

Article

Development of Paclitaxel Proliposomal Dry Powder Inhaler (PTX-PLM-DPI) by Freeze-Drying Method for Lung Cancer

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Abstract: Despite various efforts, a successful selective delivery system for chemotherapeutic agents for lung cancer is still lacking. Dry powder inhaler (DPI) systems based on proliposomes (PLMs) could be a potential system for the efficient delivery of paclitaxel to lungs. PLM-based DPI prepared with a freeze-drying method can therefore be an alternative. Paclitaxel-loaded PLM-based DPI (PTX-PLM-DPI) powders were prepared using the method of thin film deposition on a carrier followed by freeze drying. These were prepared using soya phosphatidylcholine (SPC) and cholesterol as the lipids and mannitol as the carrier. The reconstituted liposomes were evaluated in terms of size, morphology, drug entrapment, release and cytotoxicity. The DPI powders were evaluated for their flow property, surface topography, dose uniformity and in vitro lung deposition. Stable and free-flowing PTX-PLM-DPI powder was obtained that could be reconstituted into homogenous liposomal vesicles < 200 nm as confirmed by TEM and SEM studies. The liposomes showed drug entrapment of $92.64 \pm 1.4\%$ and diffusion-controlled release of up to 28% in 24 h. These liposomes showed better dose-dependent cytotoxicity in A549 cells in comparison to paclitaxel suspension with IC_{50} values of 46 ± 0.87 ng/mL and 154.9 ± 3.64 ng/mL, respectively. In vitro lung deposition studies of the PTX-PLM-DPI showed sufficient deposition with the fine particle fraction (FPF) of $50.86 \pm 2.8\%$ of particles with an aerodynamic diameter less than 5μ . Hence, it can be concluded that PLM-based DPI prepared by freeze drying can be a promising, stable, safe and free-flowing system for the enhanced lung delivery of paclitaxel.

Keywords: proliposomes; dry powder inhaler; freeze drying; lung cancer; paclitaxel; targeted delivery



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1. Introduction

Liposomes are reported to be clinically successful nanocarrier-based drug delivery systems for targeted cancer therapy [1,2]. These formulations, constituting phospholipids and cholesterol, are accepted by the physiological system hence, are regarded as safe [3]. Moreover, liposomes are particularly preferred for lung administration due to their tendency to evade alveolar macrophages and mucociliary clearance [4]. However, one of the major limitations of the liposomal formulation is its physical instability during storage. PLMs (discovered by Payne et al., 1986), as a solidified powder, are more stable than liposomes hence, are preferred as an efficient alternative for liposomes [5–7]. When proliposomes come into contact with water or any biological fluid in the body, they instantly transform from a dry, free-flowing granular substance into a liposomal dispersion [5]. These free-flowing PLM powders can be delivered as a dry powder inhaler (DPI) for improved inhalation therapy [8,9].

Lung cancer is presently the major form of cancer in terms of mortality worldwide. Despite various efforts, we still lack a suitable delivery system for the sufficient delivery of

chemotherapeutic agents to the cancerous region in the lungs with reduced toxicity [10]. Therefore, inhalation therapy, particularly DPI, can be a potential system for the efficient delivery to lungs [11]. DPIs are more popular than other inhalation systems because they have all the benefits of solid dosage forms, such as being more stable, portable and very comfortable to use. They can also be used with a wide range of active ingredients and doses [12,13]. In some cases, DPIs are prepared by spray-drying methods where avoiding aggregation of the spray-dried powders requires detailed formulation design and extensive evaluations [13]. Marketed DPI products prepared by spray drying methods include the Novartis TOBI[®]Podhaler[™] approved by the US FDA in 2013 for the treatment of *Pseudomonas aeruginosa* bacteria in the lungs in people with cystic fibrosis and Aridol/Osmohale[™] and Bronchitol[™] (the spray-dried inhalable mannitol powders by Pharmaxis) used to diagnose asthma [14,15]. PLM-based DPI can therefore be a better alternative as the use of phospholipids and cholesterol may result in a good flow with less aggregation [16,17]. Lactose and mannitol are the most widely used carriers for such systems. Mannitol because of its inherent cryoprotectant property may be more suitable for the film deposition on carrier method using a rotary evaporator followed by freeze drying. Moreover, the hygroscopic nature of mannitol may also result in excipient-enhanced particle growth (EEG) on the passage through lung airways and may result in higher deposition due to enhanced sedimentation velocity [13].

The chemotherapeutic drug paclitaxel (PTX) is frequently employed [18–20] for treating lung cancer. Parenteral dosing, however, is likely to cause severe toxicity. Moreover, the water insolubility of PTX also limits its bioavailability. Although a number of novel PTX formulations, including albumin-bound PTX (Abraxane[®]) and PTX liposomes, have been developed, none have achieved the expected tumor targeting benefits in patients [21,22]. Hence, a lot of effort has been made to develop nanocarriers of PTX as liposomes, nanoemulsions and polymeric micelles for more site-specific delivery [22–24]. The drugs can be administered directly to the lung through oral inhalation, limiting systemic exposure, making it a promising noninvasive delivery route [7]. There are reports of DPIs of PTX prepared by spray-drying methods for lung cancer [22,25]. These systems have been found to be effective in the delivery of the drug through this system. However, these DPIs suffer from particle aggregation and insufficient flow properties [16,17]. PLM-based DPI of PTX prepared by freeze drying can therefore be a potential alternative for enhanced and safe delivery in lung cancer [9].

For enhanced delivery to the lungs, the DPI system should be able to produce a majority of particles within a size of 1–5 μ as these particles have a higher tendency to become engulfed in the bronchiole and lung alveoli [26]. This requires achieving the optimum aerodynamic diameter of the particles, sufficient fine particle fraction and flow property [7]. Particle aggregation due to the cohesive forces is a major obstruction in achieving this property and a major hurdle for the reported DPI systems [13]. Use of anti-adherents such as stearates can reduce this problem; however, the safety of such hydrophobic excipients for use in the lungs has yet to be established [16]. Use of PLMs with excipients such as phospholipids and cholesterol can therefore be a suitable alternative as these excipients have been reported to have good anti-adherent properties [16,17]. Moreover, they provide a more stable alternative for liposomal delivery with all the advantages of liposomes in vivo for enhanced lung delivery.

Hence, in this study, PTX-loaded PLM-based DPI systems were prepared by the method of thin film deposition on carrier followed by freeze drying. The PLM comprised SPC and cholesterol as the lipids and mannitol as the carrier. These free-flowing PTX-PLM-DPI powders were used for enhanced delivery for lung cancer using gelatin-based capsules and a Rotahaler[®] device. The reconstituted liposomes were evaluated in terms of their size distribution, surface morphology, drug entrapment, release and cytotoxicity. The DPI was evaluated for its flow property, surface topography, dose uniformity and in vitro lung deposition.

