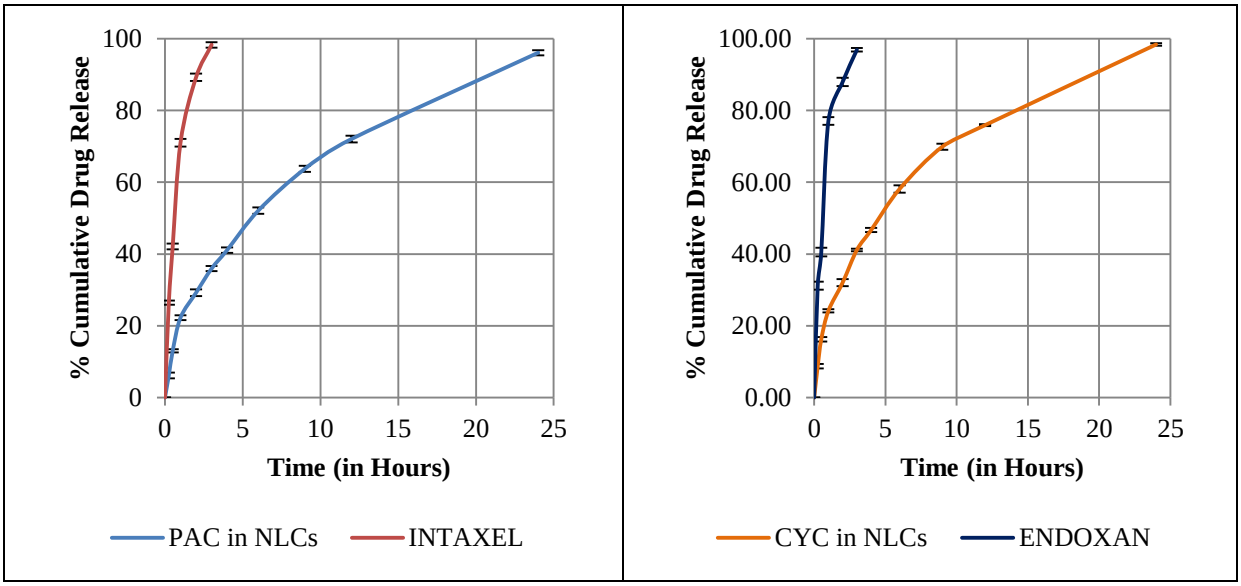

Figure 5.11: Graph of % cumulative drug release vs. time in hours for Paclitaxel & Cyclophosphamide loaded NLCs, INTAXEL, and ENDOXAN

Cumulative drug release of 98.27 ± 0.75 % and 96.87 ± 0.50 % was observed for both the INTAXEL and ENDOXAN within 3 hours respectively whereas, Paclitaxel and Cyclophosphamide loaded in NLCs required 24 hours for cumulative drug release of 96.07 ± 0.70 % and 98.37 ± 0.35 % respectively. NLCs are composed of a blend of solid and liquid lipids, which create a matrix that encapsulates the drug, leading to a slower release rate as compared to pure drugs. This matrix structure is responsible for the extended release profile observed in the study as compared to pure drugs in the in-vitro release media. The regression coefficients for various drug release models are presented in Table 5.26.

Table 5.26: Regression coefficients for various drug release models

Sr. No.	Drug Release model	INTAXEL	ENDOXAN	PAC in NLCs	CYC in NLCs
1	Higuchi Model	0.975	0.954	0.994	0.986
2	Korsmeyer- Peppas Model	0.964	0.938	0.974	0.983
3	Hixson Crowell Model	0.986	0.951	0.993	0.991
4	Zero Order	0.847	0.805	0.868	0.833
5	First Order	0.987	0.984	0.983	0.969

The First order drug release model best describes the release profile for INTAXEL and ENDOXAN indicating that the drug release rate is proportional to the remaining concentration of the drug. The First Order model suggests that the rate of drug release is concentration-dependent. This implies that the release rate decreases exponentially with time as the drug concentration in the formulation decreases. The release of Cyclophosphamide also follows a pattern where the rate decreases over time as the drug is released from the formulation.

The Higuchi and Hixson Crowell models are the most appropriate for Paclitaxel and Cyclophosphamide loaded in PAC-CYC NLCs. The Higuchi model is based on the diffusion of the drug through a porous matrix, which is a common characteristic of NLCs. This model assumes that the drug release rate is proportional to the square root of time, which is typical for systems where the drug is dispersed in a solid matrix and diffuses out over time [70]. In the case of Paclitaxel and Cyclophosphamide loaded NLCs, the lipid matrix provides a controlled environment where the drug can diffuse slowly, aligning with the assumptions of

the Higuchi model [168]. The Hixson-Crowell model considers the change in surface area and particle size as the drug is released, which is relevant for NLCs as the lipid matrix erodes over time. This model is particularly useful for systems where the dissolution of the carrier affects the release rate [169]. For Paclitaxel and Cyclophosphamide encapsulated in NLCs, the erosion of the lipid matrix leads to a change in surface area, which is captured by the Hixson-Crowell model, providing a more accurate description of the release kinetics [170].

