A Thesis

entitled

Formulation and Evaluation of Paclitaxel-loaded Nanoemulsion for Pulmonary Administration

By

Ahmed S. Fahad

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Pharmaceutical Sciences

Industrial Pharmacy

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August 2016
An Abstract of

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Purpose: The purpose of this study was to develop and evaluate paclitaxel-loaded nanoemulsion (NE) for pulmonary delivery.

Methods: Based on composition, ternary phase diagram, solubility, clarity, and ease of NE formation several components were screened in trial formulations. Subsequently Labrafil® M2125CS was identified as the oil, Cremophor® RH 40 was finalized as the surfactant, and polyethylene glycol 400 was used as the co-surfactant. The final formulation had surfactant/oil blend in the ratio of 2:1. Paclitaxel was added to this mixture that was then mixed with an aliquot volume of water to prepare a translucent nanoemulsion. The formulation was evaluated for particle size, zeta potential, pH, TEM, DSC, PLM, in vitro drug release, rheology studies and conductivity. The drug entrapment efficiency was determined by HPLC. The stability and sterility of the paclitaxel (PCL) loaded formulation were evaluated.

Results: Clear and stable oil-in-water nanoemulsion that contained entrapped paclitaxel
formed immediately when formulation ingredients were mixed using a vortex mixer. The average particle size of the nanoemulsion was approximately 20 nm, the zeta potential was around 0 mV, the pH was 5 ± 0.002, and the conductivity was 144.3 ± 0.5 μSiemens/cm. The drug entrapment efficiency was 88 ± 0.001 %.

**Conclusion:** The results obtained from this study indicate formation of stable paclitaxel-loaded nanoemulsion that can be potentially used in pulmonary delivery.
Dedicated to my family
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List of Abbreviations

APIs…………………. Active pharmaceutical ingredients

BCS…………………. Biopharmaceutical Classification System

CD……………………Cyclodextrin
CFC………………….Chlorofluorocarbon
CNS………………….Central Nerves System

DI Water………………De-ionized Water
DLS……………………Dynamic Light Scattering
DPI……………………Dry powder for inhalation
DSC……………………Differential scan calorimetry

ELS……………………Electrophoretic Light Scattering

FDA…………………. Food and Drug Administration

GRAS………………. Generally Regarded As Safe

HFA…………………. Hydrofluoroalkane
HLB…………………..Hydrophilic-Lipophilic Balance
HPLC………………..High Performance Liquid Chromatography

LV……………………Liquid ventilation

MDI…………………. Meter does inhale
MH Agar……………..Müller-Hinton Agar
MMAD………………Mass media aerodynamic diameter

O/W ………………… Oil-in-Water

PBS…………………. Phosphate Buffered Saline
PCL ………………….. Paclitaxel
PEG……………………Polyethylene glycol
PFC…………………..Perfluorocarbon
PLA…………………. Poly lactic acid
PLM………………….Polarized Light Microscopy
pMDI………………..Pressurize metered does inhaler
PTH………………….Parathyroid hormone
SD ................ Standard deviation
SE .......................... Standard error
SLN .......................... Solid lipid nanoparticles

TEM .......................... Transmission Electron Microscope

W/O .......................... Water-in-Oil
Chapter 1

Introduction

Several factors favor pulmonary drug delivery such as when treating lung diseases (e.g., asthma, chronic bronchitis) over other routes of administration for certain drugs (e.g., corticosteroids) due to their systemic side effects. Using the pulmonary route to deliver drugs for local treatment of lung diseases is widely followed to produce localized therapeutic effects inside the lungs [1]. This route of drug administration allows a quick onset of action, high drug concentration at the site, lowers dose required to be administered (20-10% of the amount given orally), improves local drug release at the disease site, and bypasses hepatic first-pass metabolism [2]. Currently, more than 65 inhaled products and 20 active substances are marketed for the treatment of lung diseases [3]. In the future inhalable medications may be available for gene therapy and to deliver therapeutic proteins and polypeptides.
1.1. Design considerations

1.1.1 Anatomy of The Respiratory System

Apart from local effects within the respiratory tract, the lungs can serve as a route of administration to produce systemic absorption due to the fact that it has high surface area for absorption (140 m²) and thin (0.1-0.2 µm) mucosal membrane [4], [5]. The absorptive area in the lung is mainly the alveolar epithelium which basically includes type I pneumocytes. The respiratory system is divided into three major areas: the oropharynx, the nasopharynx, and tracheobronchial pulmonary region. The airway circulation starts with nasal cavity and sinuses and the nasopharynx, oropharynx, larynx, trachea, bronchi, and bronchioles, alveolar ducts, and alveolar sacs (figure 1.1). The airway system is responsible for the filtration, humidification and warming the inspired air. The surface area provided by respiratory tissues for exchanging gases between air and blood is through 140m² of internal surface area. The tissue that conducts the gas exchange is called pulmonary parenchyma. It includes 130,000 lobules; each one having a diameter of about 3.5 mm and containing approximately 2200 alveoli. Additional alveoli are located on the walls of the alveolar ducts and carry out around 35% of the total gas exchange. The human lung contains approximately 300 million alveoli with a total volume of 4.8L and respiratory volume of 3.15L and air – tissue interface of 81m² [1].
1.2 Site of Aerosol Deposition in The Respiratory System

1.2.1 Factors Affecting Particle Deposition

Various physico-chemical properties, physiological, and anatomical factors affect deposition of aerosol particles in the bronchial tree. Four general parameters should be considered to evaluate the size and morphology of an aerosol particle:

1. Mass Median Diameter (MMD) is the diameter of the particles for which 50% w/w of particles have lower diameter and 50% w/w have a higher diameter.

2. Percentage of weight of particles with a geometrical diameter of less then 5µm.

3. Geometric Standard Deviation (GSD) is the ratio of the diameters of particles from aerosols corresponding to 84% and 50% on cumulative distribution curve of the weights of the particles.

4. Mass Medium Aerodynamic Diameter (MMAD) which describes the size and
morphology of the aerosols particles by considering their geometrical diameter, shape, and the density: \( \text{MMAD} = \text{MMD} \times \text{Density}^{\frac{1}{2}} \) [1].

1.2.2 Mechanisms of Particle Deposition in The Respiratory Airways

Three major mechanisms are responsible for particle deposition in the lungs: inertial impaction, sedimentation, and Brownian diffusion [1]. The deposition mechanism directly correlates with the particle diameter and determines the deposition the particles in a particular area of the respiratory airways [7].

1. Inertial impaction is the most important mechanism of aerosol particles deposition with MMAD of more than 5 \( \mu \text{m} \). When velocity and mass of the particles lead to an impact on the airway track as in the case of a bifurcation or further subdivision of the airway tube, the particles tend to settle in the upper respiratory airways. Partial blockade of the respiratory airways and changes in the direction of inspired air improve the chances for deposition through this mechanism. Hyperventilation a condition in which overbreathing patterns develop due to various reasons may significantly affect particle deposition via impaction [1].

2. Sedimentation occurs in the peripheral airways and involves aerosols with an MMAD from 1 to 5 \( \mu \text{m} \). This mechanism occurs due to the action of gravitational forces on particles and is proportional to the square diameter of the particle size (Stokes law). Therefore small particles are not affected by this mechanism. Particle motion is not considered as a factor that has an effect on this type of particle deposition. However breath holding has an impact on particle sedimentation and can improve deposition.
3. Brownian diffusion is a crucial deposition mechanism for particles with an MMAD of less than or equal to approximately 0.5µm. These particles move arbitrarily with gas molecules and collide against the airways walls. Around 80% of particles with an MMAD of less than or equal to 0.5µm are removed out during the exhalation process.

![Diagram of aerosolized drug particle pathways](image)

Figure 1.2 Pathway of aerosolized drug particles in the body [1].

### 1.2.3 Effect of Particle Size

The particle size of an aerosol has a crucial impact on its mechanism and site of deposition in the lung. Large size particles with diameters of ( >10 µm ) that come in contact with the upper airways tract are rapidly removed by mucociliary clearance.
Aerosols with particle diameter range from 0.5 to 5 µm deposit through sedimentation mechanism [1]. Aerosol particles that are intended to penetrate the lung was determined to be in the size range around 2 to 3 µm [8]. Particles with very small diameter may be exhaled before depositing in the lung, however holding the breath can prevent this. Extremely small diameter particles (< 0.1 µm) are not easily prepared, though they efficiently settle by Brownian diffusion mechanism. Nevertheless, researchers have not been able to confirm an exact geometrical diameter that results in deposition of inhaled particles because even large particles that have a porous internal structure can penetrate and deposit in the lungs [9].

1.2.4 Lung permeability

High permeability to water, gases, and to lipophilic materials is a characteristic feature of both alveolar epithelium and capillary endothelium. However, many hydrophilic materials, big size molecules, and ionic substances cannot pass through because of an effective barrier. Furthermore the alveolar type I cells limit the entry of molecules of size less than 1.2 nm diameter due to the presence of tight junctions, while endothelial junctions have larger gaps ranging from 4 to 6 nm [1]. Either by absorptive transcytosis or paracellular transport solubilized macromolecules can pass through the lung [10]. Proteins and small solutes cannot pass through normal alveolar epithelium. Large intracellular gaps within microvascular epithelium are more permeable and they allow proteins and all molecular sizes to enter systemic circulation. It has also been scientifically established that regular smoking and presence of pulmonary disease increases lung permeability [1].
1.2.5 Respiratory Clearance

Mucociliary clearance or a combination of mucociliary and alveolar clearance mechanisms are responsible for removing settled particles that have not entered the lung epithelium and other unwanted particles from entering the respiratory system [1]. The clearance mechanisms offers an important challenge that has to be overcome when formulating aerosol products [3].

1. Mucociliary Clearance

This is respiratory systems unique mechanism against materials that enter the respiratory tract from outside environment during breathing. The *mucociliary clearance* is an efficient self-respiratory cleaning beside other clearance mechanisms such as cough and alveolar clearance [1]. From the trachea to the terminal bronchioles, the ciliated epithelium is extended and covered by double-layered mucus blanket: a low-viscosity periciliary sol layer covered by a high-viscosity gel layer. The mucus is secreted by airway epithelial goblets cells and submucosal glands. The upward movement of the mucus clears the trapped insoluble particles toward the pharynx from where it can go into the gastrointestinal tract [3]. The efficiency of mucociliary clearance is significantly diminished in respiratory diseases like asthma and cystic fibrosis, [11].