2. Results

2.1. Formulation and Optimization of PTX-PLM-DPI

From the studies, PTX-PLM-DPI was successfully prepared by the thin film deposition on carrier method followed by freeze drying. Lipid phase to carrier at 1:5, SPC:cholesterol ratio of 9:1 with mannitol as carrier and evaporation time of 1 h were selected as optimized parameters for the preparation of optimized PTX-PLM-DPI as shown in Table 1. The PTX-PLM-DPI could be reconstituted into homogenous and spherical liposomal vesicles on hydration followed by sonication.

Table 1. Formulation and characteristics of optimized PTX-PLM-DPI and its reconstituted liposomes.

Formulation Number	Lipid:Carrier	SPC:Chol	Vesicle Size nm \pm SD	Entrapment % \pm SD	Release % \pm SD (in 24 h)
F ₁₆	1:5	9:1	196.20 \pm 4.99	92.64 \pm 1.40%	27.26 \pm 4.20

2.2. Evaluation of Optimized PTX-PLM-DPI

The particle size and PDI of the reconstituted liposomes from the optimized PTX-PLM-DPI powder were found to be 196 ± 5 nm and 0.335 ± 0.06 , respectively. The obtained size and homogeneity were satisfactory for lung delivery. The yield of the optimized PTX-PLM-DPI using mannitol as a carrier with 1:5 lipid phase/carrier ratio and 9:1 as SPC/cholesterol ratio was found to be 95.26% *w/w*. The high percentage yield shows the suitability of the process and use of the selected excipients for preparation of the PLM. The surface morphology of the PTX-PLM-DPI powders was examined using SEM. The surface morphology of pre- and post-lyophilized PLMs showed no major differences and showed formation of small, uniform size nanoparticles absorbed on the surface of the carriers with no sign of aggregation (Figure 1A,B). TEM analysis of the reconstituted liposomal formulation confirmed the presence of spherical vesicles with less than 200 nm size and uniform dispersion as shown in Figure 2.

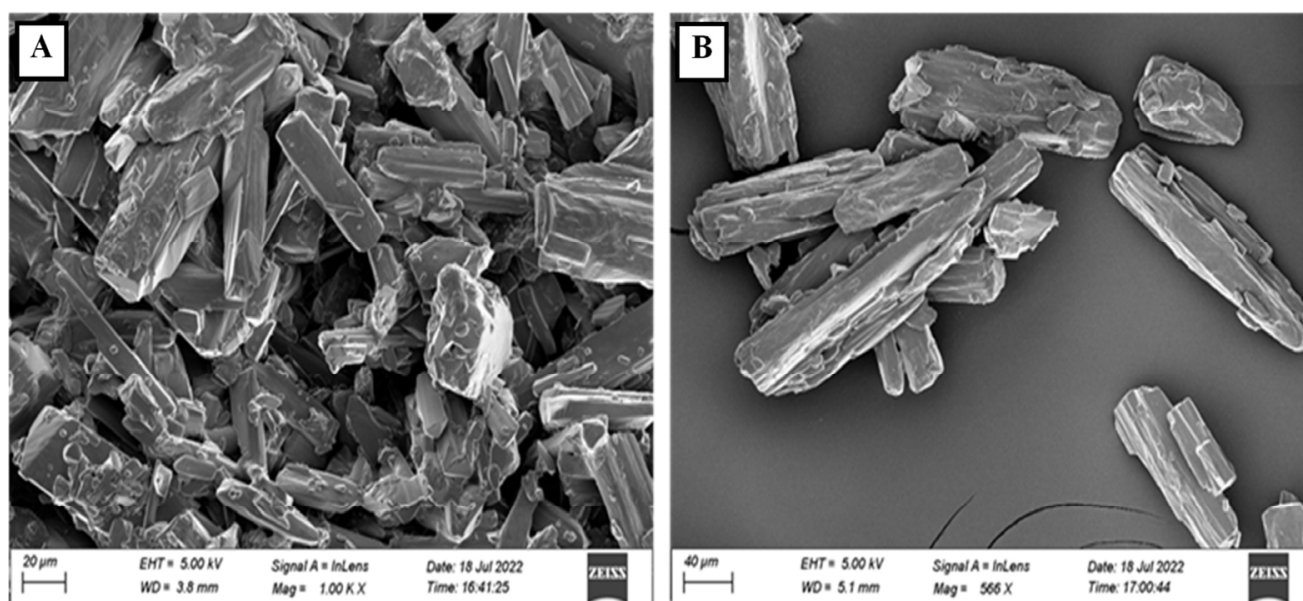


Figure 1. (A) SEM images of PTX-PLM-DPI powder (pre-lyophilization) at a magnification of $561\times$ [Scale: 20 μm]; (B) SEM photographs of PTX-PLM-DPI powder (post-lyophilization) at a magnification of $532\times$ [Scale: 40 μm].

