DEVELOPMENT AND CHARACTERIZATION OF PACLITAXEL LOADED NANOLIPOSOMES FOR LUNG CANCER THERAPY : AN IN-VITRO INVESTIGATION

THESIS SUBMITTED IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHARMACY

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By

Jayawardane Athukorallage Chamindika Perera, B.Pharm
Examination Roll No. : M4PHA19003
Registration No. : 140829 of 2017-2018

Under the guidance of

Prof. (Dr.) Biswajit Mukherjee
DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY
JADAVPUR UNIVERSITY
KOLKATA-700032
INDIA

Department of Pharmaceutical Technology
Faculty of Engineering and Technology
Jadavpur University
Kolkata-700032, India.

CERTIFICATE OF APPROVAL

This is to certify that **Ms. Jayawardane Athukorallage Chamindika Perera** (Class Roll No: 00171402001, Examination Roll No: M4PHA19003 and Registration No: 140829 of 2017-2018) has carried out the research work entitled **"*Development and Characterization of Paclitaxel loaded nanoliposomes For Lung cancer therapy ; an invitro investigation*"** independently with proper care and attention under my direct supervision and guidance in the Pharmaceutical Research Laboratory in the Department of Pharmaceutical Technology, Jadavpur University,Kolkata,India. She has incorporated her findings into this thesis of the same title, being submitted by her, in partial fulfillment of the requirements for the degree of **Master of Pharmacy** from Jadavpur University. I am satisfied that she has completed her work with proper care & confidence to my entire satisfaction.

.....

Prof. (Dr.) Biswajit Mukherjee
Project Guide
Department of Pharmaceutica Technology
Jadavpur University
Kolkata – 700032, India.

……..

Prof. (Dr.) Pulok Kumar Mukherjee
Head of the Department
Department of Pharmaceutical Technology
Jadavpur University
Kolkata – 700032.

……..

Prof. Chiranjib Bhattacharjee
Dean
Faculty of Engineering and Technology
Jadavpur University
Kolkata – 700032.

DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of her Master of Pharmaceutical Technology studies. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

NAME : JAYAWARDANE ATHUKORALLAGE CHAMINDIKA PERERA

EXAM ROLL NUMBER : M4PHA19003

REGISTRATION NUMBER : 140829 OF 2017-2018

THESIS TITLE : DEVELOPMENT AND CHARACTERIZATION OF PACLITAXEL LOADED NANOLIPOSOMES FOR LUNG CANCER THERAPY: AN *IN-VITRO* INVESTIGATION.

SIGNATURE WITH DATE :

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…….

Jayawardane Athukorallage Chamindika Perera

Date :

Place :

DEDICATED TO

MY GUIDE

MY FAMILY AND

WELL WISHERS

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INTRODUCTION

1. INTRODUCTION

1.1. Lung Cancer

Cancer is a class of diseases, characterized by the uncontrolled growth and spread of abnormal cells. The common types of cancer in males are lung, prostate colorectum, stomach and liver cancer; and in females are breast, colorectum, lung, cervical and stomach cancer.

Lung cancer is the **uncontrolled growth of abnormal cells** in one or both lungs. These abnormal cells do not carry out the functions of normal lung cells and do not develop into healthy lung tissue. As they grow, the abnormal cells can form tumors and interfere with the functioning of the lung, which provides oxygen to the body via the blood.

The National cancer institute-America defines lung cancer as "cancer that forms in tissues of the lungs, usually in the cells lining air passages".

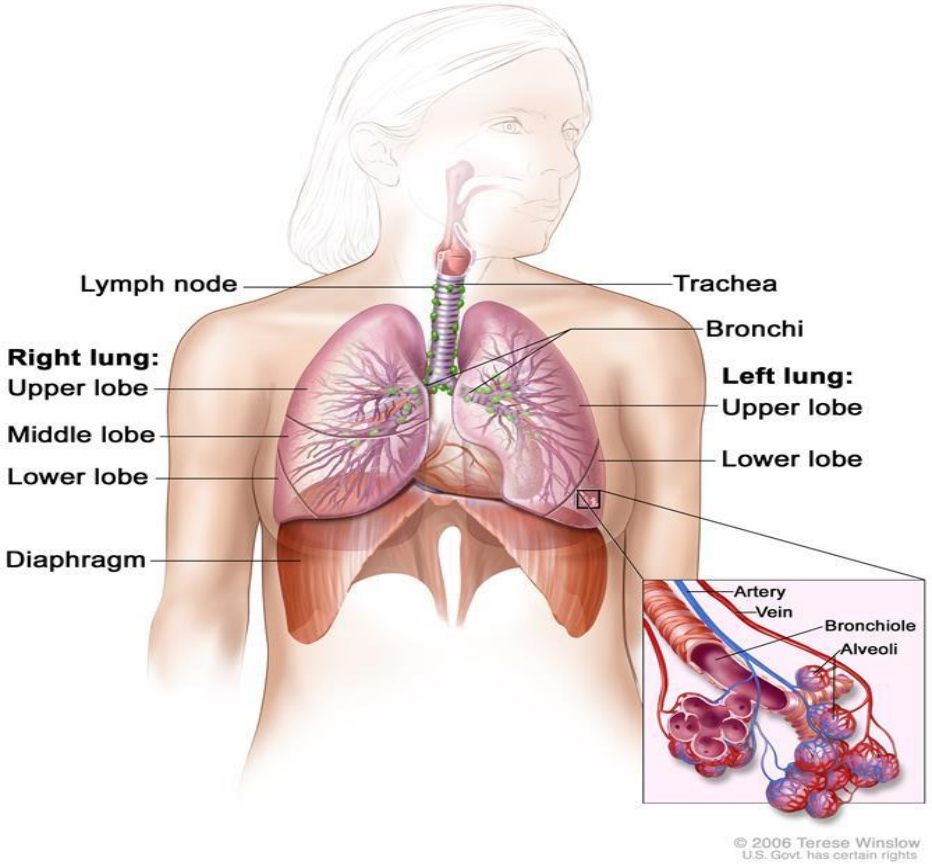

Figure 1: **Anatomy of the respiratory system :** ***(https//www.cancer.gov/types/lung/hp)***

1.1.1. Incidence and Mortality

Lung cancer is one of the major causes of malignancy-related death in the most developed counties, and the incidence of lung cancer in developing countries is increasing rapidly. The World Health Organization's Global Burden of Disease analysis projects 1,676,000 deaths from lung cancer worldwide in 2015. It predicts that this toll will continue to rise to reach a staggering 2,279,000 deaths in the year 2030. *(www.who.int/healthinfo/global_burden_diesease/projection2002)*

Lung cancer is the leading cause of cancer-related mortality in the United States. Estimated new cases and deaths from lung cancer (NSCLC and SCLC combined) in the United States in 2019 according to the American Cancer Society (Cancer Facts and Figures- last accessed 23rd January 2019) as follows.

- New cases: 228,150.
- Deaths: 142,670.

Lung cancer is a disease of modern times. At the turn of the 20th century, lung cancer was rare, accounting for less than 0.5 percent of all malignancies. Lung cancer has a poor prognosis, which means incidence closely matches mortality. Irrespective of cause, mortality from lung cancer is high : with only15% of lung cancer patients surviving for more than 5 years after diagnosis.*(Rodriguez E and Lilenbaum 2010)*

1.1.2. Types of lung cancer

Small-cell lung carcinoma (SCLC, 15% of all lung cancer) and non-small-cell lung carcinoma (NSCLC, 85%) are the two major forms of lung cancer.

NSCLC is a very invasive type of lung carcinoma and is more common.

The NSCLC is further classified into three main sub types:

- Squamous cell carcinoma
- Large cell carcinoma
- Adenocarcinoma

SCLC is any type of epithelial lung cancer other than small cell lung cancer (SCLC).

1.1.3. Non- Small- Cell Lung Cancer (NSCLC)

NSCLC arises from the epithelial cells of the lung of the central bronchi to terminal alveoli. The histological type of NSCLC correlates with site of origin, reflecting the variation in respiratory tract epithelium of the bronchi to alveoli. Squamous cell carcinoma usually starts near a central bronchus. Adenocarcinoma and bronchioloalveolar carcinoma usually originate in peripheral lung tissue. According to the American Cancer Society (ACS), non-small cell lung cancer (NSCLC) accounts for about 80 percent of lung cancer cases.

The three main subtypes of NSCLC are:

- **Adenocarcinoma**. This accounts for 40 percent of lung cancer cases. It is usually found in the outer parts of the lung. It tends to grows slower than the other two subtypes, so there is a better chance of a tumor being found before it has spread.

- **Squamous cell carcinoma**. This accounts for about 25-30 percent of lung cancers. It grows from the cells that line the insides of the lung's airways. Squamous cell carcinoma is usually found at the center of the lung.

- **Large cell carcinoma**. This accounts for 10-15 percent of lung cancers. It can be found in any part of the lung, and tends to grow faster than the other subtypes.

But there are several other types that occur less frequently, and all types can occur in unusual histologic variants.

Although NSCLCs are associated with cigarette smoke, adenocarcinomas may be found in patients who have never smoked. As a class, NSCLCs are relatively insensitive to chemotherapy and radiation therapy compared with SCLC.

However, present approaches for NSCLC therapy are still limited to chemotherapy, surgical resection, radiotherapy, or their combination, based solely on anatomic factors, the size of the tumor and where it has spread (*Horeweg N et al. 2012*). Patients with advanced metastatic disease may achieve improved survival and palliation of symptoms with chemotherapy, targeted agents, and other supportive measures.

NSCLC is most commonly staged (as stage I,II,III,IV) using a system called TNM classification:

T - tumor size and location

N - the number of nearby lymph nodes that have become involved

M - metastasis, or how far the cancer has spread

Accurate staging is the basis for predicting survival and is key to clinical trials that compare treatments among homogeneous populations of patients. For example, patients with Stage - I non-small cell lung cancer treated with surgical resection have a five-year survival of about 73 % *(Goldstraw P et al. 2007).*

1.1.4. Risk Factors for lung cancer

Increasing age is the most important risk factor for most cancers. Other risk factors for lung cancer include the following:

- History of or current tobacco use: cigarettes, pipes, and cigars.
- Exposure to cancer-causing substances in second hand smoke.
- Occupational exposure to asbestos, arsenic, chromium, beryllium, nickel, and other agents.*(Straif K et al. 2009)*
- Radiation exposure from any of the following:
    - Radiation therapy to the breast or chest.
    - Radon exposure in the home or workplace.
    - Medical imaging tests, such as computed tomography (CT) scans.
    - Atomic bomb radiation.
- Living in an area with air pollution.
- Family history of lung cancer.
- Human immunodeficiency virus infection.
- Beta carotene supplements in heavy smokers.

The single most important risk factor for the development of lung cancer is smoking. For smokers, the risk for lung cancer is on average tenfold higher than in lifetime nonsmokers (defined as a person who has smoked <100 cigarettes in his or her lifetime). The risk increases with the quantity of cigarettes, duration of smoking, and starting age.

Smoking cessation results in a decrease in precancerous lesions and a reduction in the risk of developing lung cancer. Former smokers continue to have an elevated risk of lung cancer for years after quitting. Asbestos exposure may exert a synergistic effect of cigarette smoking on the lung cancer risk. *(Wingo PA et al. 1999)*

1.1.5. Signs and symptoms of lung cancer

Sometimes lung cancer does not cause any signs or symptoms. It may be found during a chest x-ray done for another condition. Signs and symptoms may be caused by lung cancer or by other conditions.

- Chest discomfort or pain.
- A cough that doesn't go away or gets worse over time.
- Trouble breathing.
- Wheezing.
- Blood in sputum (mucus coughed up from the lungs).
- Hoarseness.
- Loss of appetite.
- Weight loss for no known reason.
- Feeling very tired.
- Trouble swallowing.
- Swelling in the face and/or veins in the neck.

1.1.6. Pathophysiology overview of lung cancer

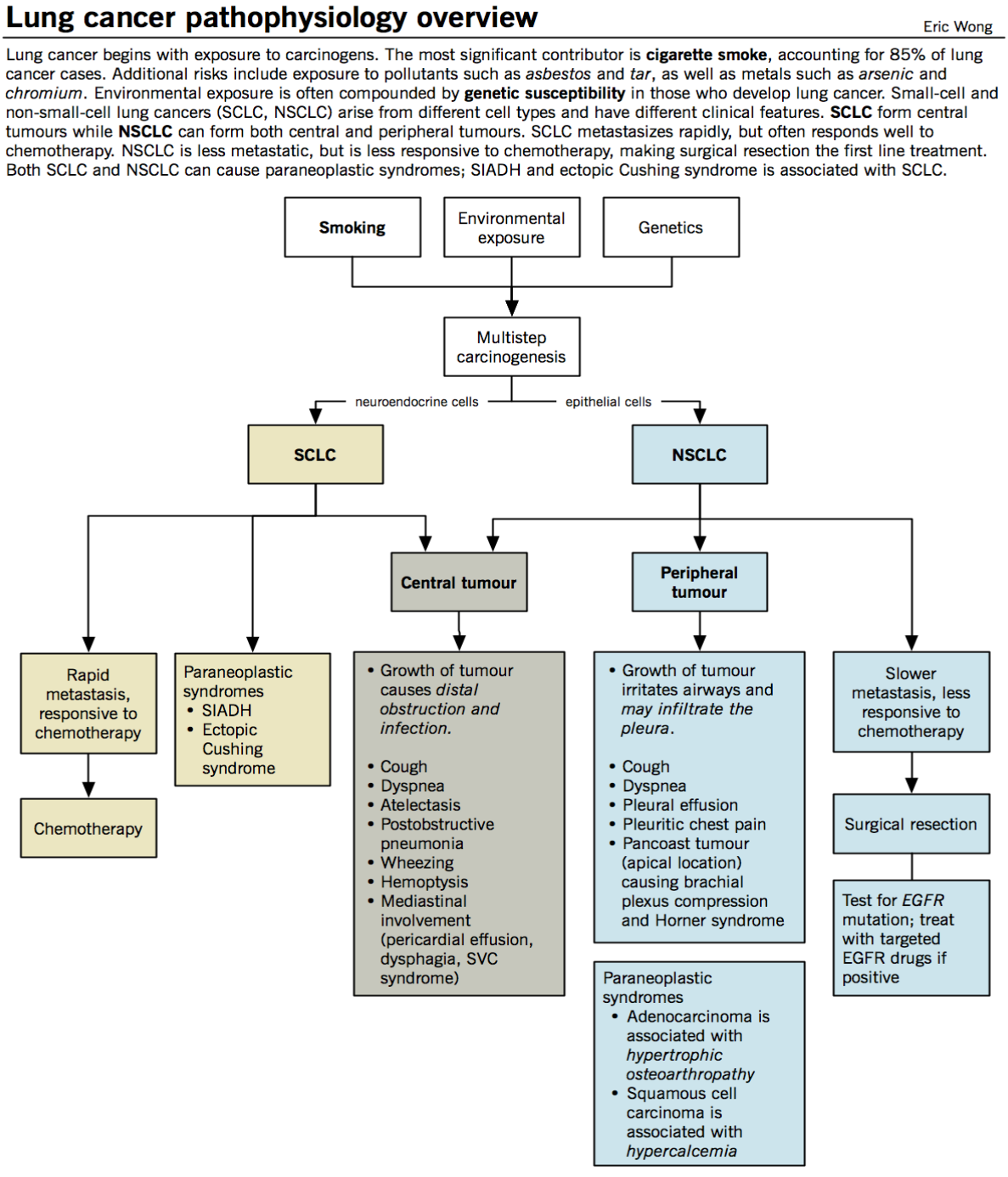

Figure 2 : Lung cancer pathophysiology overview *(McMaster Pathophysiology Review/Oncology/Lung cancer)*

1.1.7. Treatment for Lung cancer

Surgery, chemotherapy and radiation are the standard treatment options for lung cancer depending on the stage of malignancy, respectability and overall performance.*(Chang A. 2011)*

Smoking cessation

Smoking increases the risk of pulmonary complications after surgery.

Three main interventions exist in addition to counseling and support:

- Nicotine replacement therapy (NRT) : can be purchased in many forms including gum and transdermal patch; all forms increases rate of quitting by 50-70%.
- Antidepressants : Bupropion and Nortriptyline are as effective as NRT; other antidepressants such as selective serotonin reuptake inhibitors (SSRI) are not effective.
- Nicotine receptor partial agonist : Varenicline is more effective than bupropion and NRT.

Surgery

- Surgical resection of the tumor and some normal tissue around it.
- First line of choice for NSCLC who are medically fit to undergo surgery.

Radiation therapy

- Indicated for patients with stage I, II, III NSCLC.
- In lung cancer, stages I, II, and III describe various sizes of primary tumor and lymph node involvement without distant metastasis. Any distant metastasis is automatically stage IV.
- Also used in combination with surgery for NSCLC and with chemotherapy for SCLC.

Chemotherapy

Chemotherapy is a first-line treatment for advanced stage of lung cancer which drugs are usually administered intravenously for systemic circulation. The use of chemotherapeutic drug is based on the principle of toxic compounds to inhibit the proliferation of cells growing at an abnormal rate.

- First line of treatment for SCLC, which are often disseminated upon clinical presentation.
- Also indicated for patients with more advanced stage of NSCLC (to improve survival, disease control or for palliative care).

When might chemotherapy be used?

Depending on the stage of non-small cell lung cancer (NSCLC) and other factors, chemo may be used in different situations:

- **Before surgery** (sometimes along with radiation therapy) to try to shrink a tumor. This is known as ***neoadjuvant therapy***.
- **After surgery** (sometimes along with radiation therapy) to try to kill any cancer cells that might have been left behind. This is known as ***adjuvant therapy*.**
- **Along with radiation therapy** (concurrent therapy) for some cancers that can't be removed by surgery because the cancer has grown into nearby important structures
- **As the main treatment** (sometimes along with radiation therapy) for more advanced cancers or for some people who aren't healthy enough for surgery.

Chemo is often not recommended for patients in poor health, but advanced age by itself is not a barrier to getting chemo.

1.1.8. Drugs used to Treat Non-Small Cell Lung Cancer

The chemotherapeutic drugs, most often used for NSCLC include :

- Cisplatin
- Carboplatin
- **Paclitaxel (Taxol)**
- Albumin-bound **paclitaxe**l (Abraxane: Paclitaxel Albumin-stabilized Nanoparticle)
- Docetaxel (Taxotere)
- Gemcitabine (Gemzar)
- Vinorelbine (Navelbine)
- Irinotecan (Camptosar)
- Etoposide (VP-16)
- Vinblastine
- Pemetrexed (Alimta)

Drug Combinations Used to Treat NSCLC are :

- Carboplatin - Taxol
- Gemcitabine - Cisplatin

Most often, treatment for NSCLC uses a combination of 2 chemo drugs. Studies have shown that adding a third chemo drug doesn't add much benefit and is likely to cause more side effects.

Single-drug chemo is sometimes used for people who might not tolerate combination chemotherapy well, such as those in poor overall health or who are elderly.

If a combination is used, it often includes cisplatin or carboplatin plus one other drug. Sometimes combinations that do not include these drugs, such as gemcitabine with vinorelbine or paclitaxel ,may be used. For people with advanced lung cancers who meet certain criteria, a targeted therapy drug such as bevacizumab (Avastin), ramucirumab (Cyramza), or necitumumab (Portrazza) may be added to treatment as well. Doctors give chemo in cycles, with a period of treatment (usually 1 to 3 days) followed by a rest period to allow the body time to recover. Some chemo drugs, though, are given every day. Chemo cycles generally last about 3 to 4 weeks.*(The diagnosis and treatment of lung cancer (http://guidance.nice.org.uk/cg121) -National Institute for Health and Clinical Excellence, 2011)*

1.1.9. Possible Side effects of Chemotherapeutics

Chemo drugs attack cells that are dividing quickly, which is why they work against cancer cells.But other cells in the body, such as those in the bone marrow (where new blood cells are made),the lining of the mouth and intestines, and the hair follicles, also divide quickly. These cells are also likely to be affected by chemo, which can lead to certain side effects.

The side effects of chemo depend on the type and dose of drugs given and how long they are taken.

Some common side effects include:

- Hair loss
- Mouth sores
- Loss of appetite
- Nausea and vomiting
- Diarrhea or constipation
- Increased chance of infections (from having too few white blood cells)
- Easy bruising or bleeding (from having too few blood platelets)
- Fatigue (from having too few red blood cells)

These side effects usually go away after treatment is finished. There are often ways to lessen these side effects. For example, drugs can be given to help prevent or reduce nausea and vomiting.