2. Alveolar Clearance

Absorptive and nonabsorptive clearance mechanisms clear deposited particles from the terminal airways [12]. The absorptive mechanism involves either direct penetration to the epithelium cells or uptake and clearance by alveolar, interstitial,
intravascular, and airway microphages. The nonabsorptive mechanism carries 
particles from the alveoli to the ciliated area from which the mucociliary 
clearance process further removes the particles from the conducting airways [1].

1.3 Advantages of pulmonary drug delivery for treatment of respiratory and local 
diseases

For many years inhaled drugs have been used for treatment common respiratory diseases 
such as asthma, chronic obstructive pulmonary disease (COPD) and chronic bronchitis. 
Several advantages of inhaled drugs over systemic drug delivery are explained in Table 1 [13].
Table. 1 advantages of pulmonary drug delivery for treatment of respiratory and systemic diseases.

<table>
<thead>
<tr>
<th>Respiratory diseases</th>
<th>Systemic diseases</th>
</tr>
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<tr>
<td>Reduce risk of systemic side-effects</td>
<td>A noninvasive “needle free system”</td>
</tr>
<tr>
<td>Rapid onset of action</td>
<td>Compatibility with wide range of substances range from small to very large molecules [14, 15]</td>
</tr>
<tr>
<td>Avoid harsh gastrointestinal environment and hepatic first-pass metabolism</td>
<td>Enormous absorptive surface area for absorption and highly preamble membrane in the alveolar area [10]</td>
</tr>
<tr>
<td>Ability to deliver high drug concentration to the site of disease</td>
<td>Less harsh environment, low enzymatic activity and bypass hepatic firs-pass metabolism</td>
</tr>
<tr>
<td>Achieve therapeutic effect at a fraction of the systemic dose</td>
<td>Prolong residence time due to slow mucociliary clearance mechanism [16]</td>
</tr>
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<td>Sustained release effect</td>
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1.4 Pulmonary drug delivery formulations

Pulmonary delivery is commonly used for treatment or prophylaxis of respiratory airways diseases and for systemic absorption of drugs delivered to the respiratory tract [17].
Aerosol formulations are stable dispersions of solid materials and liquid droplets in a
gaseous phase [18]. The most common pharmaceutical aerosol formulations are: jet or
ultrasonic nebulizers, metered dose inhalers (MDIs), and dry powder inhalers (DPIs)
[19].

1.4.1 Nebulizers
Relatively large volumes of drug solutions or suspensions of drugs that are not easily
formulated as MDIs or DPIs can be delivered successfully to the lung using nebulizers. In
addition, for high dose protein drugs in which the dose is very large to be delivered as
MDIs or DPIs, nebulizers can be used to achieve respiratory therapeutic levels of protein
based drug [20].
There are two type of nebulizers available in the market:

- Jet Nebulizers: utilize compressed gases to produce aerosols droplets
  within respiratory range and they are commonly used in clinical
  applications.
- Ultrasonic Nebulizers: use ultrasonic energy to produce aerosol form
  liquid [20].

1.4.2 Metered does inhalers (MDIs)
A pharmaceutical MDI is a pressurized dosage form that can deliver therapeutic agents
into the respiratory system. MDI components include active ingredients in a solution or
suspension, propellant system, and at least one liquefied gas in a container under pressure that is sealed with a metering valve. After actuation, the MDI delivers metered doses of the therapeutic agent in the form of an aerosol, which is administered by using a suitable oral or facial adapter to reach the mist ultimately to the pulmonary tract [17]. MDIs may contain up to 200 doses in a small cylinder shaped container and drug delivery is highly reproducible [21]. Major advantages of MDIs include portability, low cost, non-invasive process and disposability. On the other hand, MDIs have few limitations, that are mostly related to the drug delivery device and components used in the system [17]. The first propellants developed for MDIs produced high velocity droplets that can exceed 30 m/s resulted in high drug loss in oropharyngeal areas [21]. However, after introduction of HFA based formulations the lung deposition of drug particles improved to reach up to about 53% of delivered dose in a solution type aerosol formulation [22].

1.4.3 Dry powder for inhalation (DPIs)

DPIs are aerosol pharmaceutical dosage forms that deliver medicaments as fine particles. The active pharmaceutical ingredients are either pre-loaded or filled into hard capsules or foil blister during manufacture [22]. DPIs have several benefits over nebulizers and MDIs particularly in the administration of peptide and protein base medications to the respiratory system [17]. DPIs are portable, propellant free, have low cost, easy to use and operate, and because the ingredients are loaded as a dry state inside the device they have the highest stability of all aerosol formulations [23]. Moreover, novel and new generation of DPIs such as from Vectura and Inhalace™ from Nektar have improved systemic delivery of DPI formulations [24]. However, DPI formulations are limited
because of being prepared using hygroscopic components even when processed to reduce aggregation through use of micronized compounds [19]. Further research is needed in this area to overcome this issue [17].

1.5 Advances in pulmonary drug carriers

1.5.1 Liposomes

Liposomes are most extensively investigated carriers for pulmonary drug delivery because they can be fabricated from pulmonary surfactants and endogenous phospholipids and as a result they are biocompatible, biodegradable, and relatively non-toxic [1]. Liposomes are produced in broad size range and both hydrophilic as well as hydrophobic drugs can be incorporated, these drugs include cytotoxic agents, antimicrobial and antiviral drugs, asthma medications and drugs intended for systemic absorption [25]. Researchers have been investigating liposomes as drug carriers for 30 years, and many therapeutic agents have been incorporated into liposomes as a strategy to develop pulmonary drug delivery leading to animal and human studies [26]. Liposomal aerosols have several advantages over other traditional pulmonary aerosol carriers, which are: extended duration of release, no significant local irritation, minimal toxicity and improved drug stability. A recent application of liposomes is the pulmonary delivery of macromolecules such as DNA to the respiratory system via inhalation [27].

1.5.2 Polymeric Microspheres and Nanospheres

1.5.2.1 Microspheres

The term Microparticle is used to describe three major categories: microspheres
(uniformly distributed spheres), microcapsules (central core surrounded by an outer layer of polymeric membrane), and particles with unidentified shape [28]. They have particle size between 1 to 999 µm, and can be prepared using a variety of natural and synthetic polymers. Hydrophobic as well as hydrophilic drug molecules can be incorporated inside microspheres in which they improve physiochemical stability with the drug protected from enzymatic degradation and provide sustained drug release of the incorporated molecule. Several polymers have been used in fabricating microspheres: polylactic acid (PLA), albumin, polyglycolic co-lactic acid (PLGA), etc. [1]. Albumin microspheres are biocompatible and biodegradable so they may be appropriate as a carrier for pulmonary drug delivery [29]. Morphological requirements such as shape and size can be easily modified and adjusted to meet certain parameters thorough microspheres drug carriers [1]. Furthermore, particle size of the drug incorporated microspheres is less sensitive to hygroscopic growth within the lung [30]. Mucoadhesive properties of some polymers like highly viscous hydroxypropylcellulose (HPC) can be utilized for the formulation of microspheres to enhance pulmonary absorption by increasing lung retention time and reducing mucociliary clearance within the lung [31]. Microspheres can play an important role in the delivery of drug molecules to target alveolar macrophages to treat certain conditions, such as tuberculosis. Moreover, an in vitro study suggested that microspheres can target alveolar macrophages without potentially activating an inflammatory response [32].

1.5.2.2 Nanoparticles

Nanoparticles share the same features with microparticles, drug molecules are either
being uniformly distributed within the matrix surface or loaded inside the nano-carrier core utilizing natural or synthetic biodegradable polymers, providing enzymatic protection and enhancing bioavailability of therapeutic agents through an extended release profile [33]. Nanoparticle based drug carriers and have been used in drug delivery and diagnostic purposes [34]. Mucoadhesive polymers can be utilized in fabricating nanoparticles to enhance bioavailability by increasing the retention time of nanoparticles within pulmonary mucosa and minimizing the action of mucociliary clearance mechanism [1]. However, in vitro/vivo studies have demonstrated some limitations of polymeric micro- and nanoparticles included difficulties in producing microspheres of size below 10 um, evidence of hemorrhage due to microsphere clustering within the lung tissue, and significant inflammatory response leading to cytotoxic effect [30, 35].

1.5.3 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) offer an alternative to conventional colloidal systems such as liposomes and polymeric micro and nanoparticles [36]. In fact, SLNs have advantages such as high safety and ability to scale-up production [36]. Drug molecules like Prednisolone® and Diazepam® have been successfully loaded in SLNs. Drug loading capacity of SLNs depends on factors like miscibility and solubility of the drug in the SLN lipid, physiochemical properties of the lipid solid matrix, and the polymorphic state of the lipid component within the SLNs [1]. Controlled release profile can be achieved using SLNs as a function of ratio of lipid/ surfactant concentration, and through modifying production parameters. Release of the drug can be modulated to achieve prolonged release up to 5 to 7 weeks [1]. SLNs dispersion can be nebulized or used as
solid powder in a DPI device for pulmonary administration [1].

1.5.4 Cyclodextrins

Cyclodextrins (CDs) first discovered in 1891 [37], are cyclic oligosaccharides consisting of six (α-cyclodextrin), seven (β-cyclodextrin), or eight (γ-cyclodextrin) glycosyl units [38]. They are also known as cycloamyloses, cyclomaltoses and Schardinger dextrins [39]. Cyclodextrins are produced by intramolecular transglycosylation reaction upon degradation of starch by cyclodextrin glucanotransferase (CGTase) enzyme [40]. The exterior surface of CD is hydrophilic due to hydroxyl groups, whereas the central cavity is hydrophobic [41], as a result CD solubilizes hydrophobic drugs into solution and delivers them to the cell membrane for absorption while remaining in the aqueous phase [42]. The major interest of using cyclodextrins in pharmaceuticals is its ability to form inclusion complexes with various hydrophobic drug molecules [43-47]. The complexation inside the CD cavity can include part of the drug molecule or the entire structure of the drug molecule [1]. β-cyclodextrin is the most commonly used cyclodextrin in pharmaceutical industry due to the fact that it is commercially available, relatively low priced, has a suitable cavity size, and efficient drug incorporation [41]. CDs can be incorporated with other carriers such as microparticles for further controlling the release of an incorporated drug [48]. Cyclodextrins are used for incorporation of drug molecules for pulmonary administration, they offer protection from enzymatic degradation, extend drug release profile, reduce frequency of administration, and minimize flocculation of drug concentration following single dose administration [1].
1.5.5 Aqueous and Nonaqueous Solutions and Suspensions

1.5.5.1 Aqueous solutions and suspensions

Aqueous aerosols have been studied extensively as a carrier for pulmonary delivery. An aqueous solution of morphine was used to investigate the possibility of systemic administration by pulmonary administration [49]. The result of aerosolized morphine delivery was approximately 100% bioavailability compared to intravenous infusion. In addition, insulin was administered by pulmonary route in healthy individuals [50]. When compared to subcutaneous insulin administration that showed onset in 50 to 60 minutes and hypoglycemic effect over 10 to 120 minutes, pulmonary insulin resulted in fast absorption within 7 to 20 minutes with rapid significant hypoglycemic effect 60 to 70 minutes. A colloidal suspension of beclomethasone dipropionate (Nanocrystal™) is used for respiratory distress syndrome in newborn babies [51]. In this study, a short duration
ultrasonic nebulization minimized throat deposition and increased respirable fraction of Nanocrystal® suspension of beclomethasone dipropionate than that achieved by propellant based commercially marketed product Vanceril®. According to this study, suspension of beclomethasone dipropionate improved delivery by increasing the amount of drug delivered to the site of action as well as by increasing the fraction of the discharged dose. Moreover, the study observed that a decrease in throat deposition might minimize some adverse effects related to long-term use of propellant based steroids such as candidiasis. Finally, the aqueous based formulations offer environmentally friendly products that are chlorofluorocarbon (CFC) free that are less complicated to formulate.