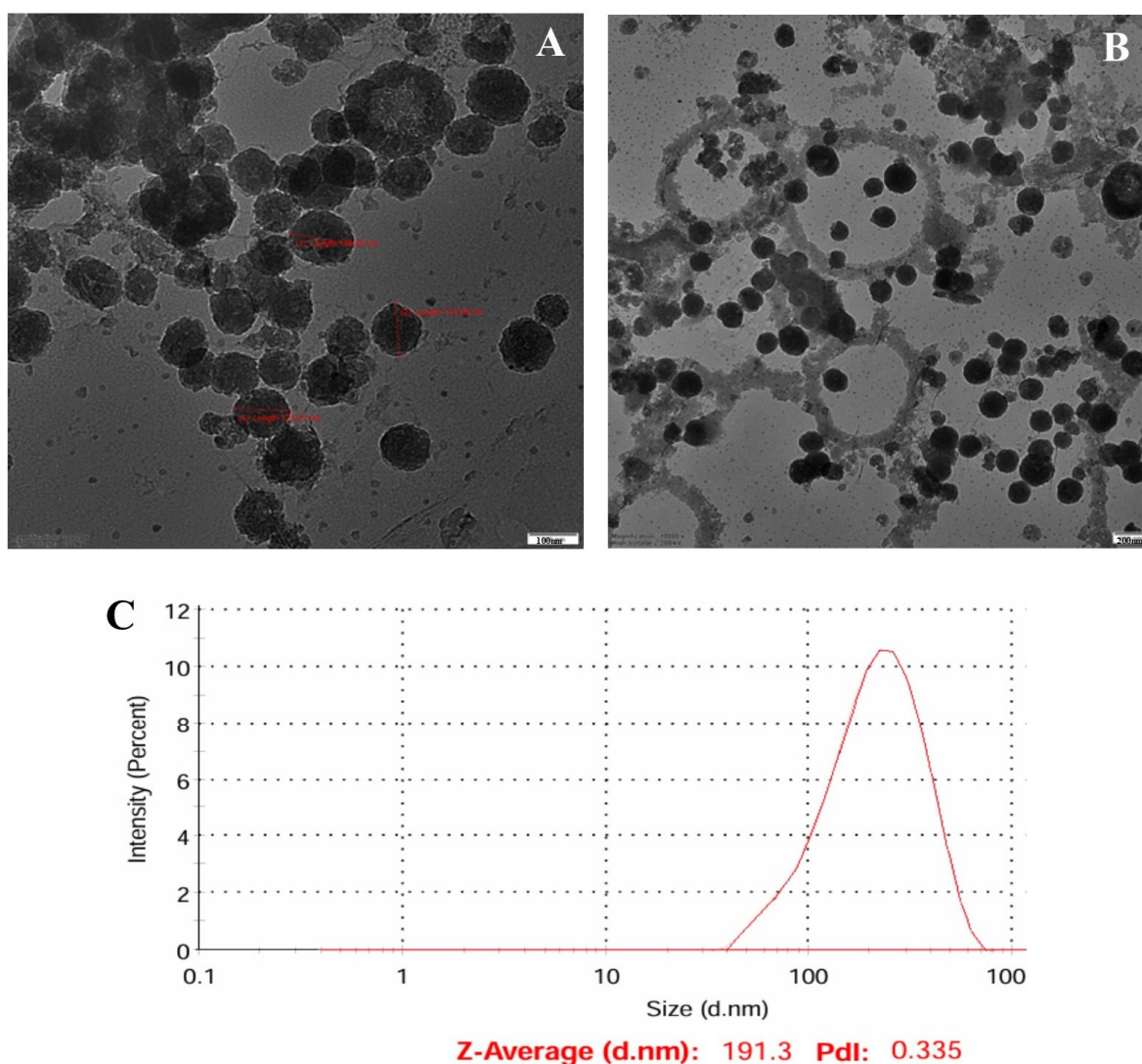


Figure 2. (A). TEM photographs of optimized PTX-PLM-DPI reconstituted liposome at a magnification of 30,000 \times with scale bar 100 nm, (B). TEM photographs of optimized PTX-PLM-DPI reconstituted liposome at a magnification of 30,000 \times with scale bar 200 nm, (C). DLS spectrum of optimized PTX-PLM-DPI reconstituted liposome.

From the analysis of flow properties of the PTX-PLM-DPI powders (pre- and post-lyophilization), it was observed that the flow property improves from passable to excellent flow after lyophilization. Carr's index improved from 24.19 to 7.93 and Hausner's ratio decreased from 1.32 to 1.08. Similarly, the angle of repose improved from 55.14 $^{\circ}$ to 28.19 $^{\circ}$. The results are shown in Table 2. The PXRD analysis of the pure drug PTX (CNM1) and the PTX-PLM-DPI powder (CNM2) showed that the characteristic PTX peaks in the PLM formulation were reduced in intensity indicating the reduced crystallinity as shown in Figure 3A,B, respectively.

Table 2. Flow characteristics of pre-and post-lyophilization PTX-PLM-DPI Powders.

Flow Parameter	Pre-Lyophilization	Post-Lyophilization
Tapped Density	0.65 \pm 0.02	0.52 \pm 0.01
Carr's Index	24.19 \pm 0.05	7.93 \pm 0.03
Hausner's Ratio	1.32 \pm 0.06	1.08 \pm 0.03
Angle of Repose	55.14 $^{\circ}$ \pm 2.10	28.19 $^{\circ}$ \pm 2.60

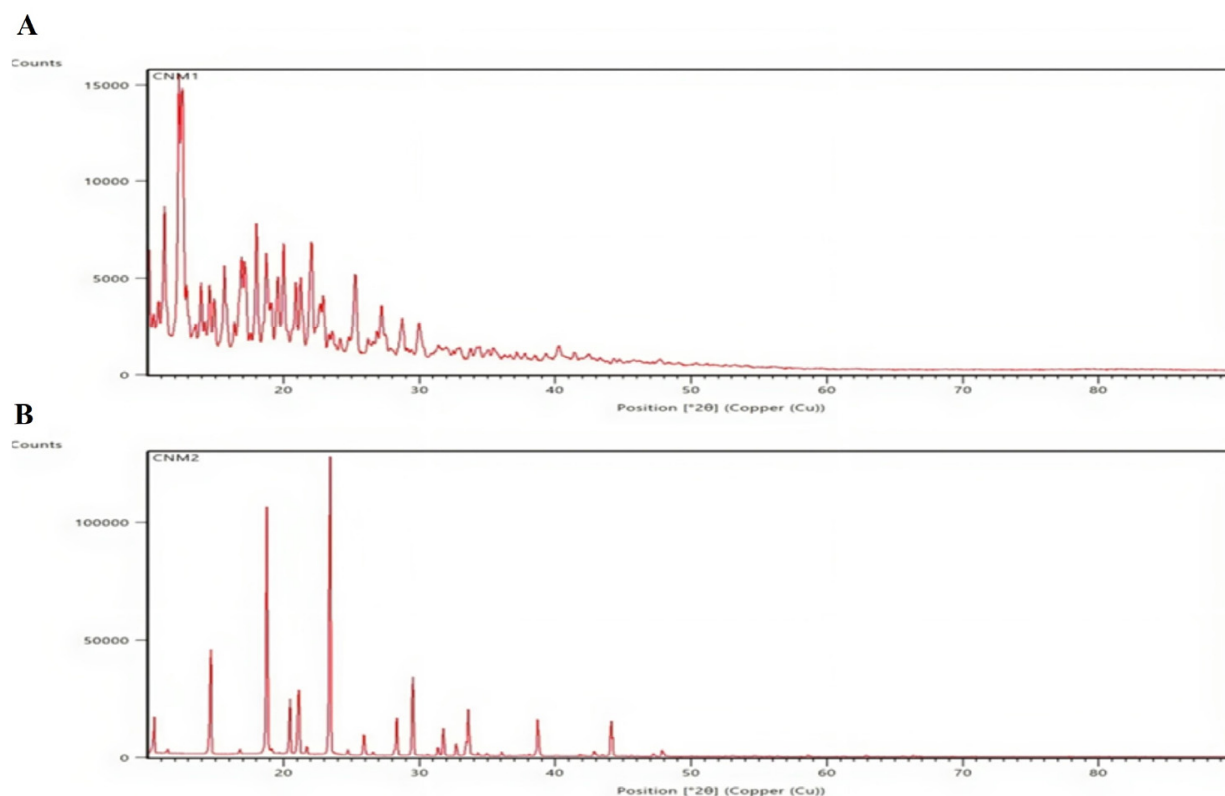


Figure 3. (A) X-ray diffractogram of pure PTX; (B) X-ray diffractogram of PTX-PLM-DPI powder.