5.9.10 Sterility

At the end of incubation period of 14 days, the test tube containing negative control, positive control, and test sample (NLCs) were observed for any signs of microbial growth. No growth was observed in Negative control and test sample, whereas growth was observed for Positive control. Therefore, the optimized NLCs passed the test for sterility.

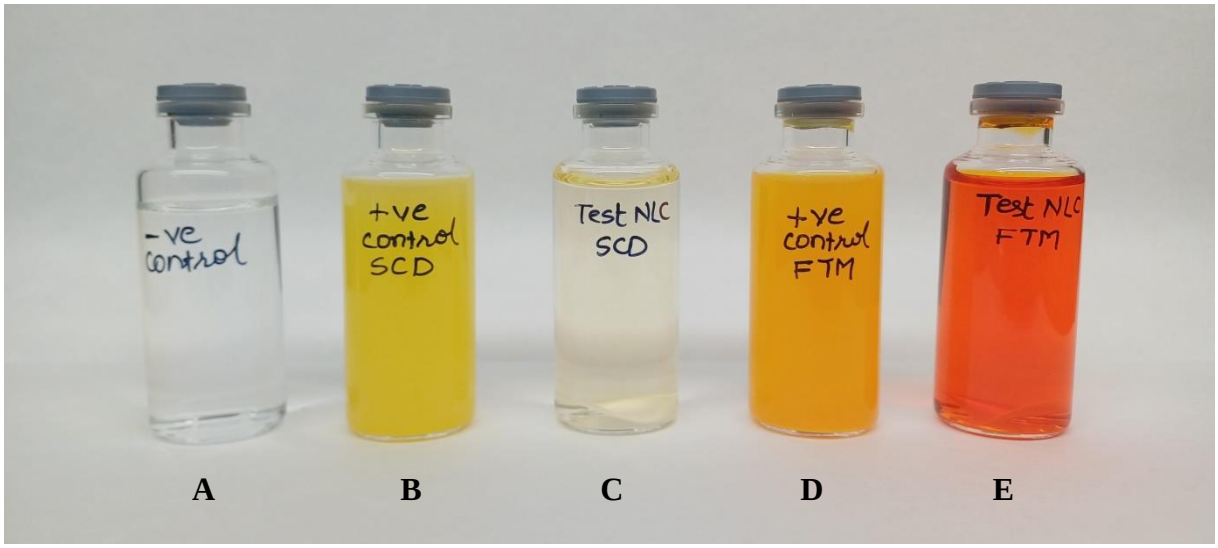

Figure 5.12: Sterility testing [A] Negative Control (Water for Injection) [B] Positive Control for Soybean casein digest medium (SCDM) [C] Test NLCs in SCDM [D] Positive control for Fluid Thioglycolate Medium (FTM) [E] Test NLCs in FTM

5.10 STABILITY STUDIES

Stability testing is a critical component in the pharmaceutical industry, ensuring that drug products maintain their safety, efficacy, and quality throughout their shelf life. This process involves evaluating how various environmental factors such as temperature, humidity, and light affect a drug's chemical and physical properties over time [171].

Results and Discussion:

The results of stability studies are presented in Table 5.27.

Table 5.27: Results of Stability study batch 01 for PAC-CYC NLCs

Sr. No.	Stability Condition	2 to 8°C				25±2°C/ 60±5%RH
	Tests	Initial	3 Months	6 Months	12 Months	15 days
1	Reconstitution time	69 seconds	76 seconds	71 seconds	79 seconds	83 seconds
2	Z-average (nm)	74.3 ± 3.7	84.0 ± 1.4	70.9 ± 2.7	80.4 ± 0.9	86.1 ± 1.5
3	PDI	0.072 ± 0.004	0.100 ± 0.002	0.065 ± 0.007	0.081 ± 0.005	0.085 ± 0.006
4	Zeta potential (mV)	-5.3 ± 2.7	-4.8 ± 1.9	-6.0 ± 0.5	-5.0 ± 1.2	-4.3 ± 2.2
5	Entrapment efficiency of Paclitaxel (%)	100.0±0.4	99.4±0.4	98.9±0.7	99.1±0.2	98.1±0.8
6	Entrapment efficiency of Cyclophosphamide (%)	99.0±0.8	98.7±0.3	97.4±0.3	96.9±0.5	97.0±1.1
7	Water content (%)	0.5± 0.1	0.6±0.0	0.5± 0.2	0.4± 0.2	0.3± 0.1
8	Sterility	Sterile	NA	NA	Sterile	NA

PAC-CYC loaded NLCs were observed to be stable for 12 months when stored at 2 to 8°C and stable for 15 days when stored at 25±2°C/60±5%RH. There was no significant change observed in the reconstitution time, water content, particle size (Z-average), PDI, and Zeta potential during the storage. Entrapment efficiency of Paclitaxel and Cyclophosphamide was observed to be constant during 12 months storage. Sterility of the product was maintained even after 12 months storage at 2 to 8°C.