1.5.5.2 Nonaqueous solutions and suspensions

1.5.5.2.1 Solutions and suspensions

Liquid ventilation of neat F-octyl bromide (LV) was used to evaluate the treatment of acute respiratory distress syndrome and acute lung injury [52]. Perfluorocarbon (PFC) liquids introduced to the lung reopened the collapsed alveoli, facilitating gas exchange, and protecting the lung from side effects of conventional mechanical ventilation such as barotrauma or volutrauma. The study reported that Phase I and Phase II trials have improved the lung compliance, oxygenation status and anti-inflammatory effects [52-54]. Pulmonary drug administration of solid and liquid suspensions in PFCs have been reported to be highly effective formulation for the respiratory route [55, 56]. For treatment and inhibition of acute respiratory syndrome, exogenous surfactants have been used as carriers for antibiotics and corticosteroids as a solution or suspension [57, 58]. As long as there are no interactions between the drug and the surfactants that could
compromise pharmacological activity of the drug, the surfactants are effective drug carriers and have a high potential for pulmonary targeting when combined with mechanical ventilation [1].

1.5.5.2.2 Solid dispersed system (Dry powder)
MDI formulations have several limitations such as poor patient adherence and problems related to new propellant gases. On the other hand DPIs can overcome several limitations related to MDIs [59]. DPIs can be used to deliver vaccines such as measles vaccine to enhance stability by formulating the vaccine as a dry powder and minimize the risk of contamination by using syringes for administration [60]. Furthermore, calcitonin and parathyroid hormone PTH was delivered by the pulmonary route for treatment of bone disorders such as osteoporosis [61]. In this study, inhaled dry powder formulations of calcitonin and PTH demonstrated between 40 and 66% bioactivity and approximately 29% bioavailability when compared to subcutaneous injections, which can make dry powder delivery of proteins and peptides a possible approach in the near future.

1.5.6 Micellar Solutions, Emulsions, and Microemulsions
1. Micellar Solutions
Cyclosporine A (CysA) pharmacokinetics and delivery were examined by the respiratory or intravenous route in adults and young rats [62]. In this experiment, intratracheal (i.t.) administration of saline suspension of CysA was used, the obtained bioavailability was found to be 78.1 ±/6.9% with a plasma peak level at 30 minutes. Whereas the i.t. instillation of CysA with micelles forming surfactant Cremophor® EL, the bioavailability
for adult and rats was reported to be $77.4 \pm 7.2\%$ and $66.3 \pm 4.3\%$, respectively. The plasma level peak was attained after 5 minutes with the micellar formulation. In addition, ethanol solution of CysA delivered by aerosol showed bioavailability of $80.1 \pm 4.1\%$ and the plasma peak level at 20 minutes. Form the data it can be seen that the micelle formulation was the fastest among all formulations evaluated and the effect is attributed to the fact that micelles must have an impact on pulmonary permeability. In conclusion, pulmonary delivery can be utilized as an efficient route for delivery of CysA to limit autoimmune diseases and allergic reactions in transplantation procedure [62].

2. Microemulsion

Pulmonary drug delivery using emulsion or microemulsion formulations have been investigated in a few studies [63]. Pulmonary delivery of a microemulsion that contained water in hydrofluoroalkane HFA with nonionic fluorinated surfactant has been investigated [64]. Aerosolized form of reverse water in chlorofluorocarbon CFC micelles stabilized by lecithin and loaded with peptides have been also been studied [65]. This system is stable and efficient to deliver peptides and proteins to the respiratory tract. However, its use is limited because CFCs are banned from commercial products. These formulations have the potential to be modified to utilize ozone friendly propellants such as HFA, hydrocarbons or fluorocarbons in MDI applications [1]. Researches have studied reverse microemulsion stabilized by lecithin and utilizing propane and dimethyl ether as propellant gases [66]. These microemulsion based formulations were reported to be stable for more than 4 weeks at room temperature with internal aqueous diameter around $3 \pm 2$ micrometer and respirable fraction of approximately 36%. This study was the first that
used lecithin reverse microemulsion for lung delivery of polar drug molecules [1]. In conclusion, using reverse microemulsion (versus micelles) might enhance solubility and increase drug loading of a wide range of polar drug molecules. Aerosolized microemulsion delivered using MDIs are still under extensive evaluation.

1.5.7 Transition to CFC free inhalers

1.5.7.1 Aerosol generators

Aerosol formulations in which liquefied gases under pressure is placed inside containers, the aerosolization of the drug particles is governed by the vapor pressure at the temperature of use [67]. Aerosol generators allow to deliver predetermined amount of drug into the lungs [1]. Extensive studies have been done to improve targeting of the drug using these devices, with steady technical improvements of over the last ten years [68]. Aerosol generator devices are comprised of aerosol generator, drug powder, auto-activated aerosols, and spray diffuser [69]. Pressurized metered dose inhalers (pMDI) are the most common inhalers. They represent approximately around 80% of prescribed aerosol inhalers, although they are complicated to use, in which the patient coordinates between activation of the dose and inspiration (hand – mouth coordination) to maximize drug delivery and minimize drug loss. However, the main advantage of the metered dose inhaler system is that it is convenient for outpatient use, and for this particular advantage, they are the most popular inhaler type to deliver therapeutic substances to the respiratory system [1]. Chlorofluorocarbons have been used as propellants in pressurized inhalers for lung administration [70], due to the fact that they are non toxic, stable, nonflammable, and technically suitable to be incorporated for pressurized aerosol formulations. On the
other hand, having chlorine molecules within the CFC formulation and because their long half time in the atmosphere approximately 75 to 120 years, researchers investigated alternative propellants to minimize the damage to the ozone-layer by the harmful effects of CFCs [71]. Furthermore, the international community signed the (Montreal Protocol) which imposes limitations in the production and use of CFCs and substances that deplete the ozone-layer in 1989 [72]. The Hydrofluoroalkanes (HFAs) were the potential alternative propellants, which are chlorine-free and therefore, ozone-layer friendly [73, 74]. Extensive toxicological investigations have reported that HFAs are not toxic, not carcinogenic, not mutagenic, and normally cleared out from the body [75, 76]. HFA - 134a is rapidly absorbed in the systemic circulation and is rapidly eliminated with a half life of 5.1 minutes [77]. The first non-CFC pMDI was developed using HFA 227 as a propellant [1]. In addition, the reformulation process of CFC MDIs with HFA is a potential approach to improve system handling, industrial compliance, dosing, and reliable drug disposition in the lung [78, 79]. The new formulations should offer improvement in term of dose uniformity and increase in the percentage of fine aerosolized particles reaching deeper areas in the lung due to the fact that manufacturers have adopted new industrial technology and improved valves and actuators [80].

1.5.7.2 Reformulation

The new pMDIs utilizing HFA propellants have operation techniques and ingredients similar to those utilized in CFC propellant containing formulations [1]. The difference between the two formulations are in terms of modification of the composition of formula, the valve, coating in the inner side of the canister, and the industrial manufacturing
processes [81]. For instance, conventional surfactants used in CFCs formulation do not have enough solubility in HFAs [82, 83]. Furthermore, in pMDIs containing suspension formulation, changing the propellant affects the physical stability of the suspension and the solubility of the drug in the new propellant may be also effected in some cases [82, 84]. Three concepts can be examined for reformulation process: surfactant compatibility with the formulation, addition of extra ingredients to improve solubility of surfactant, or designing new surfactants [85]. All these three concepts may have to be tested for each drug individually for better understanding the substitution process that can differ form one molecule to another [80]. An example of currently marketed alternative to CFC aerosol is albuterol pMDI used for treatment of asthmatic patients that was reformulated as an HFA 134a albuterol pMDI [86]. Since this formulation showed a similar pharmaceutical performance when CFC was replaced by HFA a change in the label claim regarding the drug dose was not required in the new formulation [87].