2.3. Entrapment Efficiency and In Vitro Drug Release

The percentage entrapment was measured with the standardized HPLC method. The HPLC method was standardized by varying the ratio of the acetonitrile/water system as mobile phase, different flow rate and run time. From the studies, mobile phase ratio was selected as acetonitrile/water (75:25 *v/v*) and flow rate at 1 mL/min was found to be optimized. Calibration curve at various dilutions from 3 mg/mL to 200 mg/mL was obtained with $y = 93,443x + 138,544$ and linearity $R^2 = 0.9978$. Intra day, inter day variation, accuracy and precision studies were found to have $RSD \leq 2.0$ which is well accepted as per the ICH guidelines. The entrapment of the optimized formulation was found to be $95.64 \pm 1.4\%$ *w/v*, indicating sufficient entrapment of the drug.

In vitro drug release was observed using the dialysis bag method using simulated lung fluid containing phosphate-buffered saline 7.4 (PBS 7.4) containing 0.05% SLS as the release media. The drug concentration was measured by the standardized HPLC method. The drug release from in vitro samples displayed a biphasic pattern, with an early burst release that was followed by sustained release up to 27.27% *w/v* release in 24 h.

2.4. Release Kinetics

By using the best curve-fitting method, drug release kinetics were found. Then, the release profile of the drug PTX from the formulation was fitted to the kinetic models to identify how the drug was released. The zero-order model, the first-order model, the Higuchi model, and the Korsmeyer–Peppas model were used to compare the correlation values of the drug release data. From this study, it was seen that the release curve fits all the kinetic models. The order of Higuchi model, first order and zero order showed the best fit with the highest correlation values. In Korsmeyer–Peppas's model, the 'n' number was found to be 0.35833. Since n is less than 0.5, this means that drug release is diffusion dependent. Table 3 and Figure 4 show the findings.

Table 3. Various parameters of the in vitro release kinetics models.

Zero Order Model		First Order Model		Higuchi Model		Korsmeyer–Peppas Model	
K_0	R^2	K_1	R^2	K_H	R^2	K	n
0.0425	0.9696	−0.0002	0.9789	1.0506	0.9836	0.9707	0.3583

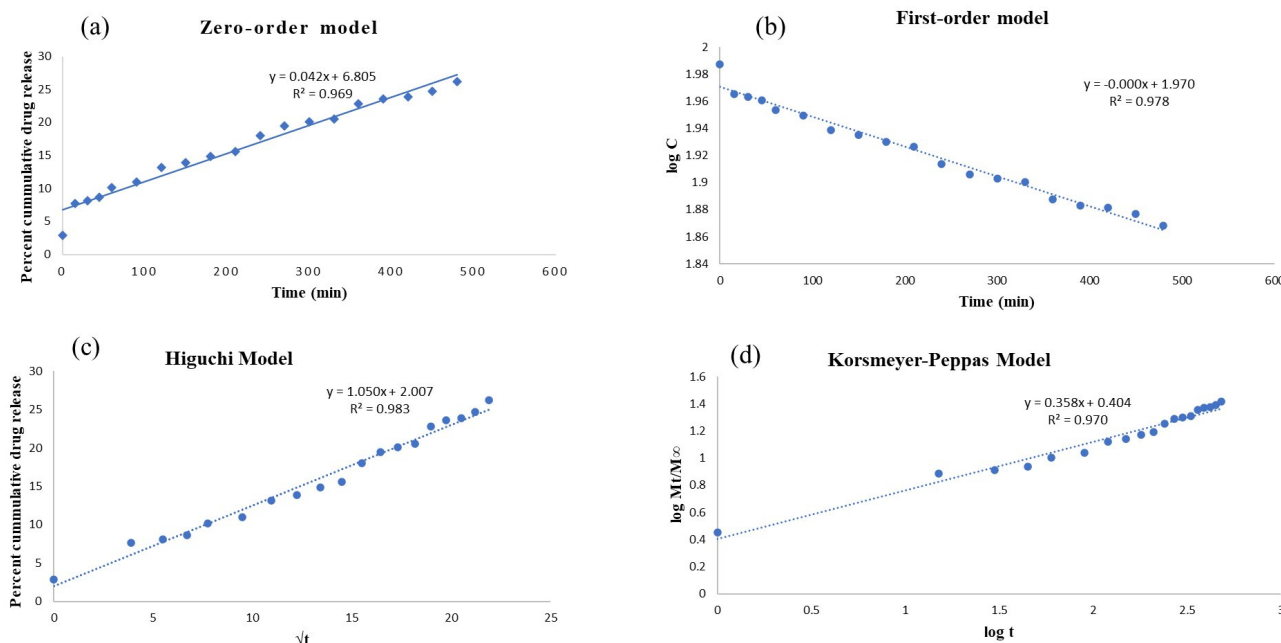


Figure 4. Release kinetics model figures (a) zero order; (b) first order; (c) Higuchi; (d) Korsmeyer–Peppas.

2.5. Delivered Dose Uniformity

From this study, the dose delivered by the device was found to be uniform when examined by loading the formulation in threehard gelatin capsules. The average drug delivered from the three capsules was $500.03 \pm 3.3 \mu\text{g}$. The results indicated the delivery of a sufficient dose homogenously.

2.6. In Vitro Lung Deposition Study

The concentration of drug collected from each plate was calculated. From this study, it was observed that the particle deposition was more at pre-separator and distributed up to stage 7 indicating that the particles were able to pass up to the lowest stage in sufficient quantity. The drug delivered to the lungs in stage 2 to 7 was $233 \mu\text{g}$. The fine particle fraction (FPF) (aerodynamic diameter less than 5μ) deposited in stage 2 to 7 was found to be $50.86 \pm 2.80\%$. The results are shown in Table 4.

Table 4. In vitro aerosol performance parameters of the dry powder formulation determined with an Anderson Cascade Impactor from the Rotahaler® device (mean \pm SD, $n = 3$).

ACI Stage	Particle Size (μm)	Amount of Drug Deposited (μg) \pm SD
Device		26.17 ± 2.44
Pre-separator		103.99 ± 0.11
Stage 0	9.0+	78.13 ± 4.55
Stage 1	7.40	34.80 ± 3.47
Stage 2	5.25	68.34 ± 1.98
Stage 3	4.00	35.64 ± 4.07
Stage 4	2.70	42.99 ± 3.66
Stage 5	1.60	35.15 ± 2.13
Stage 6	0.90	25.70 ± 6.01
Stage 7	0.55	25.36 ± 5.66

2.7. In Vitro Cytotoxicity (MTT Assay)

Free PTX suspension and reconstituted PTX-PLM-DPI liposome cell cytotoxicity were studied using MTT assay on human A549 lung cancer cell line. The study was performed at different concentrations of PTX suspension and PTX-PLM-DPI liposomes (ranging between 10 and 250 ng/mL). Both the suspension and PLMs showed concentration-dependent cytotoxicity. IC_{50} values were determined after 24 h of incubation and found to be 154.90 ± 3.64 ng/mL and 46 ± 0.87 ng/mL, respectively, as shown in Table 5. Cytotoxicity was expressed in terms of % cell viability and was found to be correspondingly more for the reconstituted liposomes in comparison to the suspension, as shown in Figure 5.