Updated Risk Assessment

Based on the optimization of various factors impacting the formulation development and characterization of PAC-CYC NLCs, the updated risk assessment is presented in Table 5.28 and justification for updated risk assessment is presented in Table 5.29.

Table 5.28: Updated Risk Assessment

Drug Product CQAs	Factor				
	Environment	**Materials**	**Equipments**	**Process Parameters**	**Formulation Parameters**
Product Description after reconstitution	Low	Low	Low	Low*	Low*
Assay of Paclitaxel	Low*	Low	Low	Low*	Low*
Assay of Cyclophosphamide	Low*	Low	Low	Low*	Low*
Sterility	Low	Low	Low	Low*	Low
Particle size distribution	Low	Low	Low	Low*	Low*
Zeta Potential	Low	Low	Low	Low*	Low*
Reconstitution time of Lyophilized product	Low	Low	Low	Low*	Low*
% water content	Low	Low	Low	Low*	Low*
Entrapment efficiency	Low	Low	Low	Low*	Low*
Filterability	Low	Low	Low	Low*	Low*

**The level of risk was not reduced from the initial risk assessment

Table 5.29: Justification for Updated Risk Assessment

Drug Product CQAs	Factor	Justification
Product Description after reconstitution	Process Parameters (Previously a High risk)	Mannitol was selected as cryoprotectant at 4%w/v concentration and lyophilization cycle for PAC-CYC NLCs was developed through SMART™ lyophilization cycle development. Product description after reconstitution was achieved as per the QTPP. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	

Drug Product CQAs	Factor	Justification
Assay of Paclitaxel	Environment (Previously a Medium risk)	All the development studies and characterization of PAC-CYC NLCs was executed at controlled room temperature (25±5°C) with relative humidity below 55%RH and either sodium vapor light or no light condition to ensure minimal exposure of room light. No separate experimentation was required as necessary precaution was in place throughout the development and stability. Hence, the risk is assessed to be Low.
	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration were optimized during development studies to achieve the assay of Paclitaxel in the range of 90.0 to 110.0% for optimized PAC-CYC NLCs. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Assay of Cyclophosphamide	Environment (Previously a Medium risk)	All the development studies and characterization of PAC-CYC NLCs was executed at controlled room temperature (25±5°C) with relative humidity below 55%RH and either sodium vapor light or no light condition to ensure minimal exposure of room light. No separate experimentation was required as necessary precaution was in place throughout the development and stability. Hence, the risk is assessed to be Low.
	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration were optimized during development studies to achieve the assay of Cyclophosphamide in the range of 90.0 to 110.0% for optimized PAC-CYC NLCs. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Sterility	Process Parameters (Previously a Medium risk)	Sterilization of PAC-CYC NLCs was ensured using sterile filtration technique. The filterability of developed product was observed to be 100% when filtered through 0.2µ pore size sterile PES filters. Drug product was observed to be sterile upon tested for sterility testing. Hence, the risk is assessed to be Low.
Particle size distribution	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration were optimized during development studies to achieve Z-average below 150nm and PDI below 0.2 for the optimized formulation. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	

Drug Product CQAs	Factor	Justification
Zeta Potential	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration impacts the final formulation were optimized during development studies which led to zeta potential in between -10mV and + 10mV for final optimized product. The zeta potential was also observed to remain stable during storage stability of optimized product. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Reconstitution time of Lyophilized product	Process Parameters (Previously a High risk)	Mannitol was selected as cryoprotectant at 4%w/v concentration and lyophilization cycle for PAC-CYC NLCs was developed through SMART™ lyophilization cycle development. Reconstitution time of lyophilized product was achieved as not more than 1 minute as per the QTPP. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
% water content	Process Parameters (Previously a High risk)	Mannitol was selected as cryoprotectant at 4%w/v concentration and lyophilization cycle for PAC-CYC NLCs was developed through SMART™ lyophilization cycle development. Water content of optimized lyophilized product was achieved as not more than 2.0%. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Entrapment efficiency	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration were optimized during development studies to achieve the entrapment efficiency of not less than 90.0%. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Filterability	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration were optimized during development studies to achieve the filterability of 100% for the optimized PAC-CYC NLCs. 100% filterability also ensure that filtration achieved sterile drug product suitable for intravenous administration. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	

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