1.6 Challenges in pulmonary drug delivery

1.6.1 Improving drug absorption

A fast pharmacological effect after pulmonary delivery of pharmaceutical ingredients can benefit local respiratory diseases as well as several systemic disorders [88]. For instance, the common rapid metabolic inactivation by hepatic first-pass metabolism after oral drug delivery can be eliminated by pulmonary drug delivery. Lung delivery can result in higher bioavailability for drugs used for treating central nervous system disorders leading to reduced dose and costs necessary to achieve therapeautic effect [88]. There are several drugs used for treating CNS disorders that are in phase II clinical trials for pulmonary
delivery, such as anxiety (alprazolam), Parkinson’s disease (apomorphine), analgesia (morphine, fentanyl), and migraine (loxapine, prochlorperazine). A new drug application was filed for inhaled dihydroergotamine (Levadex, MAP Pharmaceuticals, Mountain View, CA, USA) for treating migraine [89]. Additionally, in 2012, the inhaled dibenzodiazepine loxapine (Adasuve, Alexza Pharmaceuticals, MountainView, CA, USA) was approved in USA by the FDA for treatment of agitation associated with schizophrenia or bipolar I disorder in adults [88]. Nicotine can be formulated and optimized for pulmonary delivery to treat patients with nicotine addiction, because the commercialized products for smoking cessation that are currently marketed deliver nicotine or its substitutes (such as bupropion or varenicline) very slowly compared to inhalation of cigarette smoke [90]. Pulmonary delivery of proteins and peptides is another area that has attracted biopharmaceutical formulation and development. It has been reported that macromolecules have substantially high bioavailability across pulmonary epithelium, which can be up to 200 times higher than via any other non-invasive route into the body [91]. Insulin was one among many macromolecules that has been investigated for decades to systemically deliver it through the pulmonary route. However, Technosphere insulin (fumaryl diketopiperazine-based porous micron-sized carrier particles with recombinant human insulin; MannKind Corporation, Valencia, CA, USA) is the only insulin product still in clinical development [92]. Besides insulin, heparin (anticoagulant), calcitonin and parathyroid hormone (for treatment of osteoporosis), human growth hormone (growth hormone deficiency therapy), and erythropoietin (used in anemia) are being evaluated in clinical or preclinical trials for feasibility in pulmonary delivery [93, 94]. The pulmonary route is not feasible for delivering many drug
molecules. First, drug molecule solubility and permeability affect the rate and extent of pulmonary absorption. Second, formulation design strongly impacts the pharmacokinetic profile of drug concentration and elimination half life [95]. Highly soluble drug molecules such as albuterol can dissolve as soon as it comes in contact with lung fluid. On the other hand with poorly soluble drug molecules, dissolution can affect the absorption rate through pulmonary mucosa [88]. The availability of pulmonary fluid at the site of dissolution is an important factor. An estimation of total volume of lungs fluid volume is 15 to 17 mL in humans [10]. However, the proportion of the fluid volume that an aerosol particle is exposed is unpredictable [96], and the thickness of the lining layer along with volume of the lung lining fluid vary between the central and peripheral parts of the lungs. It has been stated that particles that landed in the upper part of the lung dissolves faster than those that deposited in the alveoli due to the presence of a large solid – liquid interface in the upper respiratory system [97]. In addition phospholipids present in the pulmonary surfactant can improve solubility of drug molecules within the lungs [98, 99]. In contrast, pulmonary surfactants can also compromise the stability of active biopharmaceuticals such as peptides and proteins [91]. To conclude, the rate and extent of the dissolution process is the first challenge for aerosol particles to be absorbed by lung tissue.

1.6.2 Prolonging drug action

In general, reduced frequency of administration and duration of therapy can enhance patient adherence. Physiological clearance such as mucociliary and microphage clearance are two main challenges to maintain sustained drug levels at the site of deposition [88].
Pulmonary mucus is a thick viscoelastic hydrogel layer up to 30 µm thickness, and it mainly contains water and glycoproteins (mucin) [100, 101]. The mucociliary clearance mechanism is highly effective and not specific, with 80 to 90% of inhaled material being cleared from the upper and central lung within 24 hours of inhalation [102, 103]. An important method to improve sustained drug levels at the site of action is to minimize clearance. Pulmonary surfactant is a non-cellular barrier that mainly consists of lipids (90%) and proteins (10%) [104], is the main barrier in the peripheral lung where mucus is not present in the healthy state [88]. Little is known about the nature of the interaction between surfactant components and inhaled particles at the molecular level [88]. Several researchers reported on particle–surfactant interactions and evaluated the clearance of particles into the surfactant layer [105], and the impact of such interaction on the biophysical functionality of the surfactant film [106]. The surface properties of inhaled particles affect the occurrence as well as intensity of interactions with pulmonary surfactant components, in particular alveolar macrophages clearance [107, 108], which shows size-dependent uptake that is considered most effective for particles with a geometric diameter range of 0.5 to 5 µm [109]. When developing a formulation that can overcome or minimize the lung clearance mechanisms on droplet aerosols methods have to be devised to reduce pulmonary clearance mechanisms, improve bioavailability, and maintain sustained concentration of drugs given by inhalation route.

1.6.3 Targeted delivery

Targeting is another post-deposition challenge for drug molecules within the lung where the active pharmaceutical ingredients require being effectively available at the site of
There are three levels of targeting of drug molecules within the lungs; first level is delivery to the central or peripheral, left or right lung: second level is delivery to the site of the disease, and the third level is delivery to specific cell type [88]. Lung cancer is a common form of cancer worldwide and has poor survival rate usually associated with late diagnosis [110]. Lung cancer is usually treated by chemotherapy, surgery, radiotherapy, or a combination of more than one method. Most chemotherapeutics marketed for treatment of lung cancer are available as an intravenous injection or infusion dosage forms, with a few options that can be given orally [111]. Systemic administration of chemotherapy is not site specific with low concentration at the site of lung tumor compared with initial dose given which makes systemic adverse effects more aggressive and noticeable [88]. Furthermore, chemotherapeutic agents are expensive and used for long-term treatment. Therefore, administration of anticancer drugs for local effect by pulmonary drug delivery could have real benefits such as better patient adherence and higher treatment efficiency [111].

### 1.7 Biopharmaceutical approaches

#### 1.7.1 Enhancing pulmonary deposition

For successful pulmonary drug delivery, the aerosol particles should be efficiently deposited in the lungs. To achieve this goal, both the inhalation device and drug formulation have to be optimized. Several advancements in inhalation formulations and device technologies have been proposed [112, 113]. Dry powder inhalation is a very popular formulation used in pulmonary drug delivery due to the fact that it enhances stability of pharmaceuticals and utilizes established manufacturing techniques such as
Nanotechnology in the form of nanoparticles prepared from biodegradable polymers, such as polylactic acid and polylactic-coglycolic acid has been intensively investigated for decades [115]. The small particle size which results in a high surface area can increase the dissolution rate, because the amount of active pharmaceutical ingredients dissolving over time is inversely related to the particle diameter [88]. Therefore, nanoparticle formulations are being considered as a potential approach to enhance solubility of lipophilic drug molecules. On the other hand a severe limitation is the fact that nanoparticles are easily exhaled form the lungs after inspiration [116, 117]. To overcome this problem, a group of researchers have developed large porous particles, known as Trojan particles, these are hybrid porous particles in the micrometer range composed of nanoparticles that could improve particle deposition in the lung [118]. These porous particles once deposited in the lungs dissociate to yield nanoparticles for drug absorption. A large number of new drug molecules are lipophilic that can cross lipid cellular membrane. But the volume of the lung lining fluid is limited and that can hinder the dissolution of active pharmaceutical ingredients. Prodrug is a pharmaceutical approach used in pulmonary dosage forms to improve solubility in which the drug molecule is structurally modified to enhance solubility and dissolution [119].

Selective formation of a particular polymorph of higher solubility using crystal engineering and formation of amorphous forms have also been investigated in inhalation dosage forms [96, 97]. For macromolecules such as proteins and peptides, solubility is less challenging but they suffer form enzymatic degradation in the lung fluid. However, the enzymatic degradation of these macromolecules can be minimized by chemical modifications that block peptidases, or via linkage to create ring-shaped biomolecules.
Nature of the excipients used in a formulation also play a significant role in the absorption of drugs given through pulmonary administration [88]. Among the excipients that have been approved and commonly used for pulmonary delivery are lactose, mannitol, and glucose [114, 120]. Phospholipids, such as dipalmitoyl phosphatidylcholine, can be used to improve solubility of poorly water-soluble drug molecules [114]. On the other hand, fusion of exogenous and endogenous surfactants might hinder the free diffusion of drug molecules, leading to reduced dissolution and absorption [88]. To overcome toxicity limitations that are encountered during formulation development, combinations of different solubility enhancers may be a valid option. Nevertheless, the integrity of pulmonary air-blood barrier is a major concern during evaluation of pulmonary excipients [88, 114].

1.7.2 Controlling lung clearance

There are several strategies that attempt to increase drug residence time in the lung such as: using poorly soluble chemically modified drug molecules, introducing positive charge to drug molecules that could improve drug retention [88], and encapsulation of the molecules in a sustained release carrier system [121, 122]. Sustained release particles can be permanently removed from the respiratory system by active clearance mechanism in the lung. Therefore, many approaches have been evaluated to overcome this issue. The first approach was based on using mucoadhesive formulation, but mucus turn over can impair the efficacy of the mucoadhesive formulation where the mucus is rapidly cleared by mucociliary mechanism in the lungs [123]. However, mucoadhesive formulations
have been investigated with significant improvements in drug bioavailability in pulmonary delivery [124, 125]. In addition to mucoadhesion properties, mucoadhesive polymers such as chitosan can improve permeability across epithelial membranes [126-128]. The modification of particulate carries with molecules such as polyethylene glycol is a widely used approach [88]. Clearance by alveolar microphages still poses challenge to the efficiency of pulmonary drug delivery. Alveolar microphages are capable of internalized particles that have a diameter of 0.5 to 5 um [109]. However, as mentioned earlier, this size range is ideal for aerosolized particles to deposit in the lung. Hence, studies were performed to evaluate the differences lung residence time and clearance between large and small particles. A study conducted by a group of researchers concluded that large porous particles have appropriate aerodynamic properties for alveolar deposition without being internalized by alveolar microphages [129]. Moreover, nanoparticles encapsulated within micron-sized carriers that disintegrate on deposition could help to circumvent clearance of nanoparticles by alveolar macrophages [118]. Dehydrated hydrogel-based particles with suitable sizes might offer efficient deposition, which swell in size as soon as it comes into contact with lung fluid [130]. The particle uptake by microphages is not just dependent on particle size it is also effected by particle shape and aspect ratio. It has been reported that thin particles and spiked particles circumvent microphage uptake [131].

1.7.3 Targeting inside the lung

The concepts of active or passive targeting are used in pulmonary drug delivery [112]. In
passive targeting, the particle deposition within the lungs is strongly dependent on aerodynamic diameter of the aerosol particles as well as the patient and device factors like breathing or dose released [112, 132]. Variation of these parameters determine the site of delivery to either the alveoli or the airways in which the selectivity of the right and left sides is not applicable [88]. Furthermore, magnetic targeting is an approach utilizing nanomagnetosols (aerosol droplets containing super paramagnetic iron oxide nanoparticles) which can be guided by an external magnetic field to the desired location inside the lung [133]. Therefore this approach can be utilized in targeting chemotherapeutic agents in which localized therapy would be highly favorable. In addition, several approaches have been investigated to improve alveolar macrophage targeting to increase intracellular bioavailability during treatment of tuberculosis [134, 135]. Targeting epithelial cells in gene therapy-based therapy for cystic fibrosis is also being investigated [136, 137].