Table 5. IC_{50} values of A549 cells dosed with free paclitaxel (PTX) suspension and reconstituted liposomes of the PTX-PLM-DPI powders for 24 h.

Sl. No.	Formulations	IC_{50} Values (ng/mL) \pm SD
1	PTX Suspension	154.90 ± 3.64
2	Reconstituted liposomes of the PTX-PLM-DPI	46 ± 0.87

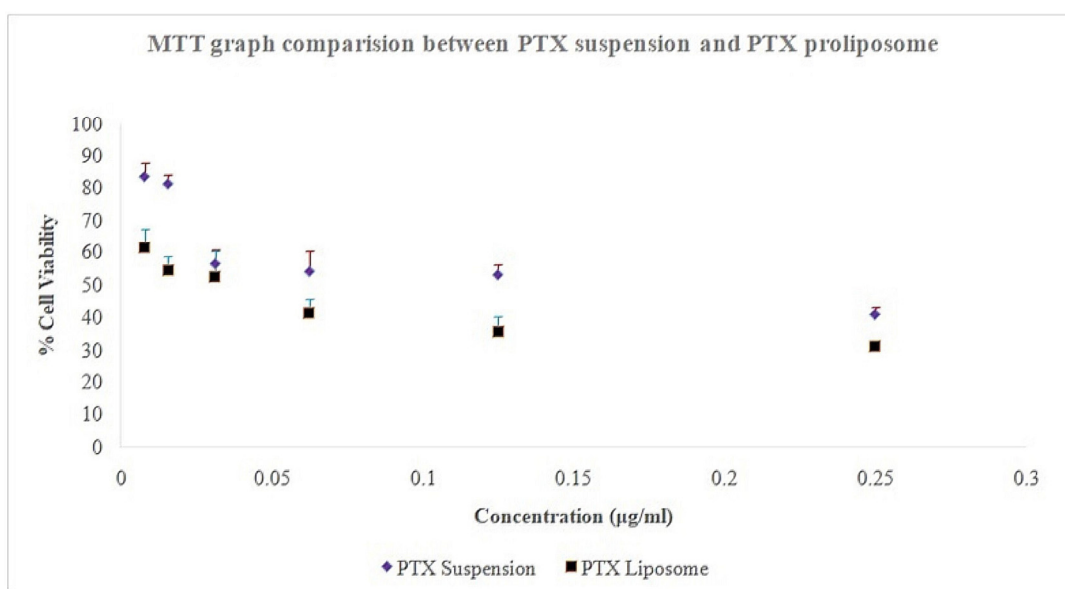


Figure 5. Cell viability results of A549 cells dosed with free paclitaxel (PTX) suspension and the optimized PTX-PLM-DPI reconstituted liposome for 24 h.

3. Discussion

Homogenous PTX-PLM-DPI powders were obtained using the film deposition on carrier method. These were optimized based on different parameters (evaporation time of organic solvent, ratio of lipid phase to carrier, ratio of phospholipid to cholesterol, type of carbohydrate carrier). LMH and mannitol were used as the carbohydrate carriers to optimize different blank formulations. Based on the size distribution and flow property of PLMs prepared with LMH and mannitol as the carrier, mannitol was found to be a better carrier. Moreover, it is a good cryoprotectant. Ethanol and chloroform in the ratio 1:1 were used as the solvent system. SPC and cholesterol constituted the lipid phase and were used in different ratios from 3:1 to 9:1. From this study, SPC/cholesterol (9:1) was found to yield better PLMs. The effect of the SPC/cholesterol ratio on the constituted liposomes from the PTX-PLM-DPI powders showed that the particle size increased on increasing the concentration of cholesterol. This may be because increased cholesterol might become incorporated into the lipid bilayer and disturb its closed structure. These might have resulted in an increased distribution of the aqueous phase inside the vesicle

which resulted in an increase in the particle size [8]. Further, the optimized combination of lipid phase to carrier phase was found to be 1:5. It was observed that PTX-PLM-DPI powders using a lower amount of carrier yielded liposomes of a larger size and those with increased amounts of mannitol showed a better size. This might be due to the poor powder flow because of increased aggregation on using insufficient amounts of carrier [8]. Hence, an optimum amount of carriers are required in the formulation of PLMs to maintain proper flow and size. During the constitution of the liposomes from the proliposomes by hydration, it was found that annealing for 1 h at -20°C showed a growth in size and sonication for 30 min post-hydration into liposomes yielded a better size distribution.

The recovery of PTX-PLM-DPI powders prepared with mannitol as a carrier with 1:5 lipid phase/carrier ratio and 9:1 as SPC/cholesterol ratio was found to be satisfactory with up to a 95.26% yield. The results indicate that the technique used for the preparation and processing of the PLMs was reliable and satisfactory. The particle size and PDI of the liposomes obtained on hydration from the optimized PTX-PLM-DPI powders was found to be 196 ± 5 nm and 0.34 ± 0.06 . Particles of a size ranging 100–200 nm are preferred for lung delivery as they produce less systemic side effects and have more tendency to avoid uptake by alveolar macrophages present in lungs and thereby have reduced clearance [8]. Hence, the obtained size below 200 nm of the liposomes was regarded as satisfactory for lung administration. Surface morphology by TEM analysis of this liposomal formulation showed spherical vesicles. The surface topography of the PTX-PLM-DPI powders was examined using scanning electron microscopy (SEM). It was observed that the PTX-PLM-DPI particles did not change due to lyophilization and the pre- and post-lyophilization showed a similar size and no aggregation was observed. This can be attributed to the use of mannitol in the formulation of PTX-PLM-DPI powders which itself is a cryoprotectant and the method of freeze drying used in the formulation [27]. The use of the PTX-PLM-DPI system involving cholesterol and SPC which have anti-adherent properties might also result in this reduced aggregation in the final carrier-based DPIs.

From this study, it was observed that the flow property improved from passable to excellent flow after lyophilization. The Carr's index of the PTX-PLM-DPI powders before lyophilization was found to be passable (24.19) which converted to 7.93 indicating conversion to an excellent flow property upon freeze drying. Similarly, Hausner's ratio changed from passable (1.32) to excellent (1.08) on lyophilization. The angle of repose showed a marked change with the flow property increasing from poor flow (55.14°) to excellent flow property (28.19°) after lyophilization. This study showed that freeze drying of the collected PTX-PLM-DPI powders is essential for increasing the flow property of the PLMs. This flow property is required for their efficient delivery to lungs as DPI through the device. Pure PTX is a crystalline material. In PXRD analysis, the characteristic PTX peaks in the PTX-PLM-DPI powder formulation were found to be reduced in intensity indicating a slight reduction in the crystallinity or amorphization of the drug during the PLM formulation [28]. This may further result in the increase in solubility of the drug that may result in better drug release after delivery to lungs.