Some drugs can have specific side effects. For example, drugs such as cisplatin, vinorelbine, docetaxel or **paclitaxel** can cause nerve damage (peripheral neuropathy).
This can sometimes lead to symptoms (mainly in the hands and feet) such as pain, burning or tingling sensations, sensitivity to cold or heat, or weakness. In most people this goes away or gets better once treatment is stopped, but it may last a long time in some people. *(http://guidance.nice.org.uk/cg121)*

1.1.9. Challenges in treating Lung cancer

Low accumulation of therapeutic agents in the tumor site, and fear of high-dose treatment due to toxicity with severe adverse effects are the main obstacles in efficient lung cancer therapy.
The bio-barriers existing in the respiratory airway systems such as mucus, ciliated cells and resident macrophages are effective to limit the localization, penetration and adsorption of drugs in the lung. Standard chemotherapeutics are administered by intravenous (IV) injection. However, IVadministration can have an increased risk of toxicity. Furthermore, it has been reported that systemically administered IV chemotherapy may be less effective than other routes of delivery for the presence of the pleural blood barrier. Therefore, more effective chemo therapeutic regimens and routes of administration with low toxicity are still needed to effectively manage these patients.

1.2. Paclitaxel : A natural chemotherapeutic agent

Paclitaxel is a naturally derived anti-cancer drug thought to inhibit tumor growth by binding to tubulin. This blocks mitosis by promoting polymerization of microtubules and simultaneously inhibiting de-polymerization (a mitotic inhibitor).
It was discovered in a US National Cancer Institute program at the Research Triangle Institute in 1967 when Monroe E. Wall and Mansukh C. Wani isolated it from the bark of the Pacific yew tree, Taxus brevifolia and named it taxol. Later it was discovered that endophytic fungi in the bark synthesize paclitaxel. When it was developed commercially by Bristol-Myers Squibb (BMS), the generic name was changed to paclitaxel.
The anticancer drug paclitaxel (marketed as *Taxol*) is a taxane diterpene amide that is widely used with good therapeutic effects against various kinds of cancers, such as ovarian, breast, NSCLC (Non-small cell lung carcinoma) and esophageal cancers *(Dang C et al., 2008).* It is recommended in NICE guidance of June 2001 that it should be used for **non-small cell lung cancer (NSCLC)** in

patients unsuitable for curative treatment. The response rate to paclitaxel in lung tumors, however, is significantly lower, averaging only 30 to 40% *(Shimomura M et al. 2012)*.

Paclitaxel is a high molecular weight drug with very limited aqueous solubility, which prevents easy absorption into the blood vessels. In patients with pleural tumors or lung cancers with pleural metastasis, paclitaxel can be sustained in the cavity for a period of 48 hours after IP injection. Moreover, the anti-cancer efficacy of paclitaxel is positively correlated with its concentration, suggesting that higher local concentrations are desirable.(*Jie Li et al.,2015)*

Paclitaxel is a natural product with antitumor activity. TAXOL (paclitaxel) is obtained via a semi-synthetic process from *Taxus baccata (*English yew plant).

The chemical name for paclitaxel is 5β,20-Epoxy-1,2α,4,7β,10β,13α-hexahydroxytax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2*R*,3*S*)-*N*-benzoyl-3-phenylisoserine. Paclitaxel is a white to off-white crystalline powder with the empirical formula as **C47H51NO14** and a molecular weight of 853.9. It is highly lipophilic, insoluble in water, and melts at around 216–217° C.

1.2.1 Mechanism of Action

Paclitaxel is one of several cytoskeletal drugs that target tubulin. Paclitaxel-treated cells have defects in mitotic spindle assembly, chromosome segregation, and cell division. Unlike other tubulin-targeting drugs such as colchicine that inhibit microtubule assembly, paclitaxel stabilizes the microtubule polymer and protects it from disassembly. Chromosomes are thus unable to achieve a metaphase spindle configuration. This blocks the progression of mitosis and prolonged activation of the mitotic checkpoint triggers apoptosis or reversion to the G-phase of the cell cycle without cell division.

The ability of paclitaxel to inhibit spindle function is generally attributed to its suppression of microtubule dynamics, but recent studies have demonstrated that suppression of dynamics occurs at concentrations lower than those needed to block mitosis. At the higher therapeutic concentrations, paclitaxel appears to suppress microtubule detachment from centrosomes, a process normally activated during mitosis. Paclitaxel binds to beta-tubulin subunits of microtubules.

1.2.2 Indications and usage

Paclitaxel (PTX) is one of the most effective anticancer drugs and has been clinically used for the treatment of a wide range of tumors, including breast, ovarian, lung, and head-and-neck cancers.

TAXOL is indicated ,

- As first-line and subsequent therapy for the treatment of advanced carcinoma of the ovary, as first-line therapy, TAXOL is indicated in combination with cisplatin.

- As the adjuvant treatment of node-positive breast cancer administered sequentially to standard doxorubicin-containing combination chemotherapy.

- As the treatment of breast cancer after failure of combination chemotherapy for metastatic disease or relapse within 6 months of adjuvant chemotherapy. Prior therapy should have included an anthracycline unless clinically contraindicated.

- Taxol in combination with cisplatin,is indicated for the first-line treatment of **non-small cell lung cancer i**n patients who are not candidates for potentially curative surgery and/or radiation therapy.

- And also it is indicated for the second-line treatment of AIDS-related Kaposi's Sarcoma.

1.2.3. The possible side effects of TAXOL (Paclitaxel)

Serious allergic reactions (anaphylaxis) can happen in people who receive TAXOL. Anaphylaxis is a serious medical emergency that can lead to death and must be treated right away.

- severe stomach pain
- low red blood cell count (anemia) feeling weak or tired
- hair loss
- numbness, tingling, or burning in hands or feet (neuropathy)
- joint and muscle pain
- nausea and vomiting
- hypersensitivity reaction - trouble breathing; sudden swelling of your face, lips, tongue
- mouth or lip sores (mucositis)
- infections
- swelling hands, face, or feet

- bleeding events
- irritation at the injection site
- low blood pressure (hypotension)

(*Summary of product characteristics of Pactitaxel Bristol-Myers Squibb Company Princeton, NJ 08543 USA)*

1.2.3 Novel formulation strategies for Paclitaxel delivery

Paclitaxel (PTX) exhibits very low solubility in water (0.01 mg/mL) as well as in most suitable pharmaceutical solvents, causing various formulation problems. Taxol®, the conventional clinical formulation of PTX consisting of Cremophor EL and ethanol, is diluted prior to administration via slow infusion to avoid PTX precipitation in the blood circula.tion. The presence of Cremophor EL, however, causes a number of side effects in cancer patients, including hypersensitivity reactions, nephrotoxicity, and neurotoxicity *(Rowinsky EK et al. 1994).* As a result significant research effort has been devoted to developing an alternative, Cremophor EL-free PTX formulation to alleviate vehicle –related side effects and at the same time improve the chemotherapeutic efficacy of paclitaxel.

Notably much interest has been focused on developing nanotechnology based paclitaxel formulations strategies such as emulsions, polymeric micelles, solid lipid nanoparticles, liposomes, dendrimers, PTX-polymer conjugates. In 2006, the FDA approved Abraxane a Cremophor EL free formulation of human serum albumin-bound paclitaxel nano- particles (average size of 130nm) for treating recurrent metastatic breast cancer. *(Gradishar WJ. 2006)*

Although, Abraxane® has been shown to improve the tumor response, the pharmacokinetic properties of Abraxane® are no better than those of the Taxol® formulation *(Sparreboom A. et al. 2005).* This may be due to the poor colloidal stability of Abraxane® during blood circulation. Indeed, upon i.v. injection and subsequent dilution in the large volume of blood, Abraxane® disassembles, allowing PTX to dissociate and resulting in a concentration curve and nonspecific bio distribution similar to those associated with free PTX *(Bernabeu E. et al 2014)*

Consequently, there is a tremendous interest in developing intelligent delivery systems that can maintain their nanostructure assembly during circulation, thus providing sustained blood levels.

Such systems would be preferentially distributed into tumor tissues by so called enhanced permeability and retention (EPR) effect and thus maximize the therapeutic efficacy of PTX while minimizing side effects.

1.3. Application of Nanotechnology in drug delivery

Nanotechnology is generally defined as the science and engineering of constructing and assembling objects on a scale smaller than one hundred nanometers (*Sajja HK et al. 2009*). In simple terms, a nanometer is one billionth of a meter and the properties of materials at this atomic or subatomic level differ significantly from properties of the same materials at larger sizes. Also the properties of the material changes when it is converted to the nanosize range and some of the prominent changes include increase in surface area, dominance of quantum effects which are associated with minute sizes, variations in materials' magnetic and electrical properties etc.

Although, the initial properties of nanomaterials studied were for its physical, mechanical, electrical, magnetic, chemical and biological applications, and now attention has been geared towards its pharmaceutical application, especially in the area of drug delivery.

In pharmaceutical field nanotechnology has revolutionized drug delivery system in the form of nanoparticles, nanoliposomes, nanoshells, nanocapsules, nanotubes, dendrimers, quantum dots,etc *(Mukherjee et al., 2014).* Nanotechnology offers drug in nanometer size range which improves the performance in a variety of dosage form. Various advantages associated with nanosizing include increased surface area, improved drug solubility, increased rate of dissolution, increased oral bioavailability, reduction in amount of drug etc.

Nanotechnology has created tremendous impact in various fields in medicine like immunology, cardiology, oncology, ophthalmology, pulmology etc. Also nanotechnology has a tremendous role in highly focussed areas like brain targeting, tumour targeting, gene delivery etc *(Martin et al.,2012)*

Nanotechnology has been firmly focusing in to the area of drug delivery. Drug delivery through the nanotechnology is continuously improved and maximize therapeutic activity and minimize undesirable side-effects and toxicities. *(Safari et al. 2014)* in their review article described the advanced drug delivery systems based on micelles, polymeric nanoparticles, and dendrimers. Polymeric nanoparticles, carbon nanotubes and many others demonstrate a broad variety of useful properties.

1.3.1. Rationale for development of nano based drug delivery systems.

- It is difficult to use large size materials in drug delivery because of their poor bioavailability, in vivo solubility, stability, intestinal absorption, sustained and targeted delivery, plasma fluctuations, therapeutic effectiveness etc. To overcome these challenges nanodrug delivery have been designed through the development and fabrication of nanostructures. Nanoparticles have the ability to penetrate tissues, and are easily taken up by cells, which allows efficient delivery of drugs to target site of action. Uptake of nanostructures has been reported to be 15–250 times greater than that of microparticles in the 1–10 um range *(Martins et al, 2012).*
- Cell-specific targeting can be achieved by attaching drugs to individually designed carriers. Various polymers have been used in the design of drug delivery system as they can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing side effects
- Recent developments in nanotechnology have shown that nanoparticals (structures smaller than 100 nm in at least one dimension) have a great potential as drug carriers. Due to their small sizes, the nanostructures exhibit unique physicochemical and biological properties (e.g., an enhanced reactive area as well as an ability to cross cell and tissue barriers) that makes them a favorable material for biomedical applications *(Agnieszka et al, 2012).*
- Nanocarriers with optimized physicochemical and biological properties are taken up by cells more easily than larger molecules, so they can be successfully used as delivery tools for currently available bioactive compounds *(Suri et al, 2007).*
- Nanoparticles can mimic or alter biological processes (e.g., infection, tissue engineering, de novo synthesis, etc.). These devices include, but not limited to, functionalized carbon nanotubes, nanofibers, self-assembling polymeric nanoconstructs, nanomembranes, and nano-sized silicon chips for drug, protein, nucleic acid, or peptide delivery and release, and biosensors and laboratory diagnostics (Singh et al, 2009).
- The controlled release (CR) of pharmacologically active agents to the specific site of action at the therapeutically optimal rate and dose regimen has been a major goal in designing such devices.

1.3.2. Contribution of Nano technology in treating cancer

Recent advances in the application of nanotechnology in medicine, often referred to as nanomedicine, may revolutionize our approach to healthcare. Cancer nanotechnology is a relatively novel interdisciplinary area of comprehensive research that combines the basic sciences, like biology and chemistry, with engineering and medicine. Nanotechnology involves creating and utilizing the constructs of variable chemistry and architecture with dimensions at the nanoscale level comparable to those of biomolecules or biological vesicles in the human body. Operating with submolecular interactions, it offers the potential for unique and novel approaches with a broad spectrum of applications in cancer treatment including areas such as diagnostics, therapeutics, and prognostics. Nanotechnology also opens pathways to developing new and efficient therapeutic approaches to cancer treatment that can overcome numerous barriers posed by the human body compared to conventional approaches. The main drawback of anticancer drugs are that they have a large volume of distribution and they are toxic to both tumor and normal cells and as a result precise drug release into specified target is highly essential in order to overcome this adverse effects. With the use of nanotechnology, targeting of anticancer drugs to specified target have become possible thereby maximizing the therapeutic effect and minimizing other unwanted adverse effects. This has been possible because of the small size of these particles which can penetrate across different barriers with the help of small capillaries into individual cells. Improvement in chemotherapeutic delivery through enhanced solubility and prolonged retention time has been the focus of research in nanomedicine. The submicroscopic size and flexibility of nanoparticles offer the promise of selective tumor access. Formulated from a variety of substances, nanoparticles are configured to transport myriad substances in a controlled and targeted fashion to malignant cells while minimizing the damage to normal cells. They are designed and developed to take advantage of the morphology and characteristics of a malignant tumor, such as leaky tumor vasculature, specific cell surface antigen expression, and rapid proliferation. Nanotechnology offers a revolutionary role in both diagnostics (imaging, immune-detection) and treatment (radiation therapy, chemotherapy, immunotherapy, thermotherapy, photodynamic therapy, and anti-angiogenesis).In recent years, nanomedicine has exhibited strong promise and progress in radically changing the approach to cancer detection and treatment (*Bhandare and Narayana, 2014).*

1.3.1. Different types of Nanocarriers used in drug delivery systems.

Nanoliposomes

Nanoliposomes are nano-sized version of liposomes. Liposomes that have vesicles in the range of nanometers are called nanoliposomes *(Zhang et al, 2006).*

Liposomes are vesicular structure with an aqueous core surrounded by a hydrophobic bilayer created by the extrusion of phospholipids. Phospholipids are GRAS (generally recognized as safe) ingredients, therefore minimizing the potential for adverse effects. Solutes, such as drugs, in the core cannot pass through the hydrophobic bilayer however hydrophobic molecules can be absorbed into the bilayer, enabling the liposome to carry both hydrophilic and hydrophobic molecules. The lipid bilayer of liposomes can fuse with other bilayers such as the cell membrane, which promotes release of its contents, making them useful for drug delivery and cosmetic delivery applications. The advantages of liposomes are they are non-toxic, flexible, biocompatible, biodegradable and nonimmunogenic for systemic and non systemic administration. Liposomes are also surface modified in order to target drugs to diseased tissue or organs.

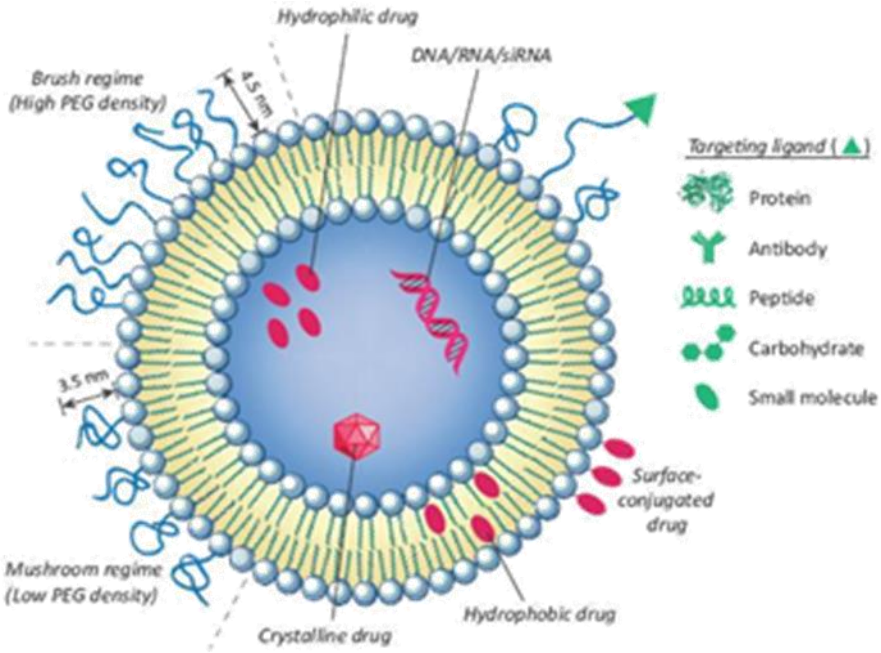

Figure 3: Schematic diagram of Nanoliposome (https:// *www.intechopen.com/books/application-of-nanotechnology-in-drug-delivery/liposomes-as-potential-drug-carrier-systems-for-drugdelivery)*

Polymeric nanoparticles.

Polymeric nanoparticles (PNPs) are structures with a diameter ranging from 10 to 100 nm. The PNPs are obtained from synthetic polymers, such as poly e-caprolactone, polyacrylamide and polyacrylate, or natural polymers, e.g., albumin, DNA, chitosan, gelatin. Based on in vivo behavior, PNPs may be classified as biodegradable, i.e., poly (L-lactide) (PLA), polyglycolide (PGA), and non-biodegradable, e.g., polyurethane. Drugs can be immobilized on PNPs surface after a polymerization reaction *(Luo et al, 2011)* or can be encapsulated on PNP structure during a polymerization step *(Mora-Huertas et al, 2010)*. Nanocarriers composed of biodegradable polymers undergo hydrolysis in the body, producing biodegradable metabolite monomers, such as lactic acid and glycolic acid. Drug-biodegradable polymeric nanocarrier conjugates used for drug delivery are stable in blood, nontoxic, and non-thrombogenic. They are also non-immunogenic as well as non pro inflammatory, and they neither activate neutrophils nor affect reticuloendothelial system (*Des Rieux et al, 2006*).

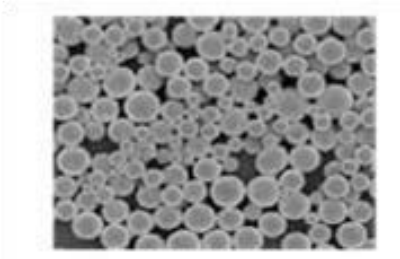

Figure 4 : Schematic diagram of nanoparticles *(Mukherjee et al., 2015)*

Solid lipid nanoparticles (SLNs)

SLNs (solid lipid nanoparticles), NLC (nanostructured lipid carriers) and LDC (lipid drug conjugates) are types of carrier systems based on solid lipid matrix, i.e., lipids solid at the body temperature *(Wissing et al, 2004)*. SLNs are particles made of solid lipids, e.g., highly purified triglycerides, complex glyceride mixtures or waxes stabilized by various surfactants (*Mukherjee et al, 2009*). The main characteristics of SLN include a good physical stability, protection of incorporated drugs from degradation, controlled drug release, and good tolerability. They have been exploited for the dermal, peroral, parenteral, ocular, pulmonary, and rectal delivery.