1.8 Conclusion
Since the marketing of the first metered does inhaler in 1956 pulmonary drug delivery has been extensively studied and developed. The application of pulmonary drug delivery is limited to inhalation therapy for various lung diseases. Currently researchers are investigating and testing more sophisticated devices and aerosolized particles. Several approaches have to be adopted to optimize pulmonary drug delivery, minimize clearance, and improve drug targeting within the lungs. Nanotechnology based formulations hold great potential to improve aerosol drug carriers with regard to biopharmaceutical and
therapeutic efficiency [88].
Chapter 2

Significance of Research

Administration of chemotherapeutic agents orally or parenterally is the most common route of administration of anticancer drugs for treatment of various kinds of malignancies. The delivery of anticancer drugs by these common routes is usually associated with severe side effects due to the fact that anticancer drugs are not target specific entering both cancer cells and normal tissue cells following absorption. The systemic adverse effects of chemotherapeutic agents are numerous such as, allergic reaction, weight loss, and bone marrow suppression, organ failure and toxicity to peripheral nerves that range from loss of sensory function and mild paresthesia to neuropathic pain, severe ataxia and weakness leading to pronounced disability [138]. These disadvantages and others that are related to systemic delivery of anticancer medications have motivated researchers to look for alternative approaches for more efficient drug delivery systems for treatment of cancer while minimizing systemic side effects. Pulmonary drug delivery has attracted immense attention in recent years, particularly to use lungs as a means of delivering drugs systemically [113]. The pulmonary route is non-invasive and can be used for both systemic and local applications
As more efficient pulmonary delivery devices and sophisticated formulations become available, physicians and health professionals will have a choice of a wide variety of devices and formulation combinations that will target specific cells or regions of the lung, avoid the lung clearance mechanisms, and maintain sustained drug levels within the lung for longer period [113]. In this research a nanoemulsion formulation loaded with the anticancer drug paclitaxel that has the potential for pulmonary delivery was developed and evaluated. The nanoemulsion was prepared using linoleoyl polyoxyl-6 glycerides NF Labrafil® M2125CS as the oil phase, polyethoxylated-hydrogenated castor oil Cremophor® RH 40 as the surfactant, and polyethylene glycol PEG 400 as the co-surfactant. Cremophor® RH 40 is used to solubilize poorly water-soluble drug molecules; at the same time it is able to enhance bioavailability of various hydrophobic drug molecules either by altering permeability of the plasma membrane or by inhibition of p-glycoprotein (P-gp) efflux mechanism for drugs that are substrates of this protein transporter. Paclitaxel was selected as drug candidate and loaded in the nanoemulsion formulation of Labrafil® M2125CS, Cremophor® RH 40 and polyethylene glycol PEG 400.
Chapter 3

Formulation and evaluation of paclitaxel-loaded nanoemulsion for pulmonary administration

3.1. Abstract

*Purpose:* The purpose of this study was to develop and evaluate paclitaxel-loaded nanoemulsion for pulmonary delivery.

*Methods:* Based on composition, ternary phase diagram, solubility, clarity, and ease of nanoemulsion formation several components were screened in trial formulations. Subsequently Labrafil® M2125CS was identified as the oil, Cremophor® RH 40 was finalized as the surfactant, and polyethylene glycol PEG 400 was used as the co-surfactant. The final formula had surfactant/oil blend in the ratio of 2:1. Paclitaxel was added to this mixture that was then mixed with an aliquot volume of water to prepare a translucent nanoemulsion. The formulation was evaluated for particle size, zeta potential, pH, and conductivity. The drug entrapment efficiency was determined by HPLC.

*Results:* Clear and stable oil-in-water nanoemulsion that contained entrapped paclitaxel formed immediately when formulation ingredients were mixed using a vortex mixer. The average particle size of the nanoemulsion was approximately 20 nm, the zeta potential
was around 0 mV, the pH was 5 ± 0.002, and the conductivity was 144.3 ± 0.5 μSiemens/cm. The drug entrapment efficiency was 88 ± 0.001 %.

**Conclusion**: The results obtained from this study indicate formation of stable paclitaxel-loaded nanoemulsion that can be potentially used in pulmonary delivery.

### 3.2. Introduction

In 1963, Monroe E. Wall discovered that bark extract from the Pacific yew possessed antitumor activities. In 1967, Monroe E. Wall and Mansukh C. Wani isolated the active ingredient, paclitaxel, from bark extract of *T. brevifolia* and they reported its structure in 1971 [140]. Since that time paclitaxel has been extensively investigated due to the fact that it has significant anticancer activity against various kinds of cancer. Paclitaxel is the first of a new class of microtubule stabilizing agents, evolved from the National Cancer Institute as the most significant advancement in chemotherapy in the recent history [140]. Microtubules are structures made up of tubulin and perform functions such as mitosis, spindle formation, and shape of cells [141]. Paclitaxel acts by interfering with the function of microtubules [142]. It stabilizes microtubules by the prevention of depolymerization [143-145], which leads to cell death. Currently, paclitaxel is prescribed for patients with aggressive forms of malignancies such as ovarian, lung and breast cancers, head and neck, esophagus, bladder, endometrium, hematological, pediatric malignancies and also, AIDS-related Kaposi’s sarcoma [146, 147]. Challenges encountered during formulation of paclitaxel are related to its hydrophobicity, poor aqueous solubility, and low oral bioavailability [148]. Paclitaxel (Taxol®) was first clinically marketed as an intravenous infusion dosage form consisting of cremophor EL
and ethanol as solubilizers in which paclitaxel powder needs to be dissolved before administration.

Pulmonary drug delivery system has become an important dosage form for pharmaceutical and biomedical researchers since the lung is capable of absorbing pharmaceuticals either for local therapy or for systemic absorption. Some of the advantages of this method include non-invasive nature, high permeability of respiratory mucosa, large absorptive surface area, and active blood supply to the lungs [149-151]. Further advantages are low enzymatic activity, rapid absorption of drug, reduced dose administered to the patients, and circumventing first-pass hepatic metabolism [2]. Medical conditions such as asthma and chronic obstructive pulmonary disease (COPD) are treated using the pulmonary route of administration. Several therapeutic agents currently injected intravenously, such as growth hormones, or insulin, can possibly be delivered to humans by inhalation where the efficiency and safety are greater.

In this study, we developed an oil-in-water nanoemulsion using spontaneous emulsification method that has the potential for pulmonary delivery of the anticancer drug paclitaxel (PCL). PCL was successfully incorporated in a self-emulsifying mixture comprised of Labrafil® M2125CS as the oil phase, Cremophor® RH 40 as the surfactant and polyethylene glycol PEG 400 as the co-surfactant. These ingredients are considered non-ionic, biocompatible, and safe in animals and humans. The efficiency and suitability of PCL loaded nanoemulsion were evaluated and characterized extensively through series
of tests and experiments such as particle size, pH, zeta potential, sterility study, thermal and stability characteristics, and *in vitro* release.

### 3.3. Materials and Methods

#### 3.3.1 Materials

Labrafil® M2125CS, Labrafac WL 1349, Transcutol P and, Capryol™ 90, were provided by Gattefosse, St. Priest, France. Tween 80 was purchased from Fischer Scientific, Waltham, MA. Ethyl Oleate, Isopropyl Palmitate NF and Isopropyl Mysristate NF from Spectrum Chemicals, Gardena, CA. Paclitaxel was purchased from TSZ CHEM Chemicals. Polyethylene Glycol 400 (PEG 400) was obtained from Hampton Research Company, Aliso Viejo, CA. Cremophor® RH 40 was supplied by BASF Chemicals, Livonia, MI. Sodium Lauryl Sulphate NF (SLS) was purchased from PCCA®, Houston, TX. Acetonitrile (HPLC grade) was bought from Fisher Scientific®, Pittsburgh, PA. Deionized water obtained from our laboratory.

#### 3.3.2 Methods

##### 3.3.2.1 Drug solubility determination

The solubility of paclitaxel in different oils, surfactants and co-surfactants was determined by adding an excess amount of PCL to 1ml of each vehicle in 2mL micro-centrifuge tubes and mixing using a vortex mixer for 5 minutes. Drug - vehicles blend were then placed in a water bath incubator set at 40°C for 30 minutes to enhance drug solubilization followed by continuous shaking in an isothermal shaker for 72 hours at a temperature of 30± 0.5 °C). The samples were then centrifuged at 5000 rpm for 15 min to
settle un-dissolved PCL. Aliquots of supernatants were withdrawn and diluted with appropriate volume of acetonitrile and analyzed by HPLC for PCL quantification.

3.3.2.2 HPLC method of Paclitaxel

Reversed phase-high-performance liquid chromatography (RP-HPLC) was used for the determination of PCL concentration. The HPLC system (Waters Alliance e2695 separation module, Milford, MA) was equipped with a reverse phase C18 column (Symmetry C18 column - 3.5 µm, 4.6 × 75 mm) and photodiode array detector. The mobile phase ratio of acetonitrile and water was 50:50 at a flow rate of 1 ml/min at ambient temperature. PCL was properly diluted with acetonitrile and injected directly into the HPLC system using a run time of 6 minutes. The retention time was around 4 minutes with maximum absorption wavelength (λ max) of 220 nm. A series of standard PCL solutions were prepared in acetonitrile by serial dilution at concentrations between 0.7812 µg/ml to 100 µg/ml. The calibration curve was constructed by plotting the average peak areas versus concentrations used for quantification.

3.3.2.3 Preparation of nanoemulsion

PCL was found to have high solubility in Labrafil® M2125CS, PEG 400, and Cremophor® RH 40 among the excipients tested. Based on phase diagrams and solubility, a mixture of Cremophor® RH 40 (50%) – PEG 400 (25%) – Labrafil® M2125CS (25%) was utilized and considered as the final formula for the formulation. This formulation has a large phase diagram area, produced rapid emulsification, and formed a clear transparent mixture when mixed with aqueous medium. The formulation
was prepared by a spontaneous emulsification method. Cremophor® RH 40 (surfactant) and PEG 400 (co surfactant) were mixed in a 20 mL glass vial using magnetic stirring at 1200 rpm for 40 minutes to produce a homogenous mixture. Then the oil phase (Labrafil® M2125) was added and stirred at 600 rpm for 20 minutes to form the final self-emulsification blend. The blend was then introduced into deionized water gradually with gentle vortexing to form a clear nanoemulsion.

**3.3.2.4 Paclitaxel loaded nanoemulsion**

The PCL incorporated formulation was prepared by adding 10 mg of PCL to 2 g of self-nanoemulsifying blend followed by magnetic stirring at 1200 rpm for 24 hours at (30 ± 0.5 °C). Then 0.6 ml of drug loaded oil - surfactant blend was added to 5 ml of deionized water with vortexing. A transparent and clear PCL nanoemulsion formed rapidly. The PCL loaded formulation was placed in sealed glass vials away from light and stored at room temperature.