In the HPLC method development, mobile phase ratio was selected as acetonitrile/water (75:25 *v/v*) and flow rate at 1 mL/min was found to be optimized. It was further calibrated by determining the linearity, precision, accuracy, interday and intraday variation. The solvent system of acetonitrile and water was varied at different ratios with decreasing polarity of 50:50, 60:40, 65:35, 70:30 and 75:25. Based on the height, sharpness and width of the peaks, and retention time, mobile phase ratio with 75:25 *v/v* acetonitrile/water was selected as the optimized solvent system. The decrease in noise and increase in sharpness of peak and retention time were due to the decrease in the polarity of the system. The flow rate was similarly optimized by varying between 0.5 mL/min, 1.0 mL/min and 1.5 mL/min. By analyzing the noise, height, sharpness, width of the peaks and the retention, a flow rate of 1.0 mL/min was selected for further studies. The calibration curve was prepared with serial dilution from 3, 6, 12, 25, 50, 100 and 200 $\mu\text{g/mL}$ and linearity was observed with $R^2 = 0.997$ and $y = 93,443x + 13,854$. As high as $95.64 \pm 1.4\%$ *w/w* of drug

entrapment was observed. The high entrapment might be due to the high lipophilicity of PTX that helped in its easier entrapment in the lipid layers [29]. The higher entrapment of the formulation might also be due to the long hydrocarbon chains of the phospholipid (SPC) that has been used in a higher amount (9:1) in the formulations. A high content of lipids might result in better vesicle formation. There are reports suggesting that SPC has a tendency to accommodate a higher amount of drug [30]. The liposomes constituted from the PTX-PLM-DPI showed a sustained release of the drug showing 28–30% drug release in 24 h in the simulated lung fluid (PBS 7.4 + 0.05% SLS). The release profile followed the entire kinetics models. The Higuchi model had the greatest match with the highest correlation values, demonstrating diffusion regulated release. This was confirmed by comparing the 'n' value in the Korsmeyer–Peppas model, which revealed that the drug release mechanism is diffusion regulated because 'n' is smaller than 0.5. Initial burst release may be due to release from the surface caused by the dissolution of the free drug absorbed on the surface of the liposomes, whereas controlled release may be caused by the gradual diffusion of the drug from the vesicular structure. The drug contained within the vesicular structure released slowly and required a prolonged period to do so. This might enhance the biopharmaceutical property of the formulation and may provide a controlled therapy with reduced toxicity due to the slow release [31,32].

Delivered dose uniformity (DDU) of threehard gelatin capsules containing PTX-PLM-DPI powder was measured using a dose uniformity sampling apparatus (DUSA) followed by HPLC analysis. The concentration of drug delivered in each actuation of the inhaler and their average concentration depicts dose uniformity of the formulation. From the results, it can be observed that the dose delivered by the device is uniform when examined by loading the formulation in threehard gelatin capsules. The average drug delivered from the three capsules is 500.43 µg. The peak area of particles deposited on each plate based on their aerodynamic diameter was analyzed in an Anderson Cascade Impactor followed by HPLC analysis [8,33]. From this study, it was observed that the fine particle fraction (FPF) was $50.86 \pm 2.80\%$, which is suitable for effective delivery to the lungs [9]. These particles would likely settle in the lower region of lung airways due to sedimentation (0.5–5 µm), whereas Brownian motion (0.5 µm) would cause stage 7 particles to settle in the alveoli [25]. However, the concentration of drug retained in the pre-separator is substantial with almost 22%, indicating scope for reducing this loss further by decreasing the size of the PLMs. The aerodynamic properties of the inhaled particulates, their physicochemical properties, efficacy of the inhaler device, the inhalation behavior of the patient and lung physiology determine the successful delivery of DPIs into the lung [17]. PTX-PLM-DPI powder preparation using SPC and cholesterol have the added advantage of achieving a better aerodynamic diameter as well as good flow due to the anti-adherent property of these phospholipids; moreover, they are regarded as safe [16,17]. This might have helped in reducing the cohesiveness associated with the particles and result in better flow and deposition.

PTX has the potential to hyper-stabilize the microtubule and facilitate the assembly of alpha and beta tubulin subunits resulting in the extension of the tubulin polymer, thereby disrupting the microtubule networking to cause mitosis and cell proliferation and finally leading to cell apoptosis [34]. As shown in the results, reconstituted liposomes of PTX-PLM-DPI exhibited profound dose-dependent cytotoxicity compared to free PTX suspension. Passive diffusion can be the uptake pathway for free PTX suspension, whereas proliposomes can be taken up by the endocytic pathways of the cell [9] and have better absorption. Sustained release of the drug from the proliposomes resulted in a better cytotoxic effect than free drug suspension with IC_{50} values of 46 ± 0.87 ng/mL. Better efficacy and lower IC_{50} values were observed compared to a free drug suspension, confirming the effective nature of proliposomes.

4. Materials and Methods

4.1. Materials

Paclitaxel (PTX) was received from Fresenius Kabi Oncology Ltd., Gurgaon, Haryana, India as a gift sample. Lactose monohydrate (LMH), mannitol, phosphate buffers, acetonitrile and acetone were obtained from MERCK & Co Inc., Rahway, NJ, USA. Soya phosphatidylcholine (SPC), cholesterol, Tween 80 and dimethyl sulfoxide were obtained from HiMedia Lab Pvt Ltd., Thane (W), Maharashtra, India. Ethanol, chloroform, HPLC grade acetonitrile and acetone were obtained from Rankem Chemicals, Gurugram, Haryana, India.

Human pulmonary cell lines A549 were obtained from National Centre for Cell Science (NCCS) Pune, Maharashtra, India. Ham's F-12 K medium and Pen-Strep were purchased from HiMedia Lab Pvt Ltd., Thane (W), Maharashtra, India. Fetal bovine serum (FBS) was obtained from Gibco Inc., 347 5th Avenue, New York, NY, USA.