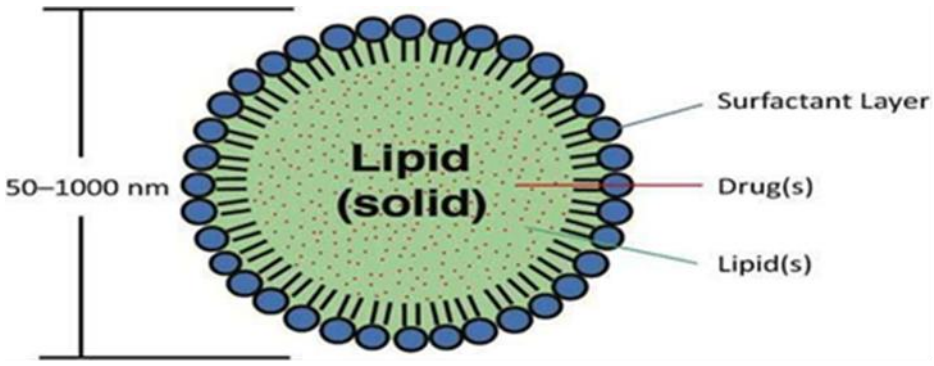

Figure 5 : Schematic diagram of solid lipid nanoparticles
(http://www.eurekaselect.com/137264/article)

Fullerenes

A fullerene is any molecule composed entirely of carbon, in the form of a hollow sphere, ellipsoid, or tube. Spherical fullerenes are also called bucky balls, and cylindrical ones are called carbon nanotubes or bucky tubes. Fullerenes are similar in structure to the graphite, which is composed of stacked grapheme sheets of linked hexagonal rings; additionally they may also contain pentagonal (or sometimes heptagonal) rings to give potentially porous molecules. (*Mudshinge et al, 2011).*

In September 1985, Robert F. Curl, Jr., Richard E. Smalley, and Harold W. Kroto discovered fullerene C_{60} during laser spectroscopy experiments at Rice University *(Pikhurov et al., 2013)*.The main disadvantage of fullerenes in drug delivery is their poor solubility. In order to overcome this barrier chemically modified fullerenes have been used as a promising vehicle for drug delivery *(Saeedfar et al., 2013).*

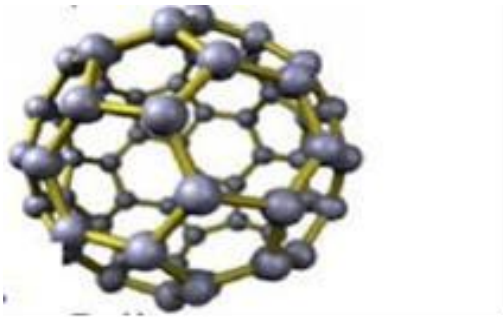

Figure 6 : Schematic diagram of Fullerene *(Mukherjee et al., 2015)*

Nanosuspension

Nanosuspension is defined as a suspension of nanoparticles in a liquid medium and the size of nanoparticle lies in between 200 to 500 nm. Some of the advantages of nanosuspension include increased saturation, solubility, increased adhesiveness to tissue. The oral administration of drug in the form of nanosuspension has been reported to enhance absorption rate and bioavailability *(Gupta et al., 2006).*

Nanomulsion

Nanoemulsions are dispersions of oil and water in which the dispersed phase droplets are in the nanosize range and it is stabilized with the help of a surfactant. It is a transparent or translucent system having a dispersed phase droplet size range of typically 20 to 200 nm (*Mishra et al., 2014).*

Nanogels

Nanogels are cross linked polymeric particles that can be considered as hydrogels if they are composed of water soluble or swellable polymer polymer chains. The unique features of nanogels are that they possess high water content, they are biocompatible and desirable mechanical properties. Also their size can be tailor made from micrometers to nanometers, they possess a large surface area for multivalent bioconjugation and an interior network for the incorporation of biomolecules *(Oh et al., 2008).*

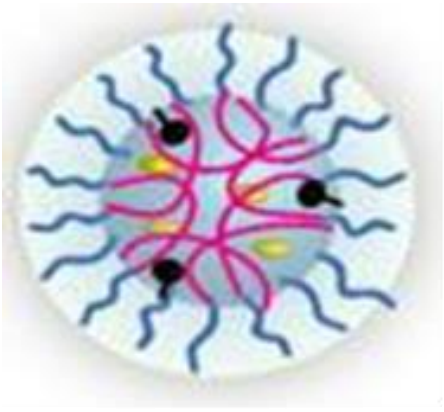

Figure 7: Schematic diagram of nanogels (*https://elements.chem.umass.edu)*

Nanoshells:

Nanoshells are spherical cores of a particular compound (concentric particles) surrounded by a shell or outer coating of thin layer of another material, which is few nanometers (1–20 nm) thick. Nanoshells are the recent modified forms of targeted therapy having core of silica with a metallic outer layer. The properties of nanoshells can be modified by adjusting the core to shell ratio.

Nanoshell materials can be synthesized from semiconductors (dielectric materials such as silica and polystyrene), metals and insulators. Usually dielectric materials such as silica and polystyrene are commonly used as core because they are highly stable *(Kalele et al, 2006).* Nanoshells possess highly favorable optical and chemical properties for biomedical imaging and therapeutic applications. Nanoshells offer other advantages over conventional organic dyes including improved optical properties and reduced susceptibility to chemical/thermal denaturation *(Loo et al, 2004).*

Quantum dots (QD)

Quantum dots are 1-10 nm in size semiconductor crystals and are made up of compounds of the element belonging to the group III to V and II to VI of the periodic table. Such elements include Hg, Cd, Ag, Pb, Zn, Se, Te etc. Tumour specific targeting agent that can bind to the active binding site of the cancerous cells can be conjugated with quantum dots for the purpose of active targeting *(Mishra et al., 2011).* DNA interacting drugs like anticancer drug doxorubicin was immobilized onto quantum dots *(Wang et al., 2010).* Many studies have also revealed that quantum dot-conjugated oligonucleotide sequences which are attached to it by specific surface carboxylic acid groups can be targeted to bind DNA or mRNA *(Pandurangan and Sravya.,2012)*

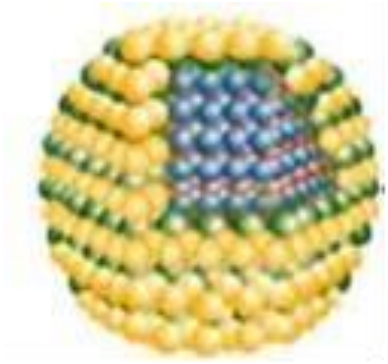

Figure 8 : Schematic diagram of quantum dots *(Mukherjee et al., 2015)*

Dendrimers

Dendrimers are branched, synthetic polymeric macromolecular nanocarriers having tree like architechture. They possess unique structural properties with a high degree of molecular uniformity, low polydispersity and size in the range of 1-10 nm *(Cameron et al., 2005)*. A dendrimer generally has three components i.e a central initiator core, the repeating units and terminal groups at the periphery. Dendrimers are synthesized from repeating units of monomers and unlike that of conventional polymerisation reaction,, dendrimers are made by step by step repetition of the monomer units (*Caminade and Turrin, 2014*). Dendrimers are synthesized by two types of method known as convergent and divergent synthesis methods. In convergent synthesis method the growth begins at the edge and is directing the production of synthesis towards the centre whereas in divergent synthesis method the development starts from the central core and is directed towards the edge radially. Dendrimers for drug delivery purpose serves as an excellent carrier for delivering both hydrophobic and hydrophilic drug and the drug is either physically entrapped inside the cavities of the dendrimer or it is covalently attached at the peripheral terminal group(s). In case of hydrophobic drugs they are normally incorporated into the non polar cavities of the dendrimer but suitable modification of the internal cavities of dendrimer also enable incorporation of hydrophilic drugs for drug delivery purpose *(Madaan et al., 2014).*

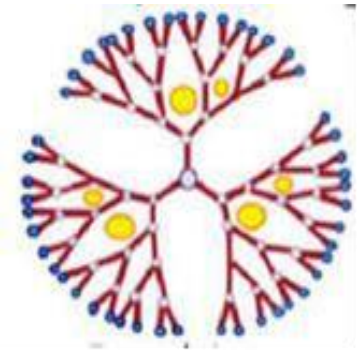

Figure 9 : Schematic diagram of **Dendrimer** *(Mukherjee et al., 2015)* **.**

Polymeric micelles

Polymeric micelles are colloidal particles of nano dimensions (usually 5-100nm) composed of an amphiphilic polymer which has both hydrophobic and hydrophilic units. In aqueous medium at low concentration these amphiphiles usually exist as monomers but at a certain concentration range these amphiphiles tend to aggregate to form unique structures called micelles (*Oerlemans et al., 2010).*

The narrow concentration range at which micelle formation takes place is referred to as critical micelle concentration.Polymeric micelles are used as drug delivery system for delivering hydrophobic anticancer drugs due to the presence of both hydrophobic and hydrophilic portion in the molecule. Since majority of the anticancer drugs are hydrophobic solubilisation of these drugs occur in the core of micelles which help to improve the bioavailability of this drugs. Many polymeric micelles for anticancer therapy are currently under clinical trials such as SP1049C, Genexol-PM etc (*Tan et al., 2012).*

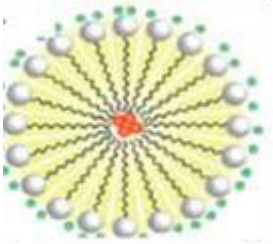

Figure 10 : Schematic diagram of Polymeric micelle *(Mukherjee et al., 2015)*

Carbon nanomaterials

Carbon nanocarriers used in DDS are differentiated into nanotubes (CNTs) and nanohorns (CNH). CNTs are characterized by unique architecture formed by rolling of single (SWNCTs – single walled carbon nanotubes) or multi (MWCNTs – multi walled carbon nanotubes) layers of graphite with an enormous surface area and an excellent electronic and thermal conductivity (*Beg et al, 2011*). There are three ways of drug immobilization in carbon nanocarriers, which are: encapsulation of a drug in the carbon nanotube (*Arsawang et al, 2011 and Tripisciano et al, 2010),* chemical adsorption on the surface or in the spaces between the nanotubes (by electrostatic, hydrophobic, π-π interactions and hydrogen bonds) (*Chen et al, 2011 ; Zhang et al, 2014),* and attachment of active agents to functionalized carbon nanotubes (f-CNTs). Nanohorns – a type of the only single-wall nanotubes – exhibit similar properties to nanotubes. Their formation process does not require a metal catalyst, thus, they can be easily prepared with very low cost and are of high purity.

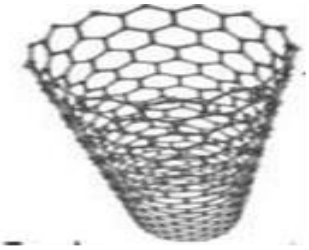

Figure 11 : Schematic diagram of carbon nanotube *(Mukherjee et al., 2015)* **.**

Superparamagnetic nanoparticles

Superparamagnetic molecules are those that are attracted to a magnetic field but do not retain residual magnetism after the field is removed. Nanoparticles of iron oxide with diameters in the 5–100 nm range have been used for selective magnetic bioseparations. Superparamagnetic nanoparticles belong to the class of inorganic based particles having an iron oxide core coated by either inorganic materials (silica, gold) or organic materials (phospholipids, fatty acids, polysaccharides, Peptides or other surfactant and polymers) *(Gupta et al.,2004;Babic et al.2008)*

In contrast to other nanoparticles, supramagnetic nanoparticles based on their inducible magnetization, their magnetic properties allow them to be directed to a defined location or heated in the presence of an externally applied AC magnetic field. These characteristics make them attractive for many applications, ranging from various separation techniques and contrast enhancing agents for MRI to drug delivery systems, magnetic hyperthermia (local heat source in the case of tumor therapy), and magnetically assisted transfection of cells (*Hora´ k, 2005; Jordan et al, 2001 and Neuberger et al, 2005).*

Nanofibres

Nanofibres have size in between 100-200nm and they are generally obtained from natural and synthetic biocompatible polymers *(Peresin et al., 2010).* There are several methods for the preparation of nanofibres such as template synthesis, drawing, self-assembly, electro-spinning, phase separation, melt-blown etc. A wide range of polymers such as gelatine, collagen, polyvinyl alcohol, chitosan and carboxymetthylcellulose can be subjected to electro-spinning techniques to produce nanofibres. Nanofibres for their application in the field of drug delivery should be made of biocompatible polymer of natural or synthetic origin. Natural polymers include chitosan, cellulose, poly(L-lactic acid) whereas synthetic polymers include polly(caprolactone), poly(vinyl alcohol) and poly(ethylene oxide) *(Vasita and Katti, 2006).*

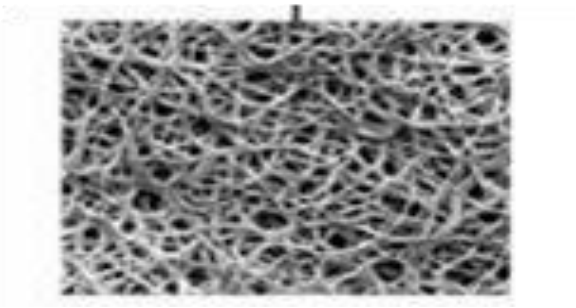

Figure 12 : Schematic diagram of nanofibres *(Mukherjee et al., 2015)*

1.4. Nanoliposome : A promising drug delivery system

Nanoliposomes can be defined as submicrometric bilayer vesicles mainly composed of phospholipid molecules. Nanoliposomes (NLs) are the nanometric version of liposomes (Ls). The word nanoliposome is derived from an older terminology known as "liposome", which means "lipid structure" (i.e. lipos: fat, and soma: body). With the advancement in the scientific field of nanotechnology, the word nanolipo-some has been introduced to exclusively refer to nanoscale lipid vesicles, since liposome is a general word covering many classes of phospholipid vesicles with diameters in the size range of tens of nanometers to several micrometers *(Khorasani S et al 2018).* Though Ls in the size range between 1-1000 nm are generally considered under NLs, but for medical and neuroscience applications, NLs of size within 10-100 nm (preferably in ~50 nm range) are the preferred range. Nanoliposomes (NLs) are nanometric version of Ls, that are composed of one or more bilayers of phospholipids enclosing equal number of aqueous compartments *(Santra et al., 2010*).

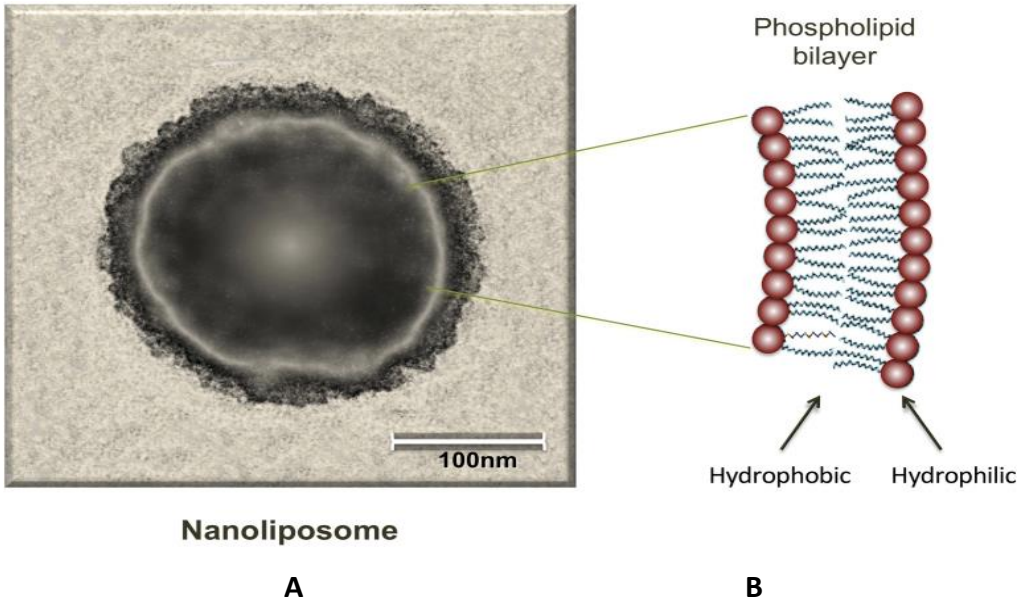

Figure 13 : A) A single nanoliposome visualized by energy filtered transmission electron microscopy (EFTEM). B) Schematic enlargement of a section of the phospholipid bilayer of the nanoliposome shows positions of the hydrophilic and hydrophobic regions of the bilayer. (*https://doi.org/10.1016/j.heliyon.2018.e01088).*

The internal compartment of nanoliposomes is filled with an aqueous media such as de-ionized water, a buffer or an isotonic saline solution in which one or more hydrophilic compound(s) can be dissolved. Such vesicles which have one or more phospholipid bilayer membranes with inner aqueous core can transport both aqueous or lipid drugs, depending on the nature of those drugs *(Yang, 2010).* Usually hydrophilic drugs can be incorporated in aqueous compartment and lipophilic drugs are incorporated in the phospholipid layer.

Due to their unique structures and superior biocompatibility, nanoliposomes are being used extensively as nanocarriers for drug delivery, protein and peptide delivery, food fortification, cosmetics and gene therapy applications (*Maherani et al ., 2011, Sharma et al 2018 and Amoabediny et al.,2018).* They improve the efficiency of a vast variety of bioactive agents, including pharmaceutical, nutraceutical and cosmeceutical compounds, by preserving the functionality of the encapsulated materials as well as targeting them to particular cells or tissues *(Maherani et al ., 2011)*. In addition to phospholipids, nanoliposomes can incorporate other molecules such as cholesterol, antigens, polymers and antioxidants in their structure. These excipients assist in improving the stability and shelf life of the formulation or targeting the nanoliposomes where their effect is needed in vitro or in vivo *(Amoabediny et al.,2018 ; M R Mozafari et al .,2018)*.

Medical and pharmaceutical applications of nanoliposomes can be mainly classified into diagnostic and therapeutic applications. These could be achieved by the employment of nanoliposomes containing various markers or incorporating different drugs or vaccines.

Nanoliposomes can also be used as a tool, a model for cell membranes, or a reagent in the basic studies of cellular interactions, recognition processes, and mode of action of certain therapeutic agents (*Mozafari and Mortazavi, 2005*; *Sharma et al., 2018; Danaei M et al., 2018*). In addition to the medical and pharmaceutical applications, nanoliposomes are being used for the encapsula-tion, delivery and controlled release of food material and nutraceuticals. These include omega fatty acids ,various dairy products as well as vitamins and other health benefit compounds. (*Khorasani et al., 2018)*

Nanoliposomes are one of the best drug delivery systems for low molecular weight drugs, imaging agents, peptides, proteins, and nucleic acids. Nanoliposomes are able to enhance the performance of bioactive agents by improving their bioavailability, in vitro and in vivo stability, as well as preventing their unwanted interactions with other molecules *(Mozafari., 2010).*

1.4.1. Nanoliposomes as carriers for anticancer drug delivery

It is believed that the efficient antitumor activity can be attributed to the selective delivery and the preferential accumulation of the liposome nanocarrier in the tumor tissue via the enhanced permeability and retention effect. Among all the nanomedicine platforms, liposomes have demonstrated one of the most established nanoplatforms, with several FDA-approved formulations because of their size, biodegradability, hydrophobic and hydrophilic character, low toxicity and immunogenicity (*Jie et al., 2011).* Several anticancer drugs like **paclitaxel,** doxorubicin have been successfully formulated using liposomes and biodegradable polymers as drug delivery system *(Mansour et al., 2009).*

Nanoliposomes as carriers have certain advantages,

- **Biocompatible ingredients:** The formulation ingredients (excipients) of liposome preparation are mainly phospholipids and cholesterol, which are the components of biological system, so they are easily accepted by the body.
- **Improved pharmacokinetic profile:** Liposomal drug delivery increases circulation lifetime of therapeutic agents and reduces their elimination especially for PEGylated liposomes.
- **Stability:** As drug is encapsulated into the vesicles (for hydrophilic drug) or stored into the bilayer (for hydrophobic drug) they are protected against degradative action of many enzymes.
- **Targeted delivery:** It provides passive targeting to the selected tissues.
- **Active targeting:** It is possible to attach several targeting ligands such as antibody, aptamer with liposome to achieve active targeting.
- **Wide range of drug entrapment:** Both hydrophilic and lipophilic drugs can be incorporated into the liposome which allows delivery of a wide range of drug.
- **Low drug requirement:** Liposomal delivery requires relatively very low amount of drug which increases therapeutic index & reduces adverse effects.
- **Cellular drug delivery:** Liposome is capable of entering into the cell through endocytosis that allows intracellular drug delivery. Intracellular drug delivery is also possible through fusion of liposome i.e., merging of vescicular membrane with cellular membrane.
- **Site avoidance effect:** Site avoidance effect can be achieved easily by liposomes.

Nanoliposomes can also provide slow release of an encapsulated drug, resulting in sustained exposure to the site of action and enhanced efficacy. On the other hand unlike liposome nanoliposome does not undergo rapid degradation and clearance by liver macrophages.

Further, nanoliposomes at the size range (10-100 nm) show many added advantages such as higher internalization in tumor tissue (EPR effect), improved pharmacokinetics profile, smart escape from reticuloendothelial system (RES), better *in vivo* stability, prolonged and site specific delivery etc. *(Wang et al, 2011).*

For the targeted drug delivery, nanoliposome plays a vital role. It can be used for passive targeting or active targeting. Surface of NLs can be easily modified with targeting ligands such as small molecules, peptides, monoclonal antibodies for site-specific drug delivery, with an overall increase in therapeutic window with reduced toxicity to healthy cells (*Chu et al, 2013).*

NLs are generally considered to be pharmacologically safe with minimal/negligible toxicity, as they are mostly composed of phospholipids, which are the natural components of cell membrane. Such cell-membrane mimicking nature of Liposomess further helps their passive targeting to cells/tissue via EPR effect (*Maeda et al, 2000).*

1.4.2. Preparation of Nanoliposomal formulations

The term nanoliposome has recently been introduced to exclusively refer to nanoscale lipid vesicles. Nanoliposomes have the same physical, structural, thermodynamic properties manufacturing and mechanism of formation as the liposomes. The underlying mechanism for the formation of liposomes and nanoliposomes is basically the hydrophilic-hydrophobic interaction between phospholipids and water molecules *(Khosravi-Darani and Mozafari., 2010).*

Mechanism of Formation

Liposome forming materials like phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) etc. have a hydrophilic polar head group (choline, inositol, ethanolamine, serine etc.) and a nonpolar tail group consisting of two fatty acid chains. These two fatty acid chains give the molecule an overall cylindrical shape. These phospholipid molecules when exposed to aqueous media, their polar head groups arrange themselves towards aqueous media and the hydrophobic tail portions attach to each other side by side to reduce oil-water interaction.