Table 3.1 The composition of the optimized formulation

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Quantity (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>10</td>
</tr>
<tr>
<td>Cremophor® RH 40</td>
<td>2000</td>
</tr>
<tr>
<td>PEG 400</td>
<td>1000</td>
</tr>
<tr>
<td>Labrafil® M2125CS</td>
<td>1000</td>
</tr>
</tbody>
</table>
3.4 Characterization of paclitaxel loaded nanoemulsion

3.4.1 Polarized Light Microscopy (PLM)

PLM utilizes a digital camera (a Nikon model TiU coupled with photometric Coolsnap EZ 20 MHz monochrome camera) at a magnification of 60x to produce images and Meta-Morph software for image analysis. This tool is used to clarify whether the active pharmaceutical ingredients or liquid formulations such as emulsions and gels are in crystalline or amorphous configuration within the formulation. In addition, it is used to differentiate between many pharmaceuticals phenomena such as crystal twining, solubility of crystals in different solvents, sublimation, and particle size distribution. The potential of using PLM over other screening tools is its high degree of sensitivity. PLM was used in our research is to confirm that the formulation is isotropic. Blank and drug-loaded formulations were prepared and investigated under PLM. A drop from each sample was taken and placed on glass slide, then observed under normal optics with and without polarizing filter.

3.4.2 Droplet size analysis

Dynamic light scattering (DLS) is a useful technique used widely for measuring particle size and distribution of particles within liquid medium. The DLS instrument (NICOMP 380 ZLS) was equipped with a 100mW He-Ne laser of wavelength 658 nm. Light scattering was measured at a scattering angle of 90°C and a temperature of 23°C. DLS utilizes small sample volume of approximately 1 ml. The sample intended for measurement should be diluted until it becomes clear or slightly hazy. Blank and PCL loaded formulations were prepared and then diluted with an appropriate volume of D.I
water for DLS measurement. All samples were transferred to disposable borosilicate glass culture tubes (Kimble Chase, Vineland, NJ). Three cycles were run at 5 minutes per cycle. Mean volume weighted diameter was then determined from the average of three runs for each sample.

3.4.3 Zeta potential analysis

Electrophoretic light scattering (ELS) is used to measure the electrophoretic mobility and zeta potential of colloidal systems. The sample prepared should be clean and dust free as dust may cause Doppler shifted peaks and change the shape and width of the observed spectrum of the measured sample [152]. Nicomp 380 ZLS instrument was used for zeta potential measurements in the ELS mode. Blank formulations and PCL loaded nanoemulsions were prepared and diluted with appropriate volume of D.I water for ELS measurements. Samples were placed in plastic cuvettes and filled to approximately \( \frac{3}{4} \)th of its volume and equilibrated in the holder for 5 minutes prior to each measurement. The measurements were taken in triplicates at a scattering angle of 14.06° and temperature of approximately 23°C.

3.4.4 Conductivity measurement

The electrical conductivity measurements were performed using a Mettler Toledo Seven Multi™ Meter equipped with a conductivity probe. Conductivity study was conducted to determine whether the emulsion is oil in water (o/w) or water in oil (w/o). The instrument was calibrated with standard solutions before sample testing. The conductivity probe was dipped in 5 ml of each standard until a stable reading was recorded. The conductivity
probe was cleaned properly after each sample reading. The measurements were done in triplicate at room temperature. Typically temperature has a unique and critical effect on the phase behavior of emulsions formulated with a non-ionic surfactant [153]. There is a proportional relationship between temperature and electrical conductivity of the emulsion. When temperature increases the conductivity increases to the extent where o/w emulsion is converted to a w/o emulsion. Phase inversion temperature (PIT) is the temperature in which the conversion occurs from o/w to w/o. The conductivity meter was used to determine the phase inversion temperature of formulations. The conductivity electrode was immersed in 20 ml of the formulation and the temperature was increased gradually using a Fisher Scientific™ Isotemp™ stirring hotplate. The conductivity values were recorded after each 5 °C increase in temperature until the PIT observed [154].

3.4.5 pH measurement

The pH was determined by immersing the pH electrode into the formulation placed in 10 ml glass vial. Standard solutions of pH 4.0, 7.0, and 10 were used to calibrate the pH meter. All measurements were done in triplicate for each sample in 25 ± 2 °C and the data were expressed as mean ± SD.

3.4.6 Differential Scanning Calorimetry (DSC)

DSC is a simple and one of the most common thermal techniques used in formulation development. Any thermal transition of the substances and materials will be associated with either absorption or release of heat [155]. DSC measurements involved placing the samples in suitable aluminum pans. Hermetically sealed pans are recommended for
volatile liquids while non-hermetic and/or open pans are used for non-volatile samples. For accurate quantitative DSC measurements, the samples and reference pan should be similar in thermal mass and type of metal used. DSC analysis was performed for the PCL loaded formulation loaded and compared to the thermograms of solid PCL powder and blank formulation using a Mettler Toledo DSC822e Star-e system. Approximately 5 to 8 mg of a particular sample was weighed and placed in standard 100µl aluminum pan. After weighing the pans were directly sealed using a mechanical crimper. An empty pan was used as a reference. The samples were scanned at a temperature range starting from 25 °C to 300 °C using a heating rate of 10°C /min under a stream of nitrogen gas. Star-e software was used for data acquisition and analysis.

3.4.7 Sterility testing
Sterility testing was performed to determine whether or not the final PCL loaded formulation enhances microbial contamination. The experiment was carried via the aseptic filtration method. 1.0 ml of the PCL formulation was passed through a 0.22 µm sterile nylon membrane filter (Millex ® syringe filter) to achieve sterility. The sterilization method was validated by using tube/direct and plate inoculation methods. Tryptic soy broth (TSB) was freshly prepared and used for the direct inoculation method. The direct inoculation method included a set of samples, which are: negative control, positive control, positive sample control, and sterile PCL formulation. The positive controls were prepared using Staphylococcus aureus (ATCC BAA 1692). This bacteria was grown and incubated in the nutrient medium (TSB) at a temperature of 37°C for 24 hours. Series of serial dilutions were made to obtain the final bacterial concentration of
10^2 CFU/ml. The negative control tube contained 0.1 ml of sterile water and 9.9 ml of the un-inoculated medium. The sterile sample tube contained 0.1 ml of the filtered PCL formulation and 9.9 ml of the un-inoculated medium. The positive control tube contained 0.1 ml of sterile water with bacteria count of 10^2 CFU/ml and 9.9 ml of un-inoculated medium while positive sample control tube contained 0.1 ml of PCL formulation, bacterial count of 10^2 CFU/ml and 9.9 ml of un-inoculated medium. Tubes were prepared in duplicate and incubated at 37°C to promote growth of bacteria. For plate method, Mueller Hinton (MH) agar plates were prepared and stored in the refrigerator then equilibrated at room temperature for 30 minutes prior to use. 100 µl sample was withdrawn from each tubes prepared by direct inoculation method on days 0, 7 and 14 and uniformly streaked onto MH agar plates. The plates were prepared in duplicate and incubated at 37°C for 24 hours. All experiments were performed under aseptic conditions in a laminar airflow hood. Washed and clean glassware’s were caped and autoclaved before being used in this study.

3.4.8 Stability study

In order to evaluate the physical and chemical stability of the final formulation, samples were subjected to long-term storage conditions (25±2 °C, 5±2 °C) for a period of 6 months. The stored samples were evaluated every month for clarity, phase separation, particle size, zeta potential, drug content, and pH values. The data obtained were expressed as mean ± SD. One-way ANOVA test was used to detect significant differences between the parameters of the formulations at day 1 and 180th day of storage.
3.4.9 *In vitro drug release study*

*In vitro* release profile of paclitaxel nanoemulsion was evaluated using a dialysis bag technique. The concentration of PCL used in the bag was 1.1mg/ml. A dialysis membrane (Spectra/Por® Dialysis Membrane) having a pore size of 2.4 nm and molecular weight cut off between 12000–14000 Da was used. The dialysis membrane was hydrated in D.I water for 24 hours before the experiment. 3 ml of freshly prepared PCL loaded formulation was placed in the dialysis bag and then both sides of the dialysis bag and the ends of the bag sealed using plastic clips. The dialysis bag was then placed in a beaker containing 125 ml of 0.5% SLS. A mechanical shaker (Thermo Scientific™ Precision Reciprocating Shaker Bath, USA) was used in this study at a rotational speed of 50 rpm/min and the temperature was maintained at 37 ± 0.5 °C. Aliquots of the release medium of about 1 ml volume were taken at various time points and replaced with fresh 0.5% SLS solution to maintain sink conditions. The experiment was done in triplicates. The drug amount, and cumulative percentage release were quantified using HPLC analysis method.

3.4.10 *Transmission Electron Microscopy (TEM)*

TEM imaging was utilized to examine the surface morphology of the final formulation. After PCL loaded formulation was prepared, one drop of the prepared sample was pipetted onto a Formvar /Carbon 400 mesh copper grid (Ted Pella, CA) and was allowed to dry for 24 hours at room temperature prior to imaging using a transmission electron microscope (JEOL JSM -7500F Field Emission Scanning Electron Microscope) operated at an acceleration voltage of 30kV.
3.4.12 Evaluation of rheological properties

The rheological properties of blank and PCL loaded nanoemulsion were studied using steady state flow and dynamic frequency sweep tests in the oscillatory mode. The tests were performed on an AR 2000 controlled stress/strain rheometer (TA Instruments, New Castle, DE). A double concentric cylinder geometry was used because the blank and PCL loaded samples have very low viscosity. The experimental conditions used during steady state flow test were 20 °C and 37 °C. The experiments were performed by increasing the shear rate from 5.314 to 531.4 s⁻¹. The frequency sweep in the oscillatory tests were from 0.1 to 10 Hz at a constant stress of 0.02 Pa. All tests were done in triplicate.
Chapter 4

Results and Discussion

4.1 Drug solubility determination

Paclitaxel is a lipophilic molecule with very low water solubility; therefore one of many pharmaceutical approaches to overcome this formulation issue is to enhance its solubility by incorporation into a lipid-based formulation. PCL belongs to the BCS class IV which includes drugs with low permeability and solubility. Oil in water (o/w) nanoemulsion is used to improve delivery of water insoluble drugs across pulmonary mucosa by increasing their solubility in the oil phase [156]. The most important features of (o/w) nanoemulsion includes submicron globule size, high fluidity, excellent solubilization capacity, and lipophilic nature [157]. Since the drug solubilizes in oil phase and the oil phase is not more than 40% of the (o/w) emulsion system the drug loading capacity in the final formulation can be significantly impacted. Certain APIs require high dose and hence it is important that the drug molecule possess high solubility in various excipients to be able to eventually provide the required dose. The aim of the solubility study was to determine appropriate excipients that have good solubility for the selected drug. The solubility of PCL in various oils, surfactants, and co-surfactants are shown in table 4.1
Table 4.1: Solubility of Paclitaxel in various excipients