4.2. Methods

4.2.1. Preparation of PTX-PLM-DPI

PTX-PLM-DPI was prepared using the film deposition on carrier method [35]. LMH and mannitol were used as the carbohydrate carriers. Different formulation ratios of lipid phase to carbohydrate carrier (mannitol or LMH) were used; 1:5, 1:10, 1:15, 1:20 and 1:25 *w/w*. Ethanol and chloroform in the ratio 1:1 was used as a solvent system and the amount of PTX used was 10 mg. Briefly, the lipid phase was prepared by dissolving SPC and cholesterol in an appropriate volume of the solvent mixture. The resultant lipid solution with or without PTX was then poured onto the carrier which was previously added in around bottom flask (RBF). The RBF was then attached to the rotary evaporator. The organic solvent was then evaporated by rotary evaporation (BUCHI Rotavapor R-3, BUCHI India Pvt. Ltd., Mumbai, Maharashtra, India) at 40 °C for one hour under negative pressure. After 1 h of evaporation, negative pressure was released and dry PLM powders were obtained in the RBF. The RBF was then detached from the rotary evaporator and dry PLMs were collected, lyophilized and stored [35,36].

The PTX-PLM-DPI were then optimized based on different variables: evaporation time of organic solvent (30–60 min), ratio of lipid phase to carrier (1:5 to 1:25), ratio of phospholipid to cholesterol (3:1–9:1) and different carbohydrate carrier (LMH or mannitol).

4.2.2. Evaluation of PTX-PLM-DPI Powder

PTX-PLM-DPI powders were evaluated for percentage yield, flow property, size distribution, X-ray diffraction (XRD), surface morphology, entrapment efficiency, in vitro drug release and delivered dose uniformity (DDU) for dry powder inhaler and it was an in vitro lung deposition study.

The PLM yield was calculated using the following formula [8]:

$$\text{Percentage yield} = \frac{\text{Total weight of PLM}}{\text{Total weight of drug} + \text{total weight of added material}} \times 100\%$$

The bulk and tapped densities, Carr's index, Hausner's ratio and angle of repose were determined to measure the flow property of the PLM-based DPI powders, pre- and post-lyophilized [37]. The bulk density and tapped density of the PLM powder sample were calculated using a graduated measuring cylinder (100 mL) in a mechanical tapped density apparatus (ROLEX). A total of 20 g of the PLM-based DPI powder was transferred to a measuring cylinder (100 mL). The initial volume (V_1) of the powder was recorded (42 mL) and the cylinder was fixed in the mechanical tapped density apparatus. The final volume (V_2) of the powder in the cylinder was recorded after tapping for 5 min with a tapping rate of 100 ± 5 taps/min.

Bulk density was calculated using the formula:

$$\text{Bulk density} = \frac{\text{Weight of powder}}{\text{Volume of powder}}$$

Tapped density was calculated using the formula:

$$\text{Tapped density} = \frac{\text{Weight of the powder}}{\text{Minimum volume of the powder after tapping}}$$

Utilizing the funnel approach, the angle of repose was computed as the tan inverse of the angle formed by the height (h) and the radius (r) of the base of the conical powder pile. The Hausner ratio was calculated as [37]:

$$\text{Hausner ratio} = \frac{\text{tapped density}}{\text{bulk density}}$$

Similarly, the Carr's Index was calculated as [38]:

$$\text{Carr's index}(\%) = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100\%$$

The morphology of the surface of PTX-PLM-DPI powder (pre- and post-lyophilization) was analyzed using SEM. The powder samples were scattered across aluminum microscope stubs of the apparatus and painted with gold. The surface of the sample was next examined with a scanning electron microscope (SIGMA VP FESEM, ZEISS, White Plains, NY, USA) to determine its morphology. A Phillips X'Pert Pro Powder XRD that used copper radiation and has a diffracted beam monochromator was used to record the X-ray powder diffraction (XRD) pattern of pure PTX and PTX-PLM-DPI powders. With a step size of 0.02 degrees and a step length of one second, the intensity of the diffraction was measured at an angle of 2θ with a range of 10 to 90 degrees. The voltage generator was set at 40 kV, and the current generator to 30 mA.

4.2.3. Reconstitution to Liposomes and Its Physicochemical Evaluation

Liposomes were constituted from the PTX-PLM-DPI powders by hydrating with PBS 7.4 as dispersion medium. A total of 100 mg of powders was dispersed in 5 mL of PBS 7.4 followed by ultrasonication (PCI Analytics, 3-SL-100, PCI Analytics Pvt. Ltd., Thane (W), Maharashtra, India) for 15 min. The average particle size and polydispersity index (PDI) of the liposome vesicles suspension in PBS pH 7.4 after diluting 10 times in milli-Q water were evaluated using a Malvern Zetasizer (Zetasizer, Malvern Nano S90, Malvern Instruments Ltd., Malvern, Worcestershire, UK) by the dynamic light scattering method (DLS). The analysis was performed at 25 °C in triplicate at a 90° scattering angle [38]. The size and morphology of liposomal vesicles surface was analyzed using TEM (JEM-2100 PLUS (HR), JEOL Ltd., Akishima, Tokyo, Japan). The liposomal vesicles were placed on carbon-coated copper grid before being stained negatively with 1% *w/v* photo tungstic acid. Images were then taken and examined.

4.2.4. HPLC Method Development

Drug concentrations for entrapment and release studies were measured by an HPLC system from Waters (Milford, MA, Waters Corporation, Milford, Massachusetts, USA) fitted with a dual pump (Waters 515), UV detector (Waters 2489) using acetonitrile/water (HPLC grade) as the solvent system. Nova-Pak[®] HR C18 60 Å (3.9 × 300 mm Prep Column) with a particle size 6 µm column was used which was held at 25.0 ± 0.5 °C. A total of 20 µL of the sample was injected manually. Empower chromatography software was used for the data collection, analysis and reporting.

Various ratios of acetonitrile/water (50:50, 60:40, 65:35, 70:30, 75:25 and 80:20 *v/v*) system at a flow rate of 1 mL/min was evaluated. The optimum solvent system was selected by considering the sharpness, height and width of the peak. The isocratic method was developed using acetonitrile/water (75:25 *v/v*) as a solvent system at three different flow rates, 0.5 mL/min, 1 mL/min and 1.5 mL/min. Peak sharpness, peak height and peak width were taken into consideration to optimize the flow rate. Linearity was determined by preparing a calibration curve with serial dilutions 3–200 µg/mL using acetonitrile/water

(75:25 *v/v*) as the mobile phase at the flow rate of 1.0 mL/min at 230 nm wavelength. A calibration curve was obtained by plotting peak area vs. concentration in µg/mL and the R^2 was determined [39–41].