Due to their tubular shape this type of arrangement results in formation of planar lamellae. When two such lamellae join to each other, facing both hydrophilic side towards water phase, a bilayer is formed. In such a bilayer oil-water interaction is minimized and present only in the terminal regions. This interaction abolishes when the terminal regions of a bilayer joins to form a spherical vesicle, enclosing an aqueous compartment. This vesicle (liposome) may be further enclosed by another bilayer to form bilamellar vesicle. Further enclosing of this liposome by a number of bilayer results in formation of plurilamellar or multilamellar vesicles.

Factors to be considered for a successful formulation

The physicochemical properties of nanoliposomes, particularly chemical composition, phase transition, morphology, size, polydispersity index, number of lamellae, surface charge, density of the ligands immobilized on their surface, and drug encapsulation efficiency, are determinative factors for their successful clinical application. An important parameter in the design and manufacture of nanoliposomal formulations is phase transition temperature (Tc) that is an indication of the thermal behavior, dynamic properties and stability of the lipid nanovesicle *(Danaei M et al., 2018)*

Selection of Materials, Ingredients and Solvents

The main characteristics of nanoliposomes strongly depend on the selection of ingredients, solvents and co-solvents for their formulation. These properties include permeability, surface activity, electrical charge, stability, and safety of the nanovesicles. The bilayer of nanoliposomes is predominantly made up of phospholipid molecules. The hydrophilic section of the phospholipids is oriented towards the internal and external aqueous phases and the hydrophobic groups associate with their counterparts on the other phospholipid molecules. Curving of the bilayer sheet into a spherical structure forms a very stable construction due to the lack of chemical interaction of the phospholipids with the aqueous medium. Therefore, it can be postulated that the mechanism of the formation of the lipidic nanovesicles is the hydrophilic-hydrophobic interactions and van der Waals forces between phospholipids and water molecules [,*Mozafari and Mortazavi.,,2005)*

Phospholipid ingredients

The most commonly employed phospholipid for the manufacture of nanoliposomes is lecithin (phosphatidylcholine), which is immiscible with water and is inexpensively isolated from egg yolk or soy. The composition of the phospholipid ingredients and the preparation method of nanoliposomes determine if a single or multiple bilayers are formed. Fatty acids also make up nanoliposomes and their degree of saturation depends on the source. Animal sources provide more saturated fatty acids.

These ingredients influence the phase transition temperature, which is the conversion from a gel to the more leaky liquid form. Sugars and large polar molecules cannot permeate through a nanoliposome bilayer. On the other hand, small lipophilic molecules can permeate through the phospholipid membrane.

Exipients

In addition to phospholipid molecules, nanoliposomes can incorporate other ingredients such as sterols in their structure. Sterols are important constituent of most natural membranes and their incorporation into nanoliposome bilayer can bring about major changes in the characteristics of the formulation. The most commonly employed sterol in the structure of the lipid vesicles is cholesterol. Cholesterol does not by itself form bilayer structures, however, it can be incorporated into phospholipid membranes in very high concentrations, e.g. up to 1:1 molar ratios of cholesterol to a phospholipid molecule (such as phosphatidylcholine) *(Maherani B and Wattraint O ,2017 ; Mozafari ,2010).* Cholesterol is used in nanoliposome formulations in order to increase their stability by modulating the fluidity of the phospholipid bilayer. It modifies membrane fluidity by preventing crystallization of the acyl chains of the phospholipid molecules and providing steric hindrance to their movement. This phenomenon contributes to the stability of nanoliposome formulation and reduces the permeability of their bilayer membrane to solutes. There are also scientific reports on the effect of cholesterol in increasing the size homogeneity and improving the polydispersity index of phospholipid vesicles *(Sebaaly C et al.,2016).*

Studies have revealed that phospholipid composition and cholesterol content are among the major parameters to be considered in the formulation of nanoliposomal products *(Mozafari et al.,2010).*

Nanoliposomes made of natural and herbal ingredients are particularly receiving increasing attention in the manufacture of medicinal and nutritional products. Phospholipids and sphingolipids, along with sterols (mainly cholesterol), are the choice of ingredients that are commonly used in the preparation of nanoliposomes. These ingredients are biocompatible, biodegradable and nontoxic. In

order to prevent or at least minimize oxidation of the phospholipid ingredients, antioxidant compounds can be incorporated into the structure of the nanovesicles.

A commonly used antioxidant in the formulation of liposome and nanoliposome products is alpha-tocopherol, which is a lipophilic molecule and as a result will be located in the lipidic phase of the vesicles. This antioxidant acts as a scavenger of free radicals and there by protects the susceptible ingredients and extends the stability and shelf life of the lipid vesicles *(,Danaei M et al., 2018;Mozafari et al.,2010).*

Solvents

Following rational selection of the nanoliposomal ingredients, appropriate solvents must be chosen based on the intended application, dosage form, route of administration, and method of preparation of the nanovesicles. The organic solvents generally employed in the classical methods of liposome and nanoliposome preparation (e.g. chlorinated solvents, diethyl ether, methanol or acetone) represent potential hazard to consumer's health due to their toxicity *[Mozafari et al., 2017].* Level of the residual organic solvents, which is acceptable in the finished product, depends on different factors such as the type of solvent and the route of administration of the therapeutic formulation. Although the organic solvents are usually removed from the product by vaccuum or evaporation, trace amounts may be present in the final formulation, potentially causing toxicity and influencing the stability of the nanovesicles. In order to solve these drawbacks, employment of alternative organic solvents is being considered by scientists and researchers in the field *(Rasti et al.,2017; Mozafari et al., 2007).*

Solvents or a detergent in nanoliposome manufacture necessitate performance of two additional steps during the manufacture process:

- Removal of these solvents and detergents, and:
- Assessment of the level of residual solvents, including the potentially toxic polar and non-polar protic and aprotic solvents, co-solvents or detergents remained in the final products.

(Mozafari et al.,2017).

The presence of unwanted chemicals and solvents, even in small amounts, may influence the efficacy, safety, and stability of the nanoliposomal products. The 'International Conference on Harmonization' (ICH) guideline, specific for residual solvents in pharmaceutical products can be used to determine acceptable levels of the mentioned chemicals remained in the finished product.

Phase transition temperature

Amphipathic molecules such as detergents and phospholipids can undergo a thermo-tropic phase transition at temperatures much lower than their melting point .When water comes in contact with the phospholipid bilayer of nanoliposomes it diffuses into the polar (ionic) region of the bilayer only when the temperature is reached at which the hydrocarbon chains of the phospholipid molecules "melt" (the transition temperature). If the temperature becomes higher than this, there will be a simultaneous dissociation of the ionic structure by the penetration of water molecules and melting of the hydrocarbon chain region of the phospholipid molecules. The temperature of transition (Tc) depends upon the nature of the hydrocarbon chains, the polar region of the molecule, the amount of water molecules present and on any solutes dissolved in the suspension medium of the vesicles. Once the water has penetrated into the vesicle bilayer and the sample is then cooled to a temperature below the Tc, the hydrocarbon chains rearrange themselves into an orderly crystal-line lattice. However, the water molecules will not necessarily be expelled from the system . Also known as "gel to liquid crystalline transition temperature", Tc is a temperature at which the phospholipid bilayers of nanoliposomes lose much of their ordered packing while their fluidity increases *(Mozafari et al 1998).*

An understanding of phase transitions and fluidity of phospholipid membranes is essential both in the manufacture and application of nanoliposomes. This is due to the fact that phase behavior of nanoliposomes determines important properties such as permeability, aggregation, fusion, deformability and protein binding, all of which can significantly affect the stability of the vesicles and their behavior in vitro and in vivo. Tc can be measured by a number of techniques including, electron spin resonance, fluores-cence probe polarization and differential scanning calorimeter (DSC). Nanoliposomes made of a pure phospholipid ingredient will not form at temperatures below Tc of the phospholipid molecule. This temperature requirement is reduced to some extent, but not eliminated, by the inclusion of cholesterol *(Mozafari et al.,2010)*.

There are adequate number of available phospholipids with different Tc values, which can be used in the manufacture of nanoliposomes.

Depending on the sensitivity of the drug or other bioactive molecules to be encapsulated, phospholipids with low Tc values can be selected to avoid the need to employ high temperatures during nanoliposome manufacturing process.

Zeta potential

Besides the phase transition property of their phospholipid ingredients, the surface charge of nanoliposomes could also be varied. They can be neutral or zwitterionic (by employing phospholipids such as phosphatidylcholine, or phosphatidylethanol-amine), negatively charged or anionic (when using acidic phospholipids such as phosphatidylserine, phosphatidylglycerol, phosphatidic acid, or dicetylphosphate) or they can be positively charged (by employing cationic lipids such as DOTAP, DOTMA, or stearylamine) in physiological pH ranges *(Mozafari, 2005)*. The net charge of the nanoliposomal formulation is an important parameter in terms of vesicle interaction with bioactive molecules. Utilizing the electrostatic attraction between oppositely charged bioactive compounds and lipid vesicles is a mean to increase encapsulation or entrapment efficiency. Therefore, for efficient entrapment of a positively charged molecule or compound an anionic nanoliposome could be employed and vice versa . From the cytotoxicity point of view, nanoliposome charge has been shown to have a very crucial role. There are many reports on the toxicity of positively-charged phospholipid vesicles. One reason for the toxicity of cationic vesicles is believed to be the interaction of the cationic lipids with cell organelle membranes, specifically the anionic lipids making up these bio-membranes. A postulated mechanism, for cationic lipid-mediated toxicity in the lung, is the involvement of reactive oxygen intermediates

(Dokka S et al.,2000). Negatively charged vesicles, however, are reported to be less cytotoxic or completely safe when compared to their cationic counterparts. Furthermore, it has been postulated that anionic vesicles, in general, associate more efficiently and are taken up more readily by the cells compared with neutral or zwitterionic vesicles , although, no clear mechanism has been pro-posed for this observation. Consequently, most FDA-approved therapeutic lipid drug formulations are negatively charged *(Kraft JC et al., 2014)*.The charge density of nanoliposomal surface and the binding affinity of various ions to the lipid vesicles can be determined by measuring a parameter called "zeta potential" (ZP). The ZP of a nanoliposome is the overall charge that the nanovesicle acquires in a particular environment or suspension medium *(Mozafari.,2010)*.

Preparation techniques of naoliposomes

Preparation of nanoliposome is more difficult than that of liposome and requires special methodology& instrumentation in most of the cases. Maintenance of particle size homogeneity and lamellarity are two major challenges of nanoliposome preparation.

There are several methods for preparation of different types of liposomes. The choice of method depends on the following parameters:

- The physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients.
- The nature of the medium in which the lipid vesicles are dispersed.
- The effective concentration of the entrapped substance and its potential toxicity.
- Additional processes involved during application/ delivery of the vesicles.
- Batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

Conventional methods

A number of classical procedures have been reported in the literature for nanoliposome preparation. These include, but not limited to, the following techniques and procedures:

- Thin-film hydration method (also known as lipid film hydration method/ Bangham method, which is the first method employed for liposome preparation synthetically)
- Solvent-injection technique
- Detergent dialysis
- Reversed phase evaporation
- Homogenization
- French pressure cell method

Downsizing procedures are performed to obtain monomodal nano-sized vesicles with narrow size distribution from a heterogeneous mixture of lipid vesicles. These methods include extrusion through filters of defined pore sizes, high-pressure homogenization, freeze-thawing and sonication. *(Maherani et al.,2011;Mozafari.,2010).*

However, the conventional method for preparing nanoliposomes including some basic steps, solubilizing the lipids in organic solvent(s), drying down the lipids from organic solvent, drying down the lipids from organic solution, dispersion of the lipids in an aqueous media, size reduction of the resultant liposomes the prepared multilamellar vesicles (MLVs) to nanosize small unilamellar vesicles (SUVs), collection of resultant SUVs and analysis of the final product(*Sandeep et al., 2013).*

Thin Film hydration / Lipid film hydration method

Of all the methods used for preparing NLs, thin film hydration method is the most simple and widely used one.

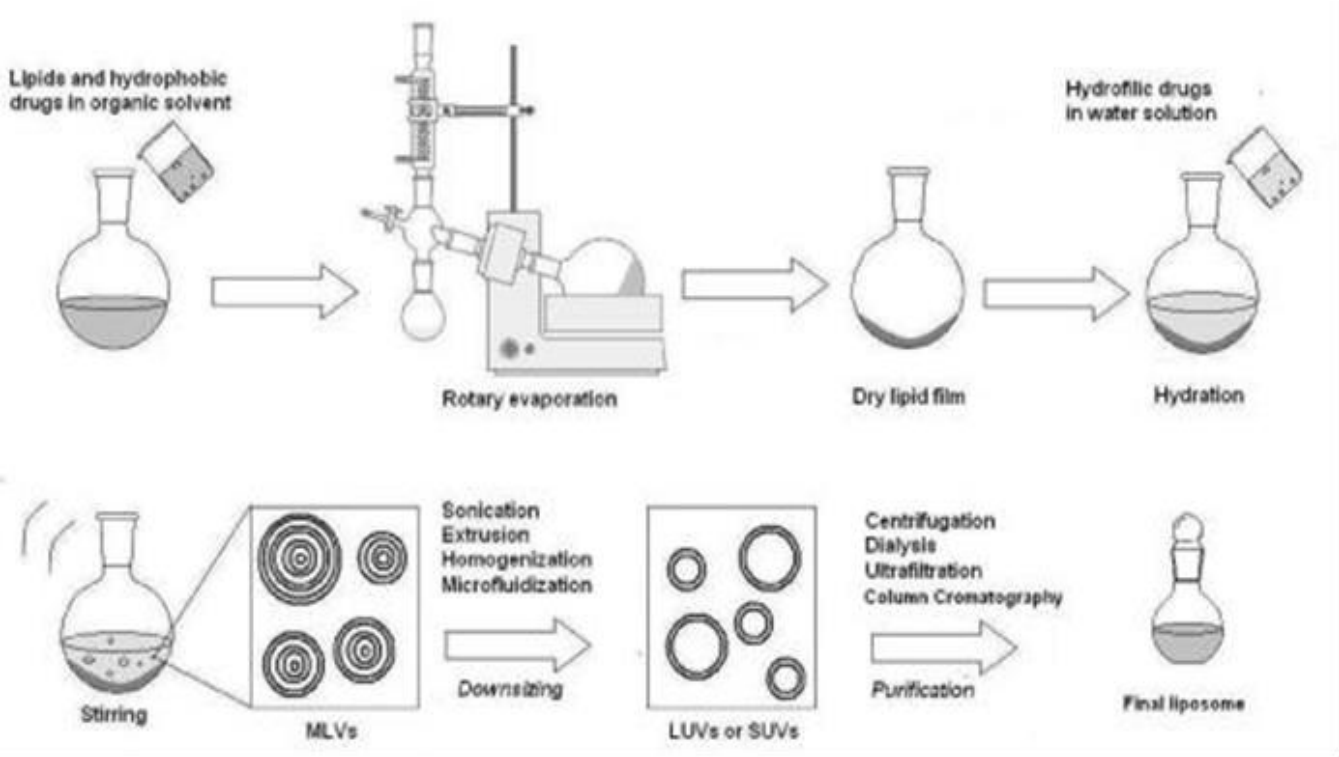

Figure 14: **Schematic diagram of liposome preparation by Lipid film hydration method**
(http://liposomes.weebly.com/the-basics.html)

In this method firstly lipids are dissolved in organic solvent to assure a homogeneous mixture of lipids and to obtain a clear lipid solution. Often it is carried out using chloroform or chloroform ethanol mixture. After the complete mixing, organic solvent is removed by evaporation under reduced pressure. After the evaporation, the dry lipid film is deposited in the flask wall. Then it is hydrated with aqueous buffer at standardized temperature above the transient temperature of lipid which results continuous vesiculisation and entrapment of aqueous phase inside the vesicle. After hydration procedure, sonication or extrusion is done to reduce the vesicle size.

If the drug is hydrophilic, it is included in the aqueous buffer and if the drug is hydrophobic, it can be included in the lipid film.

MLVs produced by this method generally have a size range of more than 1μm, which are sonicated for a definite period of time to get the desired SUVs (below 100 nm). Sonication is perhaps the most extensively used method for the preparation of SUVs from MLVs. MLVs can be sonicated either with a bath type sonicator or a probe sonicator under an inert atmosphere. In probe type sonicator, the tip of sonicator is directly engrossed into the liposomal dispersion. The energy input into lipid dispersion is very high. Also, the coupling of energy at the tip results in local hotness. Such factors may lead to breakage of delicate liposomal membrane or low drug encapsulation. In bath sonication, the liposomal dispersion in a beaker is placed into a water bath where the energy of sonication is dissipated uniformly from all around unlike the probe sonicator. Controlling the temperature of the lipid dispersion is usually easier in this method. Thus this technique of sonication is widely used for the production of NLs.

Sometimes, poor encapsulation efficiency (5 – 10% only), specifically for hydrophilic drugs is considered to be a drawback of thin film hydration method. Also, the preparation method involves the usage of organic solvents or detergents whose presence even in minute quantities in final product may lead to toxicity *(Himanshu et al., 2011)*. However, such factors can be controlled by careful optimization of critical formulation parameters (drug to lipid ratio, amount of cholesterol etc.) and process parameters (time of sonication, duration of hydration, speed and time of centrifugation etc.) during the production.

1.4.3. Encapsulation of drugs into Nanoliposomes

The methods of drug encapsulation into NLs can be divided into two sub-groups. The passive loading, in which drug encapsulation occurs during the vesicle formation process and the active loading in which drug is entrapped after the formation of vesicles.

Passive loading

Passive loading is the process, in which the drug(s) are encapsulated during the formation of Liposomes. The hydrophilic drugs are loaded within the internal aqueous core of Ls by mixing with the hydrating buffer used to hydrate the thin lipid film. Lipophilic drugs are loaded into lipid bilayers by mixing with the organic phase (chloroform/methanol) along with the other lipid components during the first step of preparation of thin film. The unentrapped drug molecules can be removed from Ls suspension by dialysis, or gel-filtration chromatography (**Tyagi et al, 2013)**.

The drug encapsulation efficiency of NLs depends on several factors, including lipid concentration, *liposome size, choice of lipids, drug to lipid ratio, method of optimization etc. Generally, the* encapsulation efficiency of water-soluble compounds, which do not interact with the lipid bilayer, is relatively low and proportional to the inner aqueous volume of NLs (*Tyagi et al, 2013*). Thus, larger size vesicles exhibit higher encapsulation efficiency than smaller size vesicles *(Akbarzadeh et al, 2013*). While the drugs that highly interact with lipid bilayer (lipid soluble drugs), normally have better encapsulation rate. Several strategies have been developed to improve the encapsulation efficiency by linking lipophilic chain to drug molecule to increase its lipophilicity and better partition into the lipid bilayer *(Sutradhar and Lutful, 2014).* Choice of lipid composition is also critical to get better loading efficiently by this method. For example, to load highly negatively charged nucleotide compounds, such as antisense or siRNA, selection of cationic lipid significantly increases the encapsulation efficiency due to enhanced drug/lipid interaction (*Bozzuto and Molinari 2015).*

Active loading

Active loading is also called remote loading method. Some weakly acidic or alkaline drug molecules can be preferably loaded into the preformed NLs by this method. This process is
driven by an electrochemical potential created by the pH, or ion gradients established across the lipid bilayer of the NLs *(Bozzuto and Molinari 2015).*

After creating the pH gradient across the liposomal membranes, drug is loaded by mixing with NLs, typically at a temperature above the phase transition temperature of the lipids to ensure the fluidity and efficient transport across the bilayer. The drug molecules interact with the ions within the NLs and get charged. The charged drug molecules are unable to come out and thus remain entrapped within liposomal core.. Doxil, a nanoliposomal doxorubicin, is one of the ideal examples of the active loading by pH gradient method. Another example of active loading by pH-gradient method is the loading of chloroquine diphosphate into Ls *(Qiu et al, 2008).*

1.4.4. Charaterization / Evaluation of nanoliposomes

Rapid, precise and reproducible quality control tests are required for characterizing the NLs after their formulation and upon storage for a predictable *in vitro* and *in vivo* behavior of the liposomal drug product.

NLs are characterized by several properties such as particle size, sizedistribution,surface charge, number of lamellae, drug encapsulation efficiency, drug release (*Mukherjee et al, 2014).*

Size and size distribution

This is the most important parameter which directly influences the *in vivo* fate of NLs along with the encapsulated drug molecules *(Mukherjee et al, 2014).* In most cases, dynamic laser light scattering technique is used, which measures the hydrodynamic diameter of the dispersed vesicles. The size distribution pattern of the vesicles can be obtained from the polydispersity index (PDI) data.

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Surface charge

Along with the size, surface charge on the liposome surface plays a key role in the *in vivo* disposition. The surface charge can be calculated by estimating the mobility of the liposomal dispersion in a suitable buffer. The lipid-cell interaction is governed by the nature and density of charge on the NLs surface *(Alexis et al, 2008).* Changing the lipid composition may alter the nature and charge on the NLs. Lack of sufficient charge on the surface of NLs can lead to their aggregation and thereby reducing their stability *(Sharma et al, 1994).* High electrostatic surface charge on the surface of NLs may provide useful results in promoting lipid-cell interaction. It has been reported that a surface charge more negative than -30 mV or more positive than +30 mV helps to form stable suspension *(Dey et al, 2015).*

Vesicle shape and lamellarity

Various electron microscopic techniques are used to assess the shape and lamellarity of NLs. The surface morphology of NLs can be assessed using field emission scanning electron microscope (FESEM), which gives clear high resolution pictures of the surface along with overall nature of the formulation regarding presence/absence of any aggregates or lumps in the sample.

The number of bilayers present in the NLs, i.e., lamellarity can be determined using freeze-fracture electron microscopy or cryo-transmission electron microscopy.

Amount of drug encapsulation

The amount of drug entrapped in NLs is a crucial factor since it decides the dose of the formulation to be administered. The formulation consists of both free and encapsulated drug. To know the actual amount of encapsulated drug, the free drug needs to be separated from the encapsulated one by suitable method such as column chromatography. Then, the fraction of NLs containing the encapsulated drug is treated with a suitable solvent/solvent mixture, so as to attain lysis of vesicles. This leads to the discharge of the drug from the vesicles into the surrounding medium, which is then assayed by a suitable technique to obtain encapsulation efficiency of the formulation *(Sandeep et al, 2013).*

Stability of NLs

During the development of liposomal drug products, the stability of the developed formulation is of major consideration. The therapeutic activity of the drug is governed by the stability of the formulation right from the manufacturing steps to storage to delivery.

A stable dosage form is the one which maintains the physical stability and chemical integrity of the active molecule during its developmental procedure and storage. A well designed stability study includes the evaluation of its physical, chemical and microbial parameters along with the assurance of product's integrity throughout its storage period. Hence a stability protocol is essential to study the physical and chemical integrity of the drug product in its storage.

1.4.5. *In- vivo* challenges with liposomal drug delivery system

Inside the body, Liposomes (Ls) usually encounter with multiple defense systems aimed at recognition, neutralization, and elimination of invading substances. These defenses include the RES (reticulo-endithelial system), opsonization, and immunogenicity. While these obstacles must be circumvented for optimal Ls function, other factors such as EPR (enhanced permeability and retention) effect can be exploited to enhance drug delivery *(Sawant and Torchilin, 2012).*The RES (reticulo-endithelial system) is the main site of Liposomes accumulation following their systemic administration. Uptake of Ls by the RES may be considered secondary to vesicle opsonization, which

involves the adsorption of plasma proteins such as immunoglobulins, lipoproteins, and/or complement proteins onto the phospholipid membrane *(Chrai et al., 2002).* Opsonization of liposomes by serum proteins depends on a variety of factors including size, surface charge and stability. However, the extent of such interaction is largely influenced with Ls size (more than 200 nm). NLs of size below 100 nm can successfully surpass opsonic activity.

This profound effect of liposomal size on complement recognition can also affect their hepatic uptake *(Chrai et al., 2002).* Generally, larger size Liposomes are eliminated more rapidly than small, neutral, or positively charged Ls. The inclusion of cholesterol is an important factor for increasing liposome stability and minimizing phospholipid exchange. Incorporation of cholesterol into the liposomal membrane abates lipid exchange with other circulating structures (e.g., red blood cells, lipoproteins etc.) that can cause the depletion of high phase transition temperature of

lipids and their replacement with less physiologically stable components *(Laverman et al., 1999; Ulrich, 2002).* Presence of required amount of cholesterol in small (less than 100 nm), electrostatically neutral/negative Liposomes has been shown to prolong circulation time in the range of several hours *(Geng et al., 2014).*

Primary organs associated with the RES include the liver, spleen, kidney, lungs, bone marrow, and lymph nodes. The liver exhibits as the main RES organ for liposomal uptake followed by the spleen, which can accumulate Liposomes up to 10-fold higher than the other RES organs *(Chrai et al., 2002).* The ability of the RES to sequester Ls from the circulation can be attributed to the fenestrations in their microvasculature. Pore diameters in these capillaries normally range from 100 to 800 nm, which are large enough for the extravasation and subsequent removal of most drug-loaded Ls, preferably in the size range of 100–1000 nm *(Sapra and Allen, 2003).* Ls are cleared in the RES by the inhabitant macrophages via direct interactions with the phagocytic cells. Previous investigation has revealed that large size Ls are cleared within few minutes by the liver and less than an hour by the spleen *(Chrai et al., 2002).* Decreasing the size of the liposomal vesicles below 100 nm, surface modification with hydrophilic polymers are some of the strategies adopted to improve circulation times and to prevent quick removal by the RES *(Oku and Namba, 1994).*

Nanoliposomes that have evaded both the RES and opsonization are subjected to the enhanced permeability and retention (EPR) effect *(Sawant and Torchilin, 2012).* The EPR effect refers to the increased vascular permeability at pathological tissues (e.g., tumors). At these sites, deregulations in angiogenesis and/or the increased expression and activation of vascular permeability factors predominates *(Nehoff et al., 2014),* leading to fenestrations that can range from 0.2 to 2μm.

This allows NLs of size below 100 nm to extravasate and accumulate by passive targeting *(Hashizume et al., 2000)*. Thus, by dint of EPR effect, NLs possess the ability for tumor tissue accumulation.

1.4.6. Types of liposomal drug delivery systems

Liposomal delivery systems can be discussed under various categories based on their size, number of bilayers, composition and method of preparation. Based on the size and number of bilayers, liposomes can be classified as multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). Based on the composition, they can be further classified as conventional liposomes, pH-sensitive liposomes, long circulating sterically-stabilized liposomes, ligand-targeted liposomes etc. Based on the method of preparation, they can be classified as reverse phase evaporation vesicles, French press vesicles, ether injection vesicles etc. The classifications based on size, number of bilayers or compositions are discussed mostly.

- **Based on size / lamellarity**

Multilamellar vesicles (MLVs)

MLVs have a size range of 0.1-1 μm and consist of two or more bilayers. Their method of preparation is rather simpler and does not require stringent optimization steps. They can be prepared by the conventional method of thin film hydration. They are mechanically stable on long storage. Due to the larger size, they are cleared rapidly by the reticulo-endithelial system (RES) cells and hence may be useful for targeting the organs of RES. The drug entrapment into the vesicles can be enhanced by slower rate of hydration and gentle mixing. Higher encapsulation efficiency is possible to achieve in MLV, due to their bigger size and multiple bilayer nature *(Sandeep et al, 2013)*. However, their size is the prime factor, which restricts their wide applications in pharmaceutical field.

Large unilamellar vesicles (LUVs)

This class of Ls consists of a single bilayer, but has a size greater than 0.1 μm. They can have higher encapsulation efficiency due to a high trapped volume and can be useful for encapsulating hydrophilic drugs. Advantage of LUVs is that less amount of lipid is required for encapsulating large quantity of drug. However, like MLVs, they are also rapidly cleared by RES cells, due to their larger size *(Sharma et al, 1997)*.

LUVs can be prepared by various techniques like ether injection, detergent dialysis, reverse phase evaporation etc.Apart from these methods, freeze thawing of Ls and slow swelling of lipids in non-electrolyte solution can also be used to prepare LUVs. Their larger size accompanied with higher rate of elimination and low blood circulation half life limit their applications in targeted delivery.

Small unilamellar vesicles (SUVs)

SUVs are smaller in size (less than 0.1 μm) when compared to MLVs and LUVs, and have a single bilayer. They are characterized by having a long circulation half-life have a low entrapped aqueous volume to lipid ratio. SUVs can be prepared by using solvent injection method (ethanol or ether injection method) or alternatively by reducing the size of MLVs or LUVs using ultrasonication or extrusion process under an inert atmosphere *(Sharma et al, 1997).*

The sonication can be performed using either a bath or probe type sonicator. For the production of SUVs by extrusion process, the prepared LUVs/MLVs are forced to pass through a narrow orifice under high pressure. Due to their desired nanosize, they are able to escape successfully from the RES and circulate for a longer period of time in blood, leadingto higher plasma half-life. Thus, these nanovesicles are considered the ideal carriers for targeted drug delivery.

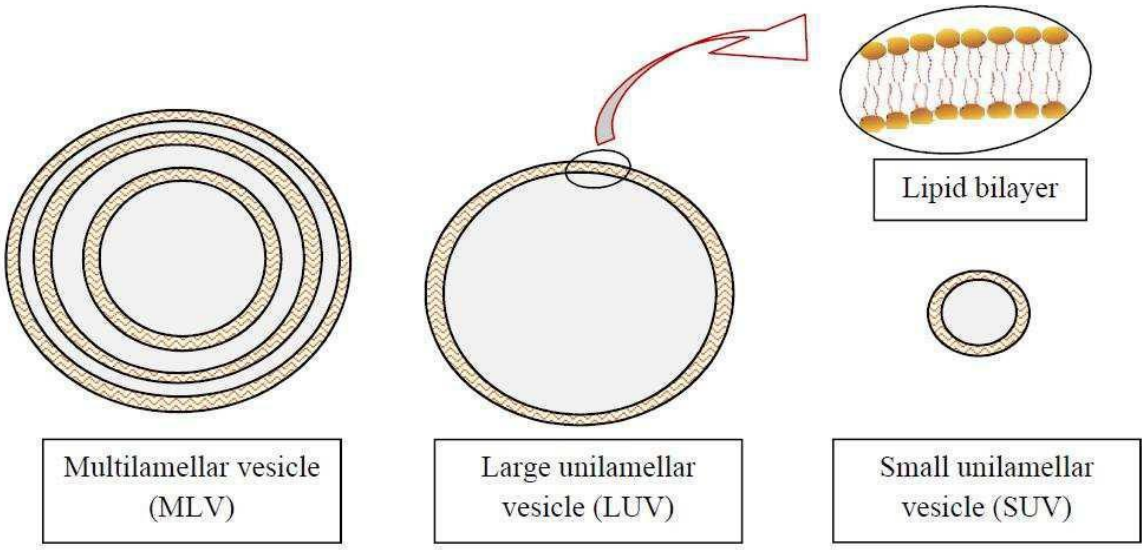


Figure 15: Classification of Liposomes based on size and number of bilayers (*Sandeep et al, 2013*)

- **Based on composition**

Conventionl liposomes

Conventional liposomes were the first generation of liposomes to be developed. They consist of lipid bilayers that are composed of cationic, anionic, or neutral phospholipids and cholesterol, enclosing an aqueous volume. Research on the clinical potential of conventional Ls began in the last few decade, whereby liposomal delivery proved useful for improving the therapeutic index of encapsulated drugs, such as doxorubicin and amphotericin *(Koning and Storm, 2003).*

However, the conventional Ls often show limited blood circulation half-life, due to their easy recognition and clearance by the cells of RES.Further, their non-specific tissue distribution leads to decreased drug availability at the target site. To improve Ls stability and enhance their circulation times in the blood, sterically-stabilized Ls were introduced. The surface coating of conventional Ls with a hydrophilic and biocompatible polymer often results in the development of modified Ls with long blood circulating property often referred to as sterically stabilized long circulating Ls. Polyethylene glycol (PEG) has been shown to be the optimal choice for obtaining such sterically-stabilized Ls. PEG polymers are considered safe and can be prepared synthetically at high purity and in large quantities, which has led to their acceptance for wide clinical applications. The establishment of a steric barrier improves the *in vivo* efficacy of encapsulated drugs by reducing *in vivo* opsonization with serum components, delayed recognition and uptake by the RES with increase in circulation half-life. This enhances drug availability and accumulation at pathological sites, with reduced side effects . Pegylation of Ls resulted in a 50-fold decrease in plasma volume of distribution as compared to non-pegylated Ls. *(Gabizon et al, 2003).* In another study, the efficacy of doxorubicin was found to increase several folds by controlled drug release from PEGylated formulations as compared to the free drug. The dose limiting cardiotoxicity was also found to reduce significantly for PEGylated formulations *(Park et al, 2009).* While coating Ls with PEG results in prolonged circulation times, there can be an offsetting reduction in the ability to interact with the intended targets *(Willis and Forssen, 1998).* Further, coating with PEG increases overall size of the Ls and imparts hydrophilicity to the Ls surface.

Ligand-targeted liposomes

Ligand-targeted liposomes offer a vast potential for site- specific delivery of drugs to intended cell types or organs, which selectively express or overexpress specific ligands (e.g., receptors or cell adhesion molecules) at the site of disease *(Willis and Forssen, 1998).*

Many types of ligands such as antibodies, peptides/proteins, carbohydrates are being coupled on Ls for specific targeting. Among these, coupling of antibodies, particularly monoclonal antibodies on Ls, to create immune-Ls represents one of the most advanced strategies for targeted cancer therapy *(Puri et al., 2009)*. Since lipid assemblies are usually dynamic structures, surface-coupled ligands have a high motional freedom to position themselves for optimal substrate-interactions *(Willis and Forssen, 1998)*. However, the *in vivo* effectiveness of immune-Ls is yet to be well established in clinics. The limited *in vivo* performance of such modified Ls may be assigned to their poor pharmacokinetics and immunogenicity, which represent major hurdle in achieving desired potential *(Puri et al., 2009)*. Further, lack of sufficient *in vivo* data, poor *in vivo*-*in vitro* correlation etc. hinder their successful technology transfer for large scale production. Therefore, constant efforts are now being made to develop newer generation of Ls utilizing a combination of the above design platforms to further improve liposomal targeting and associated drug delivery.

1.4.7. APPLICATIONS OF NANOLIPOSOMES

Nanoliposome in nanotherapy.

The bioactive materials need novel drug delivery technologies to reduce toxic effects *(Hughes., 2005)*. The delivery of some bioactive materials to special sites in the body and their release behavior is directly influenced by particle size. Nanocarriers have the potential to increase solubility, enhance bioavailability, improve time-controlled release and facilitate precision targeting of the entrapped compounds to a greater extent due to more surface area *(Mozafari, 2006)* As a result of improved stability and targeting, the amount of materials required for a specific effect when encapsulated in, is much less than the amount required when un encapsulated.

The targeted drug release improves the effectiveness of bioactive compounds. It has a wide application range and ensures optimal dosage, thus improving cost effectiveness of the product.

Oral delivery

The application of nanoliposomes as oral drug delivery system is complicated due to the poor stability of the vesicles under the physiological conditions. However, there are various studies and current publications that point out the potential of phospholipid-based nanoliposomes to improve the bioavailability of poorly soluble and low-bioavailability drugs *(Gert Fricker et al., 2010)*.

The Polymerized, microencapsulated, and polymer-coated nanoliposomes have increased in the potential of oral nanoliposomes. Using targeted liposomes and a better understanding of their cellular processing will eventually lead to effective therapies from oral liposomes.

The Cyclosporin A - lecithin vesicles with an incorporation of >98%, and its comparative study with the marketed CyA-formulation Sandimmun Neoral® in rabbits proved that the both formulations are equivalent after oral administration (*Guo et al., 2001)*.

Transdermal delivery

Transdermal drug delivery system is one of the most powerful approaches to encapsulate drugs in liposome to enhance drug efficiency.However many liposomes have been formulated to deliver a variety of drugs into the body through diffusion across the skin layers. Although, application had been limited due to the barrier properties of the stratum corneum, the outermost layer of skin. The attention in designing transdermal drug delivery systems was relaunched after the discovery of elastic vesicles i.e. transferosomes and ethosomes. Liposomes having celecoxib were prepared and were evaluated; the results showed that the maximum drug encapsulation efficiency was 43.24%. Drug release profile showed that 81.25% of the drugs released in the first 24 hours of the experiment (Moghimipour et al., 2015).

Pulmonary delivery

Targeted drug delivery to the lungs is one of the most widely investigated systemic or local drug delivery approaches. This route also makes it possible to deposit drugs more site-specific at high concentrations within the diseased lung thus reducing the overall amount of drug required to patients as well as reducing side effects. The delivery of liposome-entrapped antioxidants via the tracheobronchial route has been found to be very useful in increasing the half-times of the administered agents, thus providing a sustained release effect for prolonged drug action. The entrapment in liposomes of α-tocopherol, an exceptionally insoluble but extremely effective antioxidant, has been shown to be very effective in ameliorating oxidant-induced injuries in the lung. Pulmonary lung targeting finds applications in drug delivery to the lung itself and to other body organs via blood circulation. Perceptive pulmonary drug delivery systems towards enhancing their efficacy need *(Chattopadhyay., 2013).*

Intravitreal applications

Liposomes represent the first injectable systems for intravitreal administrations. Liposomes can give sustained release drug profile. In addition, liposomal formulation can minimize the tissue toxicity and enhance the intravitreal half-life of drugs by declining rapid clearance from vitreous cavity. The fluconazole-encapsulated liposomes which were administered intravitreally in rabbit eyes showed that entrapping of fluconazole into liposomes considerably slowed down clearance of free fluconazole after intravitreal injection and thus achieved higher fluconazole concentration in the vitreous *(Gupta et al., 2000).*

Gene therapy

The nucleic acid drugs are in the early stages of clinical trials, they can be considered as promising therapeutic agents for treatment of diseases such as hereditary disorders, cancer, neurological and cardiovascular disorders, AIDS and other viral infections *(Ulrich et al., 1999).* Cationic liposomes and nanoliposomes are the mainly used non-viral vectors that are often applied in gene therapy *(Audouy et al., 2002).* The capacity to mediate transfection was credited to spontaneous electrostatic interaction between them and negatively charged DNA molecules that ensures an efficient condensation of the polynucleotides. Modification in the lipid composition causes an appropriate charge of liposome–polynucleotide complex to increase possibility of cellular uptake. The proposed mechanism of oligonucleotide uptakes from cationic liposomes may be either fusion or endocytosis *(De Lima et al., 2001).*

Multi drug resistance therapy

The multidrug resistance (MDR) is becoming a major barrier for chemotherapeutic treatment in the fight against malignant cancers. Because of the emergence of MDR, higher doses of chemotherapeutic drugs are needed which ultimately leads to intolerable toxicity and the death of patients. Multidrug resistance diminishes the efficacies of a broad range of chemotherapeutic agents; this leads the decrease of intracellular drug concentration and the failure of killing sufficient cancer cells. When co-administration of multiple chemotherapeutic agents cannot overcome MDR due to the different pharmacokinetic properties of combined drugs and only brings limited clinical advantage.

Current development in nanomedicine and nanotechnology has enabled scientists to deliver multiple drugs of similar or different acting mechanisms into cancer cells with a predefined releasing profile. Therefore, combining nanotechnology and co-delivery technique has the great potential to selectively deliver multiple drugs to overcome MDR *(Yuan Sun et al., 2016).*

Application in vaccines production

Advantages of use of liposomes as carrier for vaccine includes;

a) non-immunogenic substance may be converted into immunogenic one

b) hydrophobic antigens may be reconstituted.

c) small amounts of antigens may be suitable as immunogens.

d) multiple antigens may be incorporated into the liposome.

e) adjuvants may be incorporated with antigens into the liposome.

f) long duration of functional antibody activity may be achieved.

g) toxicity and allergic reaction of antigens may be reduced by inclusion in liposome.

h) soluble synthetic antigen may be presented as membrane associate antigen in an insoluble liposomal matrix.

The vaccines produced by liposomal method is also known as virosomes *(Wilschut J., 2009),* that are constructed with viral surface antigens and synthetic lipids such as DOPC, DOPE or DPPC, which simulate viral membrane for vaccine delivery. When compared virosomes with conventional vaccines, virosomes exhibit the excellent immunogenicity as well as better bio-compatibility and safety. These two liposomal vaccines, Epaxal and Inflexal V, have been permitted for clinical use *(Usonis V et al., 2003).* Epaxal is a hepatitis A virus (HAV) vaccine. Inflexal V is influenza vaccine which has been used worldwide for fifteen years.

Combination therapy with nanoliposomes.

The basic principle behind combination chemotherapy is the combination of drugs with different mechanisms of action and non-overlapping side effects these can be applied for the development of nanomedicines. However different types of combinations have been use in recent years, with at least additive effects in therapeutic outcomes for the combinations compared to individual therapies (Mayer et al.,2006). Combinations involving one or two different liposomal drugs targeted against two or more different antigens on the same cells, or on two or more different types of cells.

1.4.7. Liposomal formulations available in market

Table 1: Liposomal formulations available in market and under clinical trial:

Liposomal Product	Trade name and manufacturers	Stage/clinical trial phase	Therapeutic indication
Liposomal Doxorubicin	DOXIL, JNJ	Approved	Ovarian cancer, AIDS- related Kaposi‟s sarcoma, Multiple myeloma.
Liposomal Amphoteracin B	Ambisome, Astellas Pharma U.S.	Approved	Used against fatal fungal infections with fever and low white blood cells.
Liposomal Daunorubicin	DaunoXome, NeXstar Pharmaceuticals	Approved	Used for treatment of Kaposi's sarcoma.
Liposomal Vincristine	Marqibo, CASI Pharmaceuticals	Phase III	Used for treatment of metastatic malignant uveal melanoma.
Liposomal Verteporfin	Visudyne, Baush + Lomb	Approved	Used for treatment of age-related macular degeneration, pathologic myopia and ocular histoplasmosis.
Liposomal cytarabine	DepoCyt, Sigma-Tau Pharmaceuticals.	Approved	Used by intrathecal administration for treatment of neoplastic meningitis and lymphomatous meningitis.
Liposomal morphine Sulfate	DepoDur, picara Pharmaceuticals.	Approved	Used by epidural administration for treatment of postoperative pain following major surgery.
Liposomal amikacin	Lipoplatin, Regulon Inc.	Phase III	Used for treatment of lung infections due to susceptible pathogens.
Liposomal cisplatin	Lipoplatin, Regulon Inc	Approved	Used for treatment of epithelial malignancies such as lung, head and neck, ovarian, bladder and testicular cancers
Liposomal Paclitaxel	LEP-ETU, Insys Therapeutics	Phase II	Used for treatment of ovarian, breast and lung cancer.
Hepatitis A vaccine	Epaxal , Crucell Spain S.A	Approved	Used as a vaccine adjuvant in this formulation.
Influenza vaccine	Inflexal V, Berna Biotech Ltd.	Approved	Used as a vaccine adjuvant

CHAPTER-2
LITERATURE REVIEW

2. LITERATURE REVIEW

Cancer is primarily characterized by the uncontrolled proliferation of cells and their ability to metastasize. In spite of significant advances in the health care system, cancer remains a challenging medical problem.Lung cancer is one of the major causes of malignancy-related death in most developed countries, and the incidence of lung cancer in developing countries is increasing rapidly. Metastasis occurs frequently at the lung site, and about 80% of deaths from lung cancer are attributed to the metastatic process **(Zhao et al., 2013)**. Two major types of lung carcinoma are non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC is a very invasive type of lung carcinoma and is more common. However, present approaches for NSCLC therapy are still limited to chemotherapy, surgical resection, radiotherapy, or their combinations **(Horeweg et al., 2012)**.

The anticancer drug paclitaxel (marketed as *Taxol*) is a taxane diterpene amide that is widely used with good therapeutic effects against various kinds of cancers, such as ovarian, breast, NSCLC (Non-small cell lung carcinoma) and esophageal cancers **(Dang C et al., 2008)**

Paclitaxel (PTX) is one of the most effective anticancer drugs and has been clinically used for the treatment of a wide range of tumors, including breast, ovarian, lung, and head-and-neck cancers. **(Goldspiel BR.,1997)**. PTX exhibits very low solubility in water (,0.01 mg/mL) as well as in most suitable pharmaceutical solvents, causing various formulation problems. Taxol®, the conventional clinical formulation of PTX consisting of Cremophor EL and ethanol, is diluted prior to administration via slow infusion to avoid PTX precipitation in the blood circulation. The presence of Cremophor EL, however, causes a number of side effects in cancer patients, including hypersensitivity reactions, nephrotoxicity, and neurotoxicity. Significant research effort has been devoted to developing an alternative, Cremophor EL-free PTX formulation. These formulations include nanoparticles, liposomes, polymeric micelles, bioconjugates, and dendrimers. Compared with Taxol, loading in liposomes achieves a significant increase in the maximum tolerated dose of PTX, a shorter infusion time, and a fewer side effects.**(Green MR et al, 2006 ;Yang T et al , 2007).**

Nanotechnology has been firmly focusing in to the area of drug delivery. Drug delivery through the nanotechnology are continuously improved and maximize therapeutic activity and minimize undesirable side-effects and toxicities.

In their review article described the advanced drug delivery systems based on micelles, polymeric nanoparticles, and dendrimers. Polymeric nanoparticles, carbon nanotubes and many others demonstrate a broad variety of useful properties. Engineered nano materials ranges between 1 and 100 nm have novel optical, electronic, and structural properties that are not available either in individual molecules or bulk solids such as, clusters of atoms, molecules, and molecular fragments into small particles. The concept of nanoscale devices has led to the development of biodegradable self-assembled nanoparticles, which are being engineered for the targeted delivery of anticancer drugs **(Safari et al., 2014).**

A review of nanomedicines on the market and in clinical translation reveals that the vast majority is based on Ls/NLs. The first approval of a liposomal preparation of doxorubicin (Doxil) in 1995 for treatment of Kaposi sarcoma was found to increase circulation half-life, pharmacological activity of the encapsulated drug to a significant level **(James et al., 1994).** Till now it is one of the most popular commercially available nano formulations. Following this, a number of liposomal formulations of several anti-cancer drugs such as vincristine, daunorubicin, cytarabine etc. were developed and subsequently got approval for their clinical use.

Nanoliposomes can also be used as a tool, a model for cell membranes, or a reagent in the basic studies of cellular interactions, recognition processes, and mode of action of certain therapeutic agents **(Mozafari and Mortazavi, 2005; Sharma et al., 2018; Danaei M et al., 2018).**

NLs can be prepared by several methods. Out of which, thin film hydration or solvent evaporation method is the most common type used both in pilot as well as in large scale manufacturing for passive loading of drug(s). The method is simple and does not involve complicated multi-step process. Moreover, it is also easily reproducible. By careful standardization of critical process parameters, NLs of size less than 100 nm was successfully produced by this method with a reasonable loading efficiency (**Dey et al, 2015**). Cryo-transmission electron microscopy proved formation of stable structure with intact lamellarity. The formulation showed a sustained drug release along with increased *in vitro* and *in vivo* effectiveness.

In a study by Mukherjee and coworkers, acyclovir containing nano-vesicular liposomes and niosomes based on cholesterol, soya-L-α-lecithin and non-ionic surfactant, span 20 showed improved physicochemical properties than marketed formulation and free drug.

The effort was made to study *in vitro* whether acyclovir-loaded nanovesicles could sustain the release of the drug by increasing residence time and thus, acyclovir could reduce its dose-related systemic toxicity. There were good vesicular distributions in both of the niosomes and the liposomes. The obtained vesicles were within nanosize range and about 35% of them were within a size of 100 nm. When the *in vitro* drug release data was compared, it was found that the experimental NLs released about 90 % drug in 150 min whereas the drug release was just 50 % from the marketed formulation in 200 min **(Mukherjee et al, 2007)**.

Santra K. et al. (2010) developed dexmethasone loaded liposome. The objective of the study was to develop dexmethasone-containing liposomes (DCL) based on different combonation of cholesterol and soya l-α-lecithin by lipid film hydration method with standardized process parameters such as amount of drug loading, drug-release, liposome size, etc. They carried out the study of different process parameters such as drug-excipients interaction (by FTIR study), surface morphology by scanning electron microscopy (SEM), particle size analysis and *in-vitro*drug release study. At the end of the study standard dexamethasone loaded liposomes have been successfully developed based on cholesterol and soya-L-α-lecithin with a drug loading of around 1.5%.

Layek B. et al. (2010) developed stable sustained release liposomal drug delivery system for tamoxifen citrate using soya phosphatidylcholine, cholesterol and span 20 as main ingredients by thin film hydration method. The main aim of this study was to develop a simple vesicular delivery system for tamoxifen citrate which can deliver drug at a lower concentration over a prolong period of time and thereby reducing the potential dose related side effects. Stability study at different conditions was carried out. From this study they concluded that the liposomal formulation was a useful drug delivery system for sustaining the in vitro release of tamoxifen citrate.

phosphatidylethanolamine (PE)-conjugated nanoliposome was developed by thin film hydration method and evaluated, characterized for their accumulation in liver, kidneys, and lungs in rats. They concluded that PE-conjugated nanoliposomes released the drug in a sustained manner and were capable of distributing them in various organs. So, this may be used for cell or tissue targeting therapy, attaching specific antibodies to PE **(Rudra et al., 2010).**

Formulation of Nimodipine-loaded nanoliposome was developed by the emulsion-ultrasonic method with sodium cholesterol sulfate (SCS) as the regulator and then lyophilized by adding different cryoprotectants. The main purpose of this study was to prepare nimodipine-loaded nanoliposomes for injection and evaluate their characteristics after lyophilization. **(Guan T. et al. 2011)**

Liposomal formulation of fluoxetine was constructed and characterized. The studied parameters had significant effect on physicochemical characteristics of the nanocarriers. High fluoxetine encapsulation efficiency 83 ±3 % and appropriate particle size 101±12 nm and zeta potential -9±2 mV were achieved for pegylation liposomes composed of DSPE-PEG, DSPC and cholesterol at respective molar ratio of 5:70:25. An *in vitro* fluoxetine release of about 20% in 48 h was observed from the optimum formulation. Atomic force microscopy studies confirmed homogeneous distribution of particles and spherical shape with smooth surface **(Haeri et al, 2014)**.

The formulation of a potential delivery system based on liposomes (Lips) from soy lecithin (SL) for paclitaxel (PTX) and was achieved (PTX-Lips). At first, PTX-Lips were prepared by thin film method using SL and cholesterol and then were characterized for their physiochemical properties (particle size, poly dispersity index, zeta potential, and morphology). The results indicated that PTX-Lips were spherical in shape with a dynamic light scattering (DLS) particle size of 131 ± 30.5 nm. Besides, PTX was efficiently encapsulated in Lips, 94.5 ± 3.2% for drug loading efficiency, and slowly released up to 96 h, compared with free PTX. More importantly, cell proliferation kit I (MTT) assay data showed that Lips were biocompatible nanocarriers, and in addition the incorporation of PTX into Lips has been proven successful in reducing the toxicity of PTX. As a result, development of Lips using SL may offer a stable delivery system and promising properties for loading and sustained release of PTX in cancer therapy. **(Nguyen et al., 2017)**

CHAPTER-3
OBJECTIVE OF THE STUDY

3. OBJECTIVE

Lung cancer is the leading cause of cancer related death in worldwide and Chemotherapy is a first-line treatment for advanced stage of lung cancer which drugs are usually administered intravenously for systemic circulation. Low accumulation of therapeutic agents in the tumor site, and fear of high-dose treatment due to toxicity with severe adverse effects are the main obstacles in efficient lung cancer therapy.

The bio-barriers existing in the respiratory airway systems such as mucus, ciliated cells and resident macrophages are effective to limit the localization, penetration and adsorption of drugs in the lung. Furthermore, it has been reported that systemically administered IV chemotherapy may be less effective than other routes of delivery for the presence of the pleural blood barrier. Therefore, more effective chemo therapeutic regimens and routes of administration with low toxicity are still needed to effectively manage these patients.

Paclitaxel (PTX) is one of the most effective anticancer drugs and has been clinically used for the treatment of a wide range of tumors, including breast, ovarian, **lung**, and head-and-neck cancers.

PTX is a high molecular weight drug with very limited aqueous solubility, which prevents easy absorption into the blood vessels. Moreover, the anti-cancer efficacy of paclitaxel is positively correlated with its concentration, suggesting that higher local concentrations are desirable.(*Jie Li et al.,2015).*Consequently, there is a tremendous interest in developing intelligent delivery systems that can maintain their nanostructure assembly during circulation, thus providing sustained blood levels. Such systems would be preferentially distributed into tumor tissues by so called enhanced permeability and retention (EPR) effect and thus maximize the therapeutic efficacy of PTX while minimizing side effects.

Liposomes are popularly explored for the delivery of drugs to the lungs due to their mimicking properties with the phospholipid component of lungs. Therefore, present investigation has been designed to develop a paclitaxel (PTX)-loaded nanoliposomal formulation to combat the severity of lung cancer.

To accomplish the above mentioned objectives, following studies will be performed.

(i) Pre-formulation study to check drug-excipient interaction

(ii) Preparation and physicochemical characterization of nanoliposomal formulation containing Paclitaxel in terms of morphology, drug loading and *in vitro* drug release.

(iii) *In- vitro* study in lung cancer cells to explore their curative potential.

CHAPTER-4
MATERIALS & EQUIPMENTS

4. MATERIALS & EQUIPMENTS

4.1. Chemicals

The following chemicals (in analytical grade) were used for the study.

Table -2: List of chemicals used in the study.

Name	Source
Paclitaxel (PTX)	Sigma Aldrich Chemicals Pvt Ltd.,Bangalore, India.
Cholesterol (CHL) Extra Pure	E. Merck Limited, Mumbai, India.
Soya-L-α-Lecithin (SLE)	Himedia Laboratories Pvt. Limited. Mumbai,India.
Butylated Hydroxy Toluene (BHT)	Qualigens Fine Chemicals,Mumbai,India.
Disodium hydrogen phosphate	E. Merck Limited, Mumbai,India.
Potassium dihydrogen phosphate	E. Merck Limited, Mumbai, India.
Sodium Chloride	Emplura R
Acetonitrile	E. Merck Limited, Mumbai, India.
Tween-80	S.D. Fine chemical Ltd,Mumbai,India.
Flurorescein isothiocyanate (FITC)	Sigma-Aldrich Co.,Bangalore, India.
Chloroform GR	E. Merck Limited, Mumbai, India
Ethanol	E. Merck Limited, Mumbai, India
3-[4, 5-Dimethylthiazol 2-yl]-2,5-diphenyltetrazolium bromide **(MTT**)	Sigma-Aldrich Co.,Bangalore, India.

4.2. Equipments

Various instruments used in the Paclitaxel nanoliposome preparation and the company from which they are obtained are given below.

Table 3: List of Equipments used

Equipment Name	Name of company
Rotary Vacuum Evaporator	ROTAVAP SUPERFIT (Model- PBU -6), Mumbai, India.
Aspirator A3S	Eyela, Tokyo Rikakikari Co Ltd., Taguig City, Philippines.
Cold circulating bath	Spac N Service, Kolkata, India.
Vacuum desiccator	Tarson, Kolkata, India.
Bath type Sonicator	TRANS - O- SONIC, Mumbai, India.
HPLC (High Performance Liquid Chromatography) system	SHIMADZU Corporation,Kyoto,Japan.
Cold centrifuge	3K30, SIGMA lab Centrifuge, Shrewsbury, UK.
FTIR Spectroscope (Fourier Transform Infrared Spectroscope)	JASCO Magna IR 750 series II FTIR instrument, JASCO international Co. Ltd. FTIR 4200 Tokyo, Japan
Deep freezer(-80 ^{O}C)	New Brunswick Scientific, Freshwater Boulevard Enfield, U.S.A.
Lyophilizer	Instrumentation India, Kolkata -32 ,India
FESEM (Field Emission Scanning Electron Microscope)	JSM , JEOL,Tokyo, Japan.
Zeta potential & particle size analyzer	ZETASIZER, Nano ZS 90, MALVERN Instrument Ltd, Malvern UK.
Electron Microscope	TecnaiPloloria version 4.6 FEI Tecnai G2,Netherland.
AFM (Atomic Force Microscope)	Agilent Technologies,Santa Clara,CA,USA.
Magnetic stirrer	Remi Equipment, Mumbai, India.
Digital Balance	Sartoious, Goetinge, Germany.
P^{H} meter	Eutech Instruments , Haridwar, India
CO_2 Incubator	MCO-15 AC Scanyo ElectricBiomedical Co. Ltd.,Osaka,Japan.
All Glass apparatus	Borosil,Mumbai,india.

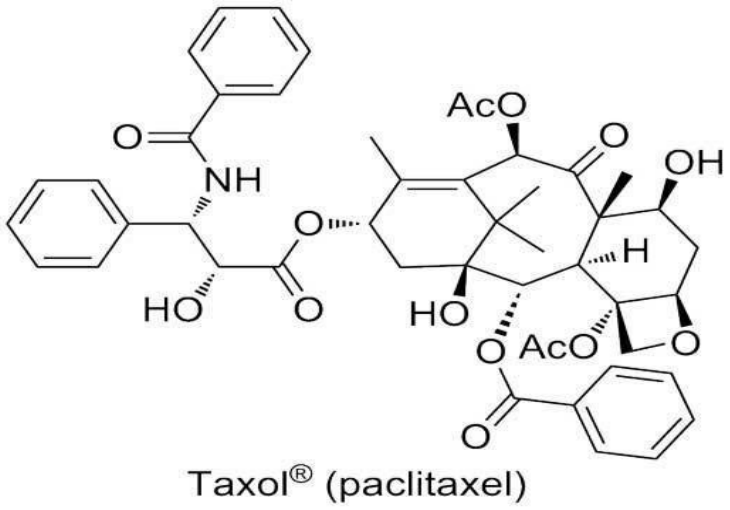
4.3 Description about ingredients used for PTX-NL.

4.3.1. Paclitaxel (PTX)

Formula: $C_{47}H_{51}NO_{14}$

Molecular mass: 853.9

IUPAC Name: (1S,2S,3R,4S,7R,9S,10S,12R,15S)-4,12-bis(acetyloxy)-1,9-dihydroxy-15-({(2R,3S)-2-hydroxy-3-phenyl-3-[(phenylcarbonyl)amino]propanoyl}oxy)-10,14,17,17-tetramethyl-11-oxo-6-oxatetracyclo[11.3.1.$0^{3,10}$.$0^{4,7}$]heptadec-13-en-2-yl benzoate



Figur 16 : Structure of Paclitaxel

Physical nature: It is highly lipophilic, insoluble in water, and melts at around 216-217° C.

Use : Treatment of breast, ovarian, lung, bladder, prostate, melanoma, esophageal, as well as other types of solid tumor cancers. It has also been used in Kaposi's sarcoma.

4.3.2. Soya-l-α-lecithin (SLE)

Lecithin is a generic term to designate any group of yellow-brownish fatty substances occurring in animal and plant tissues composed of phosphoric acids, choline, fatty acids, glycerol,
glycolipids, triglycerides and phospholipids (e,g., phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI)).

The lecithin used by us mainly contains soya-l- α-lecithin and L- α-phosphatidylcholine.

Synonym:- Plinopc; Azolectin; 16:0-18:2 PC;L-alpha lecithin; Lecithin (SOY), L- ; lecithin soybeans; Lecithin(EGG), L-; 2-linleoyl-1-palmitoyl; 2-linoleoyl-1-palmitoyl; 3-sn-phosphatidylcholine.

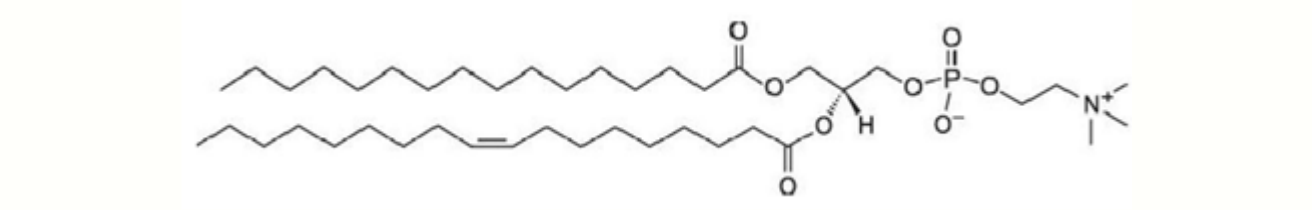


Figure17 : Structure of soya-l-α-lecithin

Molecular weight : 760.09 g/mol.

Molecular Formula : $C_{42}H_{82}NO_8P$.

Solubility : Soluble in chloroform: 50 mg/ml, clear, very faintly yellow solution. Soluble in hexane-ethanol, methanol, ethanol, toluene, ether, mineral oils, fatty acids. Sparinglysoluble in benzene. Insoluble in water (CMC < 0.001nM), cold acetone, cold vegetable and animal oils.

Sources of lecithin

Lecithin can be obtained from plants, animals and marine sources. It is usually commercially available from sources such as soy beans, eggs, milk, marine sources, rapeseed, cottonseed, and sunflower. It is a complex mixture of acetone-insoluble phosphatides that consist chiefly of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, combined with various amounts of other substances such as triglycerides, fatty acids and carbohydrates. Among refined substances, especially concentrated sources of lecithin include dehydrated (powdered) egg yolk (14-20%), natural egg yolk (7-10%), wheat germ (2.82%), soy oil (1.8%) and butter fat (1.4%). Soy oil has the highest lecithin and phosphatide content of any known oil.

Pharmacokinetics

Phosphatidylcholine is absorbed into the mucosal cells of the small intestine, mainly in the duodenum and upper jejunum, following some digestion by the pancreatic enzyme phospholipase, producing lysophosphatidylcholine (lysolecithin). Reacylation of lysolecithin takes place in the intestinal mucosal cells, reforming phosphatidylcholine, which is then transported by the lymphatics in the form of chylomicrons to the blood.

Phosphatidylcholine is transported in the blood in various lipoprotein particles, including very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL); it is

then distributed to the various tissues of the body. Some phosphatidylcholine is incorporated into cell membranes.

Side Effects

It can cause some side effects including diarrhea, nausea, abdominal pain, or fullness. Lecithin is converted by gut bacteria into trimethylamine-N-oxide (TMAO), which is released into circulation, and may with time contribute to atherosclerosis and heart attacks.

Usage

In the pharmaceutical industry, it acts as a wetting, stabilizing agent and a choline enrichment carrier, helps in emulsifications and encapsulation, and is a good dispersing agent. Lecithin is used for treating memory disorders such as dementia and Alzheimer's disease. It is also used for treating gallbladder disease, liver disease, certain types of depression, high cholesterol, anxiety, and a skin disease called eczema. Some people apply lecithin to the skin as a moisturizer. Lecithin is used as an ingredient in some eye medicines to keep the medicine in contact with the eye's cornea. Lecithin is used in a surprisingly large array of our daily foods. Perhaps most widely used in margarine (for anti-spatter and as an emulsifier), it is also used in chocolates, caramels and coatings (to control viscosity, crystallization, weepage, and sticking), in chewing gum (for its softening, plasticizing, and release effects), in instant foods such as cocoa powders, coffee creamer and instant breakfast (for wetting, dispersing, and emulsifying), in calf milk replacers (to add energy and aid digestibility and emulsification).

4.3.3. Cholesterol (CHL)

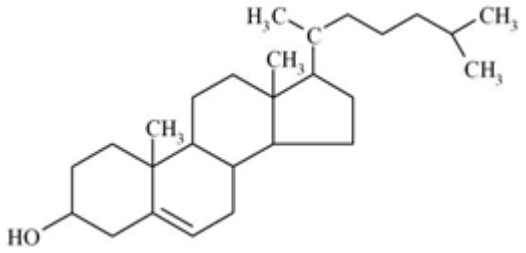


Figure 18 : Structure of cholesterol

IUPAC name : 10,13-dimethyl-17- (6-methylheptan-2-yl) -2,3,4,9,11,12,14,15,16,17-decahydro-1H-cyclopenta[a]phenanthren-3-ol.

Chemical Formula : $C_{27}H_{46}O$.

Appearance : White or faintly yellow almost odorless, pearly leaflets or granules

Molecular weight : 386.66

Melting point : 148–150 °C.

Boiling point : 360 °C (decomposes)

Solubility : Solubility in water 0.095 mg/L (30°C).Soluble in acetones, benzene, chloroform, ethanol, ethers, hexane, isopropyl myristate and methanol

Sources

From a dietary perspective, cholesterol is not found in significant amounts in plant sources. In addition, plant products such as flax seeds and peanuts contain cholesterol-like compounds called phytosterols, which are believed to compete with cholesterol for absorption in the intestines. Animal fats are complex mixtures of triglycerides, with lesser amounts of phospholipids and cholesterol. As a consequence, all foods containing animal fat contain cholesterol to varying extents. Major dietary sources of cholesterol include cheese, egg yolks, beef, pork, poultry, fish, and shrimp. Human breast milk also contains significant quantities of cholesterol.

Pharmacokinetics

Cholesterol is found in all body tissues, especially in the brain, spinal cord, and in animal fats or oils. Cholesterol is distributed universally in all animal tissues. It can be derived either from intestinal absorption of dietary cholesterol or from synthesis de novo within the body. The fraction of dietary cholesterol absorbed is dependent on the intake; after reaching a plateau, the amount absorbed decreases with increased dietary intake. The primary site of absorption of dietary cholesterol is the proximal small intestine. Cholesterol is oxidized by the liver into a

Physiological Function

Cholesterol is required to build and maintain membranes. It modulates membrane fluidity over the range of physiological temperatures. The structure of the tetracyclic ring of cholesterol contributes to the decreased fluidity of the cell membrane as the molecule is in a trans conformation making all but the side chain of cholesterol rigid and planar. In this structural role, cholesterol reduces the permeability of the plasma membrane to neutral solutes, protons (positive hydrogen ions) and sodium ions. Within the cell membrane, cholesterol also functions in intracellular transport, cell signaling and nerve conduction. Cholesterol is the precursor molecule in several biochemical pathways. In the liver, cholesterol is converted to bile, which is then stored in the gallbladder.

Bile contains bile salts which solubilise fats in the digestive tract and aid in the intestinal absorption of fat molecules as well as the fat-soluble vitamins, A, D, E and K. Cholesterol is an important precursor molecule for the synthesis of vitamin D and the steroid hormones including the adrenal gland hormones cortisol and aldosterone, as well as the sex hormones progesterone, estrogen, testosterone, and their derivatives.

Role of cholesterol in liposome

Cholesterol by itself form bilayer structure, but can be incorporated into phospholipid membranes in very high concentration upto 1:1 even 2:1 molar ratios of cholesterol to PC. Cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and aliphatic chained aligned parallel to acyl chains in the center of the bilayer. Cholesterol fills in the gaps created by imperfect packing of lipid species and modulates membrane fluidity, elasticity, and permeability. Inclusion of cholesterol stabilizes liposomal phospholipid membranes against disruption by plasma proteins and results in decreased binding of plasma opsonins responsible for rapid clearance of liposomes from circulation. Cullis and coworkers showed that cholesterol-free liposomes bound 3-4 times more protein than cholesterol containing liposomes.

High cholesterol concentrations (30 mole%) resulted in the formation of a highly ordered crystalline state, which decreased protein binding and dramatically increased circulation life times. Cholesterol incorporation increases the separation between the choline head groups and eliminates the normal electrostatic and hydrogen-bonding interactions, this results improved stability of bilayer membrane. Moreover cholesterol can prevent penetration of water molecules into the deeper region of liposome membrane thereby reduces hydrolytic degradation of lipids. However use of cholesterol presents some problem when used in human pharmaceuticals. Cholesterol is readily oxidized creating a stability problem for lipid based drug products. Some of these oxidation by-products (e.g. 7-keto-cholesterol, 7a-and 7b-hydroxycholesterol) tend to be rather toxic in biological systems. Another problem is availability of highly purified cholesterol suitable for clinical use. Most cholesterol commercially available is derived from egg or wool grease (sheep derived). These animal sources are highly prone to viral contamination and not suitable to use in human pharmaceuticals.

Pharmaceutical use

Cholesterol is used as an emulsifying agent in the pharmaceutical formulations. It is also used as humectants. Liquid crystal properties of cholesterol and its derivatives (salt & ester) make them useful in the commercial applications of cosmetics and pharmaceuticals.

Cholesterol is used in liposomes to encapsulate and deliver chemotherapeutic drugs to diseased tissues. Cholesterol-C14 is used clinically as an organ imaging agent and organs visualized by the technique include ovaries, adrenals, and spleen.

4.3.4. Butylated hydroxyl toluene (BHT)

IUPAC name : 2,6-Bis(1,1-dimethylethyl)-4-methylphenol

Synonyms : 2,6-Di-tert-butyl-4-methylphenol; 2,6-Di-tert-butyl-p-cresol; 3,5-Di-tert-butyl-4- hydroxytoluene (BHT)

CAS Registry Number: 128-37-0

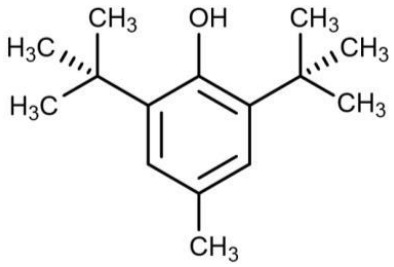


Figure19 : Structure of Butylated hydroxyl toluene

Molecular formula: $C_{15}H_{24}O$

Molar mass : 220.35 g./mol

Appearance : White to yellow powder

Density : 1.048 g/cm3

Melting point : 70 °C

Boiling point : 265 °C

Usage

BHT is primarily used as a food additive that exploits its antioxidant properties. BHT is also used as an antioxidant additive in such diverse products as cosmetics, pharmaceuticals, rubber, electrical transformer oil (at 0.35%), and embalming fluid. In the petroleum industry, where BHT is known as the fuel additive AO-29, it also finds uses in hydraulic fluids, turbine and gear oils, and jet fuels, among other applications. BHT is also used to prevent peroxide formation in diethyl ether and other laboratory chemicals

CHAPTER-5
METHODOLOGY

5. METHODOLOGY

5.1. Preparation of stock and standard solution of paclitaxel

Stock solution of paclitaxel was prepared by dissolving 10 mg of paclitaxel (PTX) in 10 ml of mixture of acetonitrile and water (HPLC grade) in a ratio of 70:40 (v/v) in a 100 ml volumetric flask. Working standard solutions were prepared by taking suitable aliquots of drug solution from standard solution of 1000µg/ml.

5.2. Preparation of calibration curve of paclitaxel

The mobile phase consisting of Acetonitrile and water (70: 40 v/v) was prepared and filtered through 0.45µm syringe filter. Prior to injection of the drug solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the systems. Different working standard solutions of paclitaxel were prepared from standard solution of paclitaxel. The concentrations of working standard solutions were as follows

10 µg/ml., 20 µg/ml., 40 µg/ml., 60 µg/ml., 80 µg/ml., 100 µg/ml. Samples were filtered through 0.45 µm filter prior to the injection into high performance liquid chromatography (HPLC) system.

system equipped with C_{18} column operated at 30 ^{0}C. Samples were analysed in triplicates. The sample volume was 40 µl and the column was eluted with a mobile phase consisting of acetonitrile: water (70:30, v/v) with a flow rate of 1 ml/min and detection was performed at 227nm.

5.3. Pre-formulation study

Various types of technique such as Fourier transform infrared spectroscopy (FTIR), Diffraction scanning colorimetry, X-ray diffraction studies etc. are employed as pre-formulation studies to access any possible interaction between drug and the excipients. Here, in our investigation, we have used FTIR technique to investigate drug- excipients interaction, if any, and stability of the formulation.

5.3.1. Study of drug-excipients interaction through Fourier Transform Infrared Spectroscopy (FTIR)

Drug-excipients interactions were investigated by FTIR spectroscopy. The pure components of experimental formulations such as paclitaxel (PTX), excipients such as cholesterol (CHL), soya lecithin (SLE), Butylated hydroxytoluene (BHT) and their physical mixture, as well as blank formulations and paclitaxel loaded nanoliposomal formulation (PTX-NL) were mixed separately with infrared grade potassium bromide (KBr) in a ratio of 1:100. Pellets were prepared using KBr press at a pressure 5.5 metric ton. The pellets were then scanned using FTIR spectrophotometer.

5.4. Preparation of nanoliposomes (NLs)

Out of several methods of liposome preparation, in our work we have used conventional lipid layer hydration method (also called thin film hydration method) to develop paclitaxel loaded nanosize liposomes (NLs) as reported in literature *(Rudra et al, 2011, Dey et al, 2016).* Requisite quantities of different components of NLs such as SPC, CHL, PTX, as specified in table - 4 and BHT (1% w/v as antioxidant) were taken into a 250 mL round bottom flask and were dissolved in minimum amount of chloroform (as 10 ml for each 10mg of PTX) . The resultant mixture was mixed by gently-shaking the content in a rotary vacuum evaporator (Rotavap, model: PBU-6, Superfit Continental Pvt. Ltd., Mumbai, India) fitted with an A3S aspirator (Eyela, Tokyo Rikakikai Co. Ltd., Taguig City, Philippines) and circulating water bath (at 4 °C) (Spac-N Service, Kolkata, India) and the organic solvent (Chloroform) was evaporated at at 40 °C in the water bath to form thin film of lipid layer on the inside-wall of the flask. For complete elimination of the residual chloroform, the flask was kept overnight in a vacuum desiccator. The thin film thus obtained was hydrated in PBS (pH,7.4) for 60 min at 60 °C at 160 rpm and sonicated at about 30 ± 3 KHz using a sonicator (Trans-o-Sonic, Mumbai, India) for 30 min. Then it was preserved overnight at 4 °C. The suspension was centrifuged at 3000 rpm for 5 min at 4 ^{0}C to separate the larger vesicles and the obtained supernatant was again centrifuged at 16,000 rpm for 1 hour in a cold centrifuge at 4 °C (3K30 Sigma Lab Centrifuge, Merrington Hall Farm, Shrewsbury, UK) to obtain the liposomes and collected in a petridish; kept in a deepfreezer at 20 ^{0}C for 6 hours and then freeze-dried using a laboratory freeze-drier (Laboratory-Freeze Dryer, Instrumentation India Ltd., Kolkata, India) for 12 hours for getting a dried mass of the sample.

Table 4 : Composition of Paclitaxel loaded nanoliposomal (PTX-NL) formulations.

PTX-NL formulation code	Composition- [PTX : SPC : CHL] (by weight)
L0	1 : 8 : 5
L1	1 : 11 : 7
L2	1 : 12 : 7
L3	1 : 13 : 7

Blank NL were prepared without incorporation of drug (PTX) during the preparation of thin film.

5.4.1. Preparation of fluorescent NLs

FITC was used as the fluorescent material and FITC-incorporated nanoliposomes were prepared to visualize the intracellular localization of NL within the cancerous cells.. The stock solution of FITC was prepared by dissolving FITC (about0.4g) in 100 ml of a mixture of chloroform: ethanol (3:1 v/v). The incorporation of FITC in the formulation was done by the addition of about 100 µL of FITC from the stock solution during the first step of preparation of the thin film *(Maji et al, 2014)*. The rest of the procedure was same as mentioned above.

5.5. Characterization of nanoliposomes

5.5.1. Physicochemical characterization of nanoliposomes

- **Determination of average particle size, size distribution, polydispersity index (PDI) and zeta potential**

To determine the size distribution, polydispersity index (PDI) and zeta potential of the prepared nanoliposomes were studied by dynamic light scattering technique (Zetasizer, nano ZS90, Malvern instrument,Malvern, UK) accompanied with Data transfer assistance software (DTS) V 4.0 (Malvern instrument Limited, Worcestshire, UK). About 1 mg of the prepared freeze-dried NLs was dispersed in 2 ml of milli-Q water in a microcentrifuge tube, vortexed, and sonicated for 15 min.

The sample is illuminated by a laser beam in the DLS-nano ZS, Zetasizer and the fluctuations of the scattered light are detected at a known scattering angle of 90º by a fast photon detector.
The analysis was performed at 25 ºC and the data were interpreted by the instrument software (DTS software version 4.0)

- **Surface morphology study by field emission scanning electron microscopy (FESEM)**

Surface morphology of prepared formulations were analysed by FESEM ((Model-JSM-6700F; JEOL, Tokyo, Japan). Lyophilized formulations were spread on to a carbon tape over a stub and a platinum coating of about 5 nm was applied at an accelerating voltage of 10 kV and 10 mA current with the help of a platinum coater (JEOL, Tokyo, Japan).

- **Cryo-transmission electron microscopy (Cryo-TEM)**

For Cryo-TEM analysis, 1 mg of lyophilized NLs was dispersed in 1 ml of Mili-Q water in a microcentrifuge tube. Then the aqueous suspension was vortexed in a cyclomixer for 15 min, followed sonication in water bath for 5 min at room temperature. About 4 µl of sample suspension was applied to a clean grid, blotted away the excess with filter paper, and immediately vitrified into liquid ethane (*Dey et al, 2015).* The grid was stored in liquid nitrogen until imaging using an electron microscope (Tecnai Polora, version 4.6 FEI Tecnai G2, Netherlands) operating at 300 kV equipped with an FEI Eagle 4K x 4K charge-coupled device (CCD) camera.

- **Surface morphology of nanoliposomes by Atomic force microscopy (AFM)**

For analysis of surface morphology by atomic force microscope (AFM), 1 mg of lyophilized NLs was dispersed in 1ml of Milli-Q water in a microcentrifuge tube, sonicated for 15 minutes and filtered through 0.22µ filter. Finally morphology of particles was visualized with an AFM ((Pico Plus 5500 ILM AFM, Agilent Technologies, Santa Clara, CA, USA).

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5.5.2. Drug Content and Entrapment Efficiency Study

Accurately weighed nanoliposome (2mg) was dissolved in a mixture of Acetonitrile and water (HPLC grade) in a ratio of 60:40 (v/v). Then it was vortexed to form uniform dispersion and sonicated and kept at incubator for 1hr. After that suspension was centrifuged at 16,000rpm and supernatant was collected and analyzed by HPLC as mentioned above. The study was performed in triplicates.

The percentage loading and loading efficiency was calculated from the following formula as described earlier.

Percentage of drug loading = Amount of PTX in NLs/ Amount of NLs taken x100

Percentage entrapment efficiency = Practical loading % / Theoretical loading % x100

Persentage Yield = Weight of NLs obtained / Total weight of (PTX+SLE+CHL) used x 100

5.5.3. In vitro drug release study

On the basis of drug loading results and the physicochemical characterization, out of four formulations the L3 coded formulation was found to be the best on the basis of parameters investigated and considered for the release study experiment.

5 mg of nanoliposomal formulation was reconstituted with 1 mL of drug release media consisting of mixture of phosphate buffer saline and tween 80 (PBS+ tween 80) and poured into a dialysis bag (molecular weight cutoff 12-14 kDa). The bag containing the formulations was immersed centrally into release medium in a glass beaker with the help of glass rod. The beaker was placed on a magnetic stirrer and maintained the content of the beaker for a rotation of 300 rpm using a magnetic bead. At different pre-determined time interval, 1 mL of release medium was withdrawn and replaced with 1mL of fresh drug release media. The experiment was performed in triplicates. Sample were analyzed by HPLC system as mentioned earlier. For HPLC analysis, samples were extracted with 2ml of dichloromethane (DCM) and DCM layers were collected. The collected layer was evaporated at 40 ^{0}C followed by the application of nitrogen steam. Finally, residues obtained after the removal of DCM layer were reconstituted with the mobile phase as mentioned above and filtered through 0.45µm syringe filter before analysis through HPLC.

.Extraction factor was determined as mentioned earlier and results obtained were corrected with the extraction factor in order to determine exact concentration of released drug from formulation at different time intervals.

5.5.4. In vitro study in lung cancer cells.

Lung cancer cells such as A-549 and NCI-H23 were procured from National Centre for Cell Science (NCCS), Pune, India. A-549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) whereas NCI-H23 were maintained in RPMI-1640 medium.

Both the media were supplemented with 10% fetal bovine serum (FBS-procured bfrom theHimedia Laboratories ,Mumbai,India) containing 1% penicillin-streptomycin. Cells were kept in humidified CO_2 incubator (Heraeus Hera cell, Germany) at 37 °C.

- **Cytotoxicity study by MTT assay**

100µl respective cell suspensions (3 ×103) per well in a log phase were seeded on a 96- well tissue culture plates for overnight. Then they were treated with L3 nanoliposomal formulation and free-drug suspension at different concentration (10-100nM) for 24, 48, 72 h at 37˚C in a humidified atmosphere containing 5% CO2 in air. The untreated cells served the purpose of control. At the end of the treatment, 20µl of MTT (4mg/ml in PBS) was added to each well and incubated for another 4 hrs.Then, 100µl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the insoluble formation of precipitate. The plates were then put on shaker and absorbance was measured at 570nm. Anti proliferative effect was evaluated by measuring the percentage of cell viability as given below and IC_{50} values were calculated.

% cell viability = Absorbance control- Absorbance of test/ absorbance of control ×100

- **Intracellular localization of nanoliposomal formulation**

Internalization of FITC-labeled nanoliposomal in time-dependent manner was quantified by flow cytometer (Becton Dickinson FACS Fortessa 4 laser Cytometer) with the help of BD FACS Diva software by following the protocol as described in literature. The quantifications are indicated by the shifting /counting of cells inside in the FITC filter area.

- **Nuclear condensation study**

Cells were seeded at a concentration of (1×10^6cells/well) on coverslips placed in the wells of 6-well tissue-culture plates for a period of 24 h. Cells were then treated with free-drug suspension and nanoliposomal formulation L3 at their IC_{50} doses and kept in an incubator for a period of 24 h. After the treatment, coverslips were washed with PBS and mounted on glass slide. Finally, slides were examined under fluorescent microscope after staining with DAPI(4.6-diamidino-2-phenylindole).

CHAPTER-6
RESULTS

6. RESULTS

6.1. HPLC spectra of paclitaxel

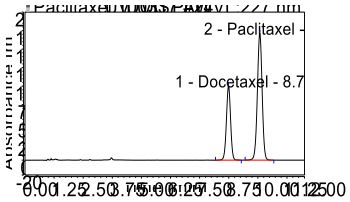


Figure 20 : HPLC spectra of **paclitaxel** and docetaxel (used as internal standard) as a representative figure

6.2. Calibration curve of paclitaxel

For the calibration curve, different concentrations of paclitaxel were taken in triplicate; respective mean areas were determined (Table 5) and calibration curve (Figure 21) was plotted. The equation obtained from calibration curve was required to determine % drug loading, drug entrapment efficiency of nanoliposome encapsulating paclitaxel as well as to determine amount of paclitaxel released in different release media at different time intervals *in vitro.*

Table: 5. The mean peak area of different concentrations of paclitaxel.

Conc. (µg/ml)	Area	Height of Peak
10	2.476	35.035
20	4.922	70.332
40	10.096	145.254
60	14.964	213.258
80	20.015	287.481
100	25.775	371.155

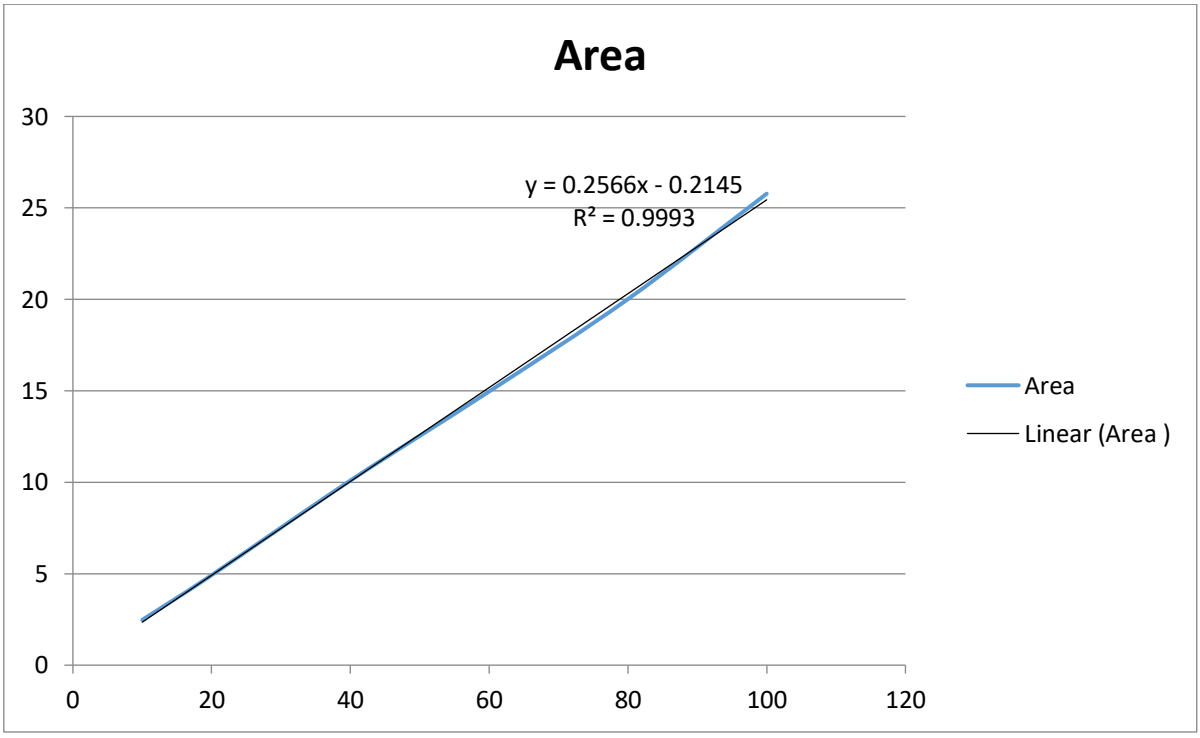


Figure 21 : Calibration curve of paclitaxel.

(X axis-Concentration (µg/ml) ; Y axis –mean peak area)

6.3. Drug excipient interaction analysis by Fourier transform infrared spectroscopy (FTIR)

Drug-excipient interactions were analysed by FTIR to analyse type and degree of interactions among the different functional groups of drug (PTX) and the excipients of the formulations. Soya-lecithin (SPC) showed its characteristic peak at 2925.50 cm^{-1} of C-H stretching, strong intensity of C=O stretching vibration at 1725 cm^{-1}, medium intensity of C-O stretching vibrations at 1350 cm^{-1} and 1050 cm^{-1} respectively. The peak of cholesterol was found at 3590 cm^{-1} due to the presence of –OH group, strong intensity band at 2930 cm^{-1} of C-H stretching as well as at 2850 cm^{-1} due to C-OH stretching. Cholesterol provided its characteristic strong intensity stretching vibration of C-O at 1060 cm^{-1}. Butylated hydroxylated toluene (BHT) showed its characteristic peaks at 1459.84 cm^{-1}. The characteristic peaks of PTX (at 3438 cm^{-1}, 2942 cm^{-1}, 1733 cm^{-1}, 1071 cm^{-1} and 709 cm^{-1} for N-H/O-H stretching, CH_3/C-H stretching, C=O stretching, C-O stretching, and C-H out of plane, respectively) were present. The peak of the drug was present in the drug – excipient physical mixture and it was absent in L3 (PTX-NL) formulation, suggesting for absence of free drug

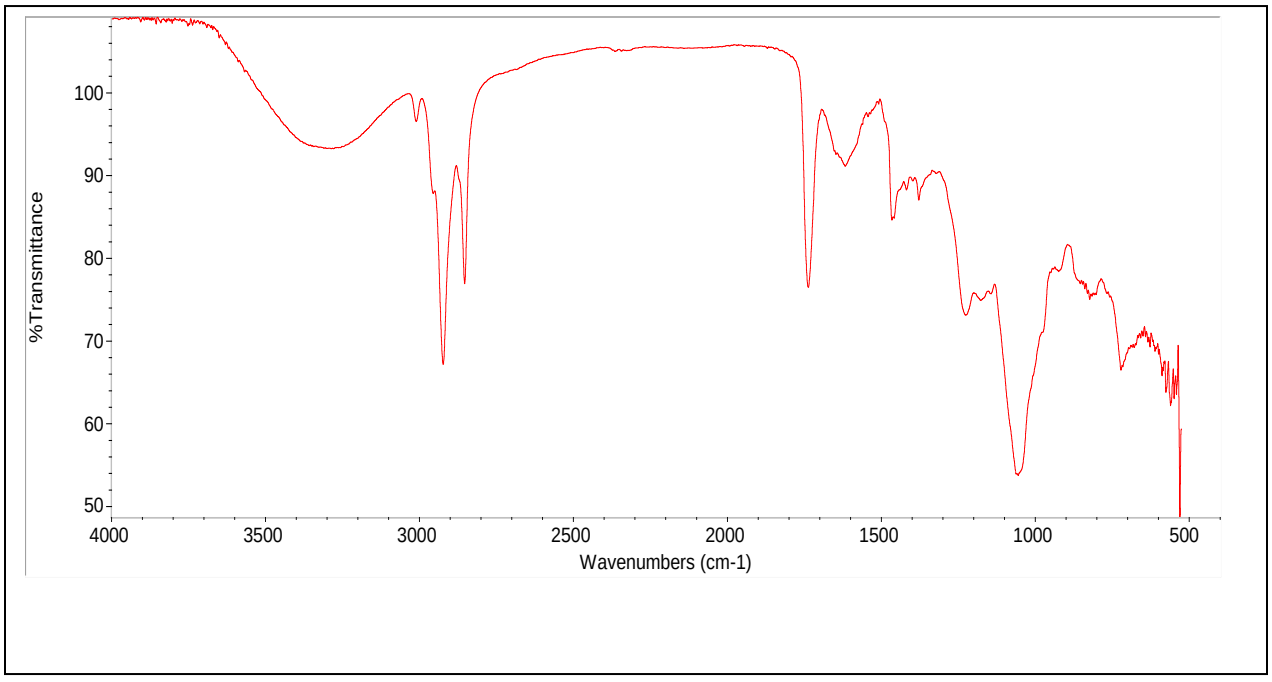


Figure 22(A) : FTIR spectra of Soya lecithin (SLE)

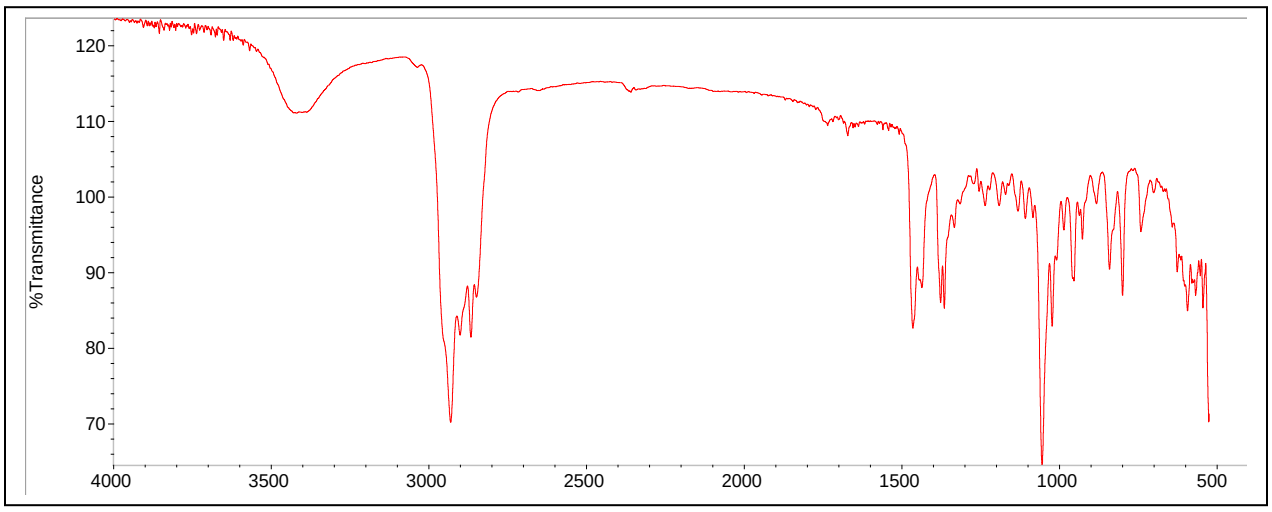


Figure 22(B) : FTIR spectra of Cholesterol (CHL)

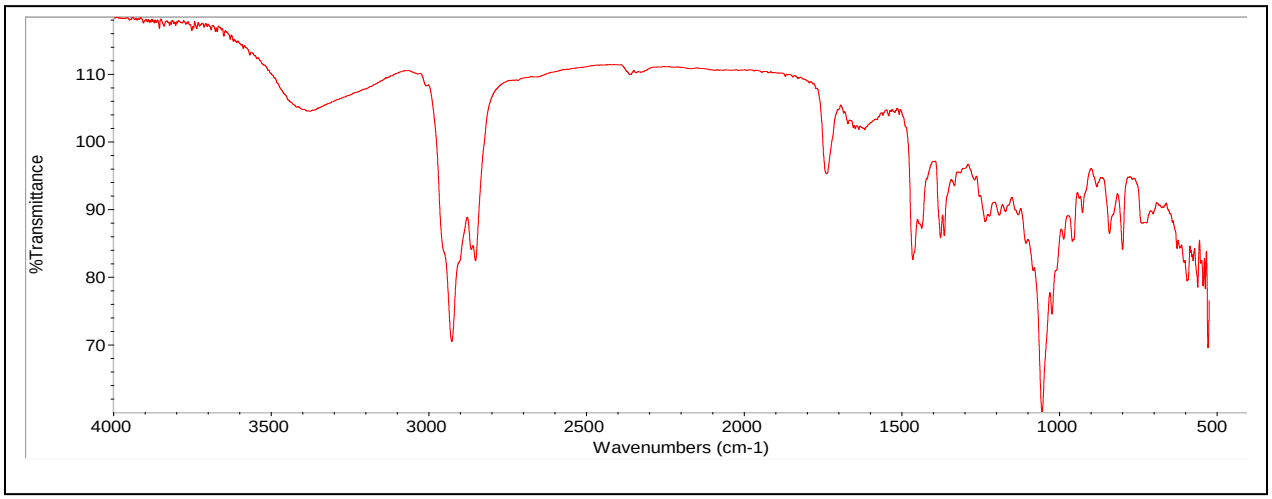


Figure 22(C) : FTIR spectra of Soyalecithin and Cholesterol.

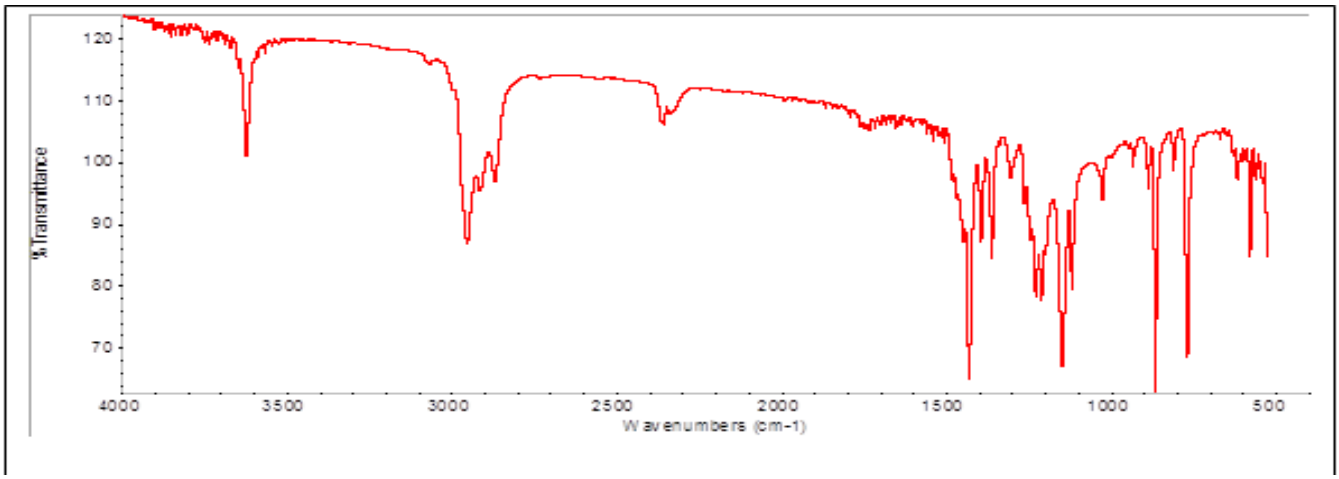


Figure 22(D) : FTIR spectra of Butylated hydroxyl toluene (BHT)

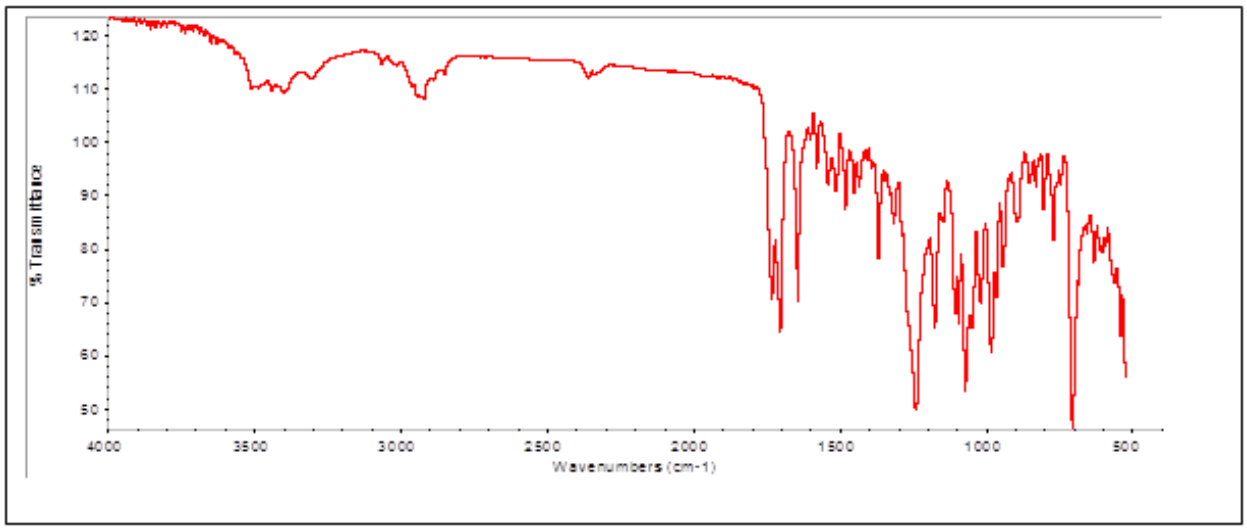


Figure 22(E) : FTIR spectra of paclitaxel (PTX)

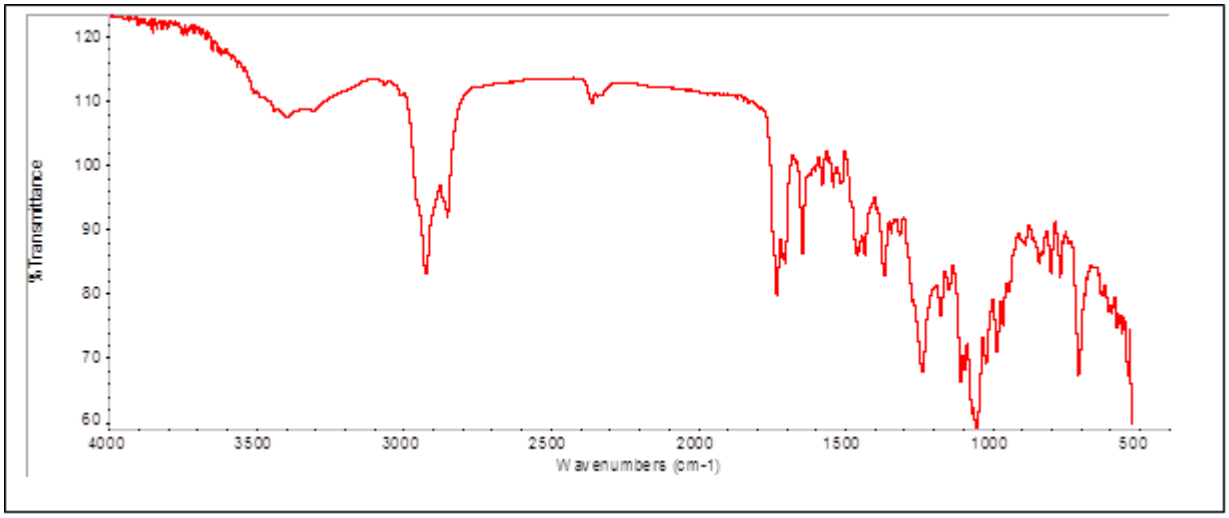


Figure 22(F) : FTIR spectra of physical mixture of SLE,CHL,BHT and PTX.

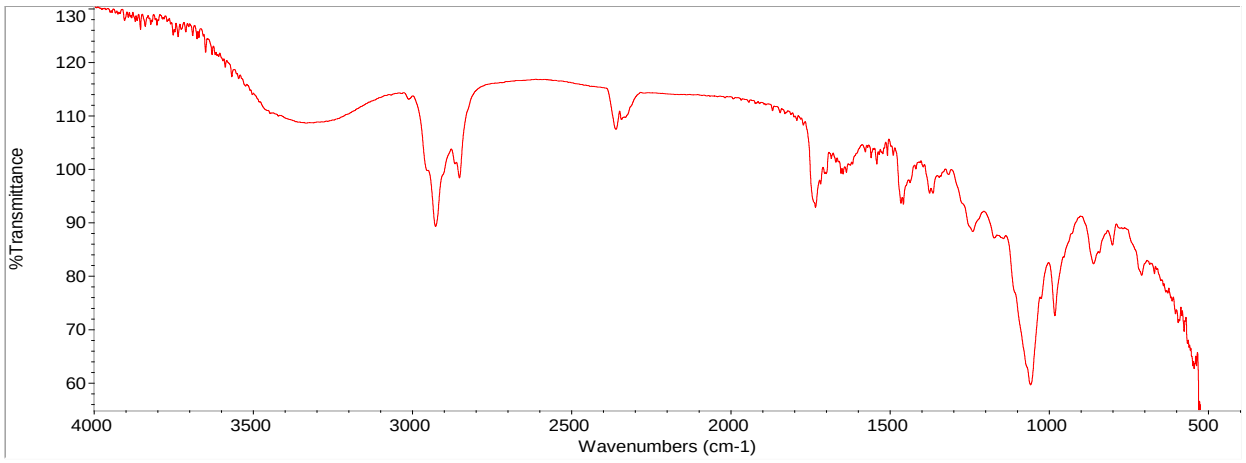


Figure 22(G) : FTIR spectra of PTX- NL formulation.