<table>
<thead>
<tr>
<th>Excipients</th>
<th>Function</th>
<th>Solubility (mg/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor RH 40</td>
<td>Surfactant</td>
<td>28.21 ±0.44</td>
</tr>
<tr>
<td>Tween 80</td>
<td>Surfactant</td>
<td>25.62 ± 0.20</td>
</tr>
<tr>
<td>PEG 400</td>
<td>Co –surfactant</td>
<td>132.3± 2.44</td>
</tr>
<tr>
<td>Caprypol 90</td>
<td>Co-surfactant</td>
<td>63.3± 1.33</td>
</tr>
<tr>
<td>Labrafil 2125 Cs</td>
<td>Oil</td>
<td>5.62±0.08</td>
</tr>
<tr>
<td>LL WL 1349</td>
<td>Oil</td>
<td>5.26±0.04</td>
</tr>
<tr>
<td>Ethyl oleate</td>
<td>Oil</td>
<td>1.18±0.006</td>
</tr>
<tr>
<td>Isopropyl P</td>
<td>Oil</td>
<td>0.49±0.01</td>
</tr>
<tr>
<td>Isopropyl M</td>
<td>Oil</td>
<td>0.61±0.009</td>
</tr>
<tr>
<td>Transcutol P</td>
<td>Surfactant</td>
<td>178.9±1.72</td>
</tr>
</tbody>
</table>

Labrafil® M2125CS was selected as an oil phase because PCL exhibited maximum solubility in this oil. Cremophor® RH 40 and PEG 400 were used as the surfactants and co-surfactant respectively. Selection of excipients in the final formulation is not only based on their drug solubility but also on their ability to form transparent and stable nanoemulsion when it is incorporated with an appropriate oil phase and introduced into the water phase. The excipients used in the formulation are non-toxic and categorized as generally regarded as safe (GRAS) materials [158]. Nonionic surfactants like Cremophor® RH 40 and PEG 400 are used in preparation of (o/w) emulsion, as they are safe, biocompatible and can tolerate pH changes. In addition, the selected surfactants and
co-surfactant materials should provide high hydrophilic-lipophilic balance (HLB) values greater than 10 which is a factor important to form appropriate (o/w) nanoemulsion [159]. The emulsification efficiency of the final formulation depends on the area occupied on the ternary phase diagram. The Cremophor® RH 40, PEG 400 demonstrated shortest emulsification time and largest emulsification area when plotted on a ternary phase diagram shown in figure 4.1 [160]. Finally, ratios of surfactant - co-surfactant - oil were as 2:1:1 and selected as the final blend for loading the anticancer drug paclitaxel.

**Figure 4.1:** Ternary phase diagrams of Cremophor RH 40 % - PEG 400 % and- Labrafil M 2125 CS % [161].
4.2 Drug loading

Self - nanoemulsifying Drug Delivery System (SNEDDS) are isotropic mixture of oil, surfactant and co - surfactant, which are able to form stable (o/w) nanoemulsion upon dilution with water with gentle agitation. The order of incorporating of oil – surfactants blend - water is critical when nanoemulsions are prepared. The process includes when the surfactant - co-surfactant mixture and oil are mixed together for a period of time followed by addition of this mixture into the aqueous phase [160]. The maximum drug - loading capacity was achieved by preparation of several SNEDDS mixtures with different concentration of the drug. The final concentration of the drug in the formulation was selected depending on the ability of the SNEDDS in maintaining the API in solubilized form without demonstrating drug precipitation or crystallization. The final formulation contained 10 mg of PCL dissolved in 2gm of SNEDDS. The drug loaded SNEDDS formed a stable nanoemulsion without phase separation over a storage period of 72 hours at room temperature. There was no difference in physiochemical properties between blank and PCL formulations.

Figure 4.2: [A] Transparent nanoemulsion       [B] Turbid or milky emulsion
4.3 Droplet size

Droplet size distribution of nanoemulsion is an important parameter to assess both stability and biopharmaceutical aspects of the formulation. The smaller the particle size the larger interfacial surface area for absorption or permeation of the drug across biological membrane and greater the bioavailability of the drug from the formulation. The higher stability and clarity of SNEDDS compared to the classic emulsion might be due to their smaller particle size [162]. Droplet size is typically affected by the type and concentration of surfactants used in the formulation. An increase in concentration of surfactant in the formulation may lead to breakdown of oil globules into smaller sizes causing decrease in droplet size [163]. The drug-loaded formulation was filtered through a 0.2 µm Nalgene® syringe filter and the particle size of the filtered formulation was determined to check for the effect of filtration on the size of the droplets. The results shown in table 4.2 concluded that there was no difference in the particle size with the filtration process or different amounts of water. This data indicated that an increase in volume of water has little or no effect on the droplet size [164]. The mean diameter of the blank and drug loaded formulation was 21.3 ± 1.85 nm and 19.6 ± 0.05 nm respectively and both samples displayed a Gaussian distribution of particle sizes (Figure: 4.3 and Figure: 4.4). Slight change in particle size of drug loaded formulation might be due to the fact that incorporation of PCL (Mol. Wt.: 853.906 g/mol) within the core of micelles [165]. No change in the droplet size and size distribution was observed between the filtered and unfiltered drug loaded formulations confirming that nanoemulsion could pass through filter media without change in droplet size.
Table 4.2: The results from the droplet size experiments for blank and PCL loaded nanoemulsion

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Volume of water (ml)</th>
<th>Particle size (nm ± SD, n=3)</th>
<th>Zeta potential (mV ± SD, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor® RH 40 PEG 400</td>
<td>10</td>
<td>21 ± 7.44</td>
<td>N/A</td>
</tr>
<tr>
<td>Labrafil® M2125CS (Blank nonoemulsion)</td>
<td>5</td>
<td>21.3 ± 1.85</td>
<td>0.05 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18 ± 1.1</td>
<td>N/A</td>
</tr>
<tr>
<td>PCL nanoemulsion</td>
<td>5</td>
<td>19.6 ± 0.05</td>
<td>0.02 ± 0.005</td>
</tr>
</tbody>
</table>

Figure 4.3: Droplet size for the blank nanoemulsion diluted with 5 mL D.I water
Figure 4.4: Droplet size of blank nanoemulsion diluted 2 mL with D.I water

Figure 4.5: Droplet size of paclitaxel loaded nanoemulsion
4.4 Zeta potential analysis

Zeta potential is the measurement of electrical charge on the surface of particles or droplets and can be measured using Nicomp 380 ZLS instrument in electrophoretic light scattering mode. Due to the fact that non-ionic surfactant and co-surfactants were used to prepare the final formulation, the zeta potential of blank and PCL loaded nanoemulsion were found to be neutral. Researchers have found that positive charged particles rapidly adhere to biological membranes [166]. Wherever this electrostatic attraction occurs, bioavailability of the drug could be enhanced. However, it may cause irritation and/or other related toxicity in the membrane [167]. The kind of charge also influences the pharmacokinetics behavior of the drug following absorption in the body. Studies have concluded that neutral nanoparticles are able to stay in blood circulation for long time compared to charged particles due to minimum protein binding [168]. A stable dispersion might be formed when the values of zeta potential are above ± 30 mV. This is due to presence of repulsion forces between particles that prevents them from aggregation [169]. Since charge-less surfactants were used in the preparation of the final formulation, a neutral zeta potential was expected as is shown in figure 4.6. Low zeta potential values can cause inter-particulate interaction between particles and that would compromise the stability of the formulation. Nevertheless, studies have reported that a stable nanoemulsion can be prepared even when non-ionic excipients are used [170, 171].
DSC was performed to investigate the nature of the drug in the formulation. The PCL powder alone exhibited a sharp endothermic peak approximately at 225°C as is shown in figure 4.7 which reflects its reported melting transition. The DSC thermogram of blank and PCL loaded formulation were free from any peaks at this particular melting temperature confirming that paclitaxel drug molecules were finely dispersed within the formulation.
**Figure 4.7:** DSC Thermogram of: (A) Pure paclitaxel, (B) Blank, (C) Paclitaxel loaded formulation

### 4.6 PLM

PLM is generally utilized to examine the isotropic properties of nanoemulsion system. The drug loaded formulation and the blank nanoemulsions were characterized by this technique. The major difference between polarized light and light microscopy is that the polarized light vibrates in only one direction while the ordinary light vibrates in different directions. The polarized light is capable to interact with various samples. The polarized light will rotate if concentrated on materials containing anisotropic structure such as liquid crystals. In such samples polarized light becomes bright and visible when viewed under a light-polarizing filter. Isotropic material will not rotate polarized light producing a dark background when viewed under polarized light. In this experiment, formulations
were free from any birefringence confirming that they are true nanoemulsions [172]. In addition, presence of any solid crystals may be recognized by presence of birefringence. The results obtained in this study for both blank and drug loaded formulations showed that PCL has not altered the optical properties of nanoemulsion system as shown in figures 4.8 and 4.9.

*Figure 4.8:* PLM optical micrograph of paclitaxel formulation without polarizing filter

*Figure 4.9:* PLM optical micrograph of paclitaxel formulation with polarizing filter at magnification of 60x
4.8 HPLC method

In this work an R-HPLC method was successfully developed and validated for quantification of paclitaxel. Validation includes determining linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), and precision. The chromatogram of paclitaxel showed a sharp peak with retention time around 4 min as shown in figure 4.10.

By plotting the areas under the curve versus standard drug concentrations, a calibration curve was obtained. A linear range form 50 µg/ml to 0.3906 µg/ml with $R^2$ value of 0.99985 was obtained as demonstrated in figure 4.11. According to the ICH Q2 (R1) recommendations [173], LOQ and LOD were calculated and found to be 2.8364 ng and 0.4680 ng, respectively. The percentage recovery values of paclitaxel ranged from 98.17% to 98.27% indicating the high accuracy of the developed HPLC method. The method also showed a good intra-day precision with RSD not exceeding 2%.