4.2.5. Entrapment Efficiency

The entrapment efficiency of optimized PTX-PLM-DPI reconstituted liposomes was studied using the standardized HPLC method following the study of Khan et al., 2020. A total of 2 mL of the reconstituted liposomes was diluted with methanol and centrifuged at 15,000 g rpm for 30 min in a cooling centrifuge (REMI, Goregaon (E), Mumbai, Maharashtra, India) at 4 °C and the drug that remained untrapped in the supernatant was measured. The supernatant was assayed using mobile phase constituting acetonitrile and HPLC grade water (75:25 *v/v*) with 20 µL volume of injection and at a flow rate of 1 mL/min to detect the amount of the untrapped drug. The detection wavelength was set at 230 nm. The concentration of PTX in the supernatant was determined from the calibration curve [37,42]. The following formula was used for determining the *entrapment efficiency* [37]:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total drug loading} - \text{untrapped drug}}{\text{Total drug loading}} \times 100\%$$

4.2.6. In Vitro Release of Paclitaxel

The *in vitro* paclitaxel release from its liposomal formulation (optimized PTX-PLM-DPI) was studied by dialysis bag method using phosphate-buffered saline pH 7.4 (PBS 7.4) with 0.05% SLS as the simulated lung fluid, at 37 °C. Prior to the experiment, the dialysis membrane (12,000–14,000 KDa molecular weight cut-off) was activated by soaking in the release fluid overnight. A total of 1 mL of reconstituted liposome was taken in the dialysis bag in 200 mL release media and magnetically stirred at 200 rpm at 37 ± 2 °C. At suitable time intervals (0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 1440 min), 2 mL of the sample was withdrawn and the drug content was estimated using the standardized HPLC method [31].

4.2.7. Release Kinetics

To predict the most appropriate pharmacokinetic model followed by the drug release profile, release data from this study were fitted to a variety of kinetic equations (zero order model, first order model, Higuchi model and Korsmeyer–Peppas model). The '*n*' value in the Korsmeyer–Peppas model denotes the drug release mechanism from the formulation [31].

4.2.8. In Vitro Evaluation of Aerosol Performance by Cascade Impactor

An eight-stage, non-viable Anderson Cascade Impactor (Copley, Nottingham, NG4 2JY, Copley Scientific Ltd. Nottingham, NG4 2JY, UK) was used to evaluate the *in vitro* inhalation properties of the prepared PTX-PLM-DPI powders. The Anderson Cascade Impactor (ACI), equipped with a USP induction port (IP) and pre-separator (PS), was coupled to a critical flow controller (CFC).

4.2.9. Delivered Dose Uniformity (DDU)

The uniformity of the delivered dose was measured using the Dose Uniformity Sampling Apparatus (DUSA) (Copley, Nottingham, NG4 2JY, Copley Scientific Ltd. Nottingham, NG4 2JY, UK) equipped with a rubber mouthpiece adapter, vacuum pump and flow meter (DFM 4). A total of 25 mg of PTX-PLM-DPI powder was filled into three hard gelatin capsules (size 3) under controlled environmental conditions. For each trial, the PTX-PLM-DPI powder-filled capsules were actuated at a constant flow rate of 60 L/min into the ACI using a Rotahaler® device. The dose accumulated was collected, dissolved in ethanol and analyzed using the standardized HPLC method at 230 nm and 1 mL/min flow rate using mobile phase constituting acetonitrile and water (75:25 *v/v*) [8,17].

4.2.10. In Vitro Lung Deposition Study

A constant flow rate (60 L/min) was set in ACI with the flow meter (Copley Scientific Ltd. Nottingham, NG4 2JY, UK) that maintained the flow throughout using the vacuum pump (Copley). A total of 25 mg of dry powder formulation was packed into three hard gelatin capsules (size 3) in a laboratory setting. The capsules were taken in the Rotahaler[®] inhaler device (38 & 39, Girija Industrial Estate, Sativali, Vasai (E) Thane, India) and attached to the induction port with the help of a rubber mouthpiece adapter. Using a Rotahaler[®] inhaler device, the formulation was actuated into the ACI at a consistent flow rate of 60 L/min for every experiment. After the aerosolization tests, the ACI was dismantled and the powder collected at various stages was quantified in ethanol using a Waters HPLC consisting of a UV detector at 230 nm and a flow rate of 1 mL/min. Acetonitrile and water was used as the mobile phase at 75:25 *v/v* [10,43]. The mass median aerodynamic diameter (MMAD) was determined, which is the aerodynamic diameter below which 50% of particles remain [44,45]. The fine particle fraction (FPF) was determined using the following formula [9]:

$$FPF = \frac{\text{Drugs in stages 2-7}}{\text{Drugs in all stages}} \times 100\%$$

4.2.11. In Vitro Cytotoxicity (MTT Assay)

The human lung adenocarcinoma cancer cell lines A549 were cultured in Ham's F-12 K culture medium having 10% fetal bovine serum (FBS) along with penicillin and streptomycin. The viable cell percentage was determined by MTT analysis. Then, 1.8×10^6 cells/well were seeded into 96-well plates and incubated for 2 h to allow them to attach to the well surfaces. After that, the free drug suspension and reconstituted PTX-PLM-DPI liposome treatments were given in triplicate and incubated for 24 h. The MTT solution was then added, and the culture was kept in the incubator for another 4 h at 37 °C. After decanting the supernatant, the formed formazan crystals were dissolved by adding 200 μ L of DMSO. The 96-well plates were then put in a microplate reader so the absorbance at 570 and 655 nm could be calculated [18].

$$\text{The \% cell viability} = \frac{\text{Abs}_{570} \text{ of treated cells}}{\text{Abs}_{570} \text{ of untreated cells}} \times 100$$

The IC₅₀ value was calculated from % cell viability vs. concentration graph.

5. Conclusions

From this study, it may be concluded that an efficient PLM-based DPI system with adequate flow properties and reduced aggregation can be prepared using the film deposition on carrier technique followed by freeze drying. The PTX-PLM-DPI system showed adequate aerodynamic profiles and in vitro deposition which may result in enhanced and site-specific delivery into lungs in vivo. The formulations showed enhanced and controlled in vitro cytotoxicity effects in A549 human lung cancer cells. Overall, although additional in vivo studies of the DPIs for its therapeutic effect in lung cancer are required for further exploration, this study highlights the potential of PLM-based DPI by freeze drying as a stable, safe and free-flowing dry powder inhalation system for the enhanced delivery of PTX for potential lung delivery.

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Abbreviations

DPI	Dry Powder Inhaler
IC ₅₀	Half Maximal Inhibitory Concentration
PLM	Proliposomes
EEG	Excipient-enhanced particle growth
PTX	Paclitaxel
LMH	Lactose monohydrate
SPC	Soya phosphatidylcholine
FBS	Fetal Bovine Serum
RBF	Round Bottom Flask
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
XRD	X-ray diffraction
DDU	Delivered dose uniformity
PDI	Polydispersity index
DLS	Dynamic light scattering
PBS	Phosphate-buffered saline
ACI	Anderson Cascade Impactor
IP	Induction Port (IP)
PS	Pre-separator
CFC	Critical Flow Controller
DUSA	Dose Uniformity Sampling Apparatus
MMAD	Mass Median Aerodynamic Diameter
FPF	Fine Particle Fraction
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Di methyl Sulfoxide

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