Figure 4.10: HPLC chromatogram for paclitaxel separation
4.9 pH

The pH of aqueous formulations delivered as an inhalation should be within physiological pH range in order to avoid mucosal irritation. The pH of blank formulation was determined to be 5. Addition of paclitaxel caused a slight change in the pH of the final drug loaded formulation. The final pH of the formulation was within range of pulmonary secretion which is 4.5 to 6.5. This test confirms that the formulation is potentially compatible with pulmonary tissue. The blank formulation remained clear and transparent even after diluting 10 to 100 times with water. The percentage transmittance %T was taken as indicator for clarity. The %T of blank formulation was found to be more than 90%. This indicates the ability of oil-surfactant mixture to form a transparent formulation when diluted with water. The observed clarity of the system is due to their extremely small droplet size of less than 100 nm which is not exceeding 1/4th of the wavelength of visible light [174].
4.10 Conductivity

The first goal of this study was to confirm that our formulation is (o/w) emulsion. High conductivity values were recorded for both blank and drug loaded formulation proving that both formulations were o/w type. The formulations reported in this work used polymeric surfactants that tend to form a turbid to milky emulsion at high temperatures [153]. We utilized polyethoxylated castor oil Cremophor® RH 40 as a hydrophilic non-ionic surfactant in preparation of o/w nanoemulsion and phase studies were done to study how temperature affected the formulations. When the system is exposed to excessive heat, the polyethoxylated derivatives become lipophilic due to evaporation of water molecules from their structures. Due to this phenomenon the phase behavior of the o/w formulation with these surfactants can change. Phase inversion temperature is defined as the temperature in which the o/w emulsion changes to w/o type as demonstrated in figure 4.12a-b. The conductivity of the system increases with increasing temperature until it reached a maximal point from which the conductivity value declined and this is referred to as the phase inversion temperature (PIT). Another goal of this study was to report the PIT and to confirm that the temperature of storage or preparation is not close to the PIT of the system to further support stability data. Formulations with PIT values close to the body temperature of 37°C may alter the biopharmaceutical properties of the formulation. The PIT data obtained for drug loaded formulation and blank were 87°C and 85 °C respectively which indicates that our final formulation is very temperature stable. The PIT for the formulation was slightly less than the blank and this can be due to the low log P drug lowering the PIT of the system [175].
Figure 4.12a: PIT for the blank

Figure 4.12b: PIT for PCL loaded formulation
4.11 Validation of sterility

The sterility of PCL loaded nanoemulsion was validated using direct inoculation and plate inoculation methods. The direct inoculation technique included samples prepared and stored as described in USP. The negative control, and sterile nanoemulsion samples were free from any sign of bacterial growth. However, positive control and positive sample control showed obvious turbidity during the 14 days study period as shown in figure. 4.13. Samples withdrawn from direct inoculation test tubes on days 0, 7, and 14 were placed on agar plates. The plates were incubated at 37°C to enhance growth of bacteria. After 1 day of incubation, the plates were observed for the presence or absence of microbial growth. The positive sample control and the positive control plates exhibited bacterial growth, while negative and sterile plated samples had no microbial growth as is shown table 4.3 and figure 4.14. The results of the sterility verification on days 0, 7, and 14 days confirmed that sterilization using aseptic filtration with a 0.22μm filter is sufficient for sterilizing the PCL nanoemulsion.

Table 4.3: Sterility test performed on MH agar plates indicating the presence (+) or absence (-) of microbial growth on days 0, 7 and 14

<table>
<thead>
<tr>
<th>Days</th>
<th>Negative control</th>
<th>Positive control</th>
<th>Positive sample control</th>
<th>Sterile sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 4.13 Direct inoculation method: (a) negative control tubes, (b) positive control tubes, (c) positive sample tubes, (d) sterile sample tubes, after 14 days of inoculation
Figure 4.14: MH agar plates (a) sterile sample (b) positive sample control, (c) negative control (d) positive control after 14 days

4.12 Stability study

The quality and quantity of the active drug loaded in a formulation must remain constant for a period of time under the influence of external factors such as light, temperature, and
humidity. Nanoemulsions are known for kinetic and thermodynamic stability, and steric stabilization since it is mostly formulated with polymeric or non-ionic surfactants. In addition, very small droplet diameter in the formulation leads to improved stability by several ways including: minimizing the effect of gravity force and Brownian motion, preventing coalescence or flocculation, and enhancing free dispersion when gently shaken or dispersed [162]. In the stability experiments the final formulation was free from drug precipitation, flocculation, or phase separation after centrifugation for 40 min. The physiochemical stability of PCL formulation was examined by measuring particle size, pH and, conductivity, % drug content, and zeta potential of all samples stored at room temperature and under refrigeration (5°C). Results from the stability study are shown in table 4.3. There was an increase in the particle size of samples stored at room temperature after four months. Based on the data reported from this study, it is concluded that the physical and chemical integrity of the drug-loaded formulation was maintained when stored at 5 °C.
Table 4.3: Stability results of PCL formulation

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Parameters</th>
<th>1 month</th>
<th>2 month</th>
<th>3 month</th>
<th>4 month</th>
<th>5 month</th>
<th>6 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Drug content %</td>
<td>102±0.02</td>
<td>88± 0.02</td>
<td>92±0.02</td>
<td>94±0.02</td>
<td>90±0.02</td>
<td>95±0.02</td>
</tr>
<tr>
<td></td>
<td>Particle size</td>
<td>25.7±0.05</td>
<td>24.6±0.05</td>
<td>27.1±1.5</td>
<td>39.9±0.1</td>
<td>52.9±1.4</td>
<td>62.5±4.2</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>4.65±0</td>
<td>6.47±0.01</td>
<td>6.84±0.01</td>
<td>6.82±0.01</td>
<td>6.81±0.01</td>
<td>7.17±0.07</td>
</tr>
<tr>
<td></td>
<td>Zeta Potential</td>
<td>-</td>
<td>0.06±0.03</td>
<td>0.1±0.04</td>
<td>0.04±0.03</td>
<td>-</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td>°C</td>
<td>Drug content %</td>
<td>105±0.02</td>
<td>90±0.002</td>
<td>100±0.001</td>
<td>100±0.001</td>
<td>100±0.001</td>
<td>84±0.01</td>
</tr>
<tr>
<td></td>
<td>Particle size</td>
<td>25.4±0.05</td>
<td>25.4±0.05</td>
<td>24.5±0.7</td>
<td>23.8±0.05</td>
<td>23.7±0.05</td>
<td>30.3±0.85</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>4.94±0.005</td>
<td>4.82±0.008</td>
<td>4.78±0.01</td>
<td>4.75±0.00</td>
<td>4.78±0.00</td>
<td>4.74±0.00</td>
</tr>
<tr>
<td></td>
<td>Zeta Potential</td>
<td>0.03±0.01</td>
<td>0.03±0.01</td>
<td>-</td>
<td>0.37±0.05</td>
<td>-</td>
<td>0.1±0.03</td>
</tr>
</tbody>
</table>

4.13 In vitro drug release studies

The results from in vitro drug release studies are shown in figure 4.15. Approximately 100% of the drug was released in 168 hours and a plateau phase was reached approximately around 80 hours after starting the experiment. The sustained release profile of the drug might be attributed to the fact that PCL is solubilized in the oil phase of the nanoemulsion formulation. Also PCL has high partition co-efficient and a low log P value. In addition the small surface area of the artificial membrane used in the experiment could have limited amount of the drug diffusing out form inside the bag to the release medium. Additionally the presence of Cremophor® RH 40 might have hindered
the release due to its ability to form strong interfacial films that surround oil globules. As a result, the rate-limiting step for drug release was its ability to diffuse across the interfacial film. Another *in vitro* release study on a nanoemulsion containing Cremophor® RH 40, Span® 80 and canola oil reported similar drug release pattern with sustained release of vitamin E acetate observed up to 10 days [176]. The high partition co-efficient of paclitaxel, low log P of approximately 3.96, dialysis membrane characteristics, and presence of Cremophor® RH 40 in the formulation can explain the sustained release of PCL from the formulation.

![Figure 4.15: In vitro release profile of paclitaxel](image)

*Figure 4.15: In vitro release profile of paclitaxel, the data represent the mean values ± S.D (n=3).*

### 4.14 TEM

TEM was used to visualize the morphology of PCL loaded nanoemulsion. Many characteristics such as particle size, shape and internal structure of the carrier system can
be efficiently evaluated by using TEM [177]. The TEM images revealed the spherical shape of the oil globules and the droplet size in nanoscale range. The droplets of PCL loaded nanoemulsion appeared as dark globules in the electron micrographs as shown in figures 4.17 (a) and (b).

![TEM images of (a) and (b) Paclitaxel loaded nanoemulsion](image)

**Figure 4.17**: TEM images of (a) and (b) Paclitaxel loaded nanoemulsion

### 4.15 Rheology

The rheological characterization of pharmaceutical preparations is a critical physical evaluation to provide insights for technical applications such as manufacturing, pumping, filling, and storage, as well as in the esthetic qualities of the final product [178]. The application and acceptance of many new pharmaceuticals and cosmetics products depend on the flow properties of the finished product [179]. The rheological characteristics of nanoemulsion loaded PCL was determined by plotting shear stress versus shear rate. The rheograms were found to be linear as is shown in figure 4.18, which indicated Newtonian
behavior. Oscillation test is used for determining the viscoelastic properties of the tested material in its rheological ground state without altering the structure of the material [180, 181]. The oscillation frequency test is a dynamic test in which the response of the tested material is measured as a function of frequency at constant stress amplitude [182]. In the dynamic test, the storage modulus $G'$ exhibits information about the elastic properties, whereas the loss modulus $G''$ is a parameter of the viscous properties. This experiment indicated that both storage modulus $G'$ and loss modulus $G''$ are linearly proportional to the oscillatory frequency. The viscous component $G''$ exceeded the elastic component $G'$ which demonstrated a Newtonian liquid behavior as is shown in figure 4.19.

![Steady State Flow of Paclitaxel](image)

**Figure 4.18:** Steady State Flow of paclitaxel loaded nanoemulsion at 20°C and 37°C.
Figure 4.19: Dynamic Frequency Sweep of paclitaxel loaded nanoemulsion at 20 °C and 37 °C.

4.16 Conclusion

In this study, paclitaxel loaded nanoemulsion was successfully prepared using cremophor RH 40, PEG 400 and labrafil M2125Cs in a ratio of 2:1:1. The particle size of the blank and drug loaded nanoemulsion was found to be less than 20 nm. The DSC results showed complete conversion of paclitaxel molecules from crystalline form a monomolecular dispersion form. The pH of the final formulation was found to be optimum for pulmonary delivery. The isotopic nature of nanoemulsion system was confirmed using polarized light microscopy. Sterilization of the final formulation with aseptic filtration was found to be sufficient for sterilization of formulation. The system
showed sustained release of paclitaxel from an oil in water nanoemulsion. The physiochemical characteristics at refrigerated conditions remained stable for up to six months. Based on these results, the nanoemulsion formulation can be a promising approach for pulmonary delivery of the chemotherapeutic drug paclitaxel for the treatment of lung cancer. Future studies like characterization of the mist and animal biodistribution studies might further support the final results.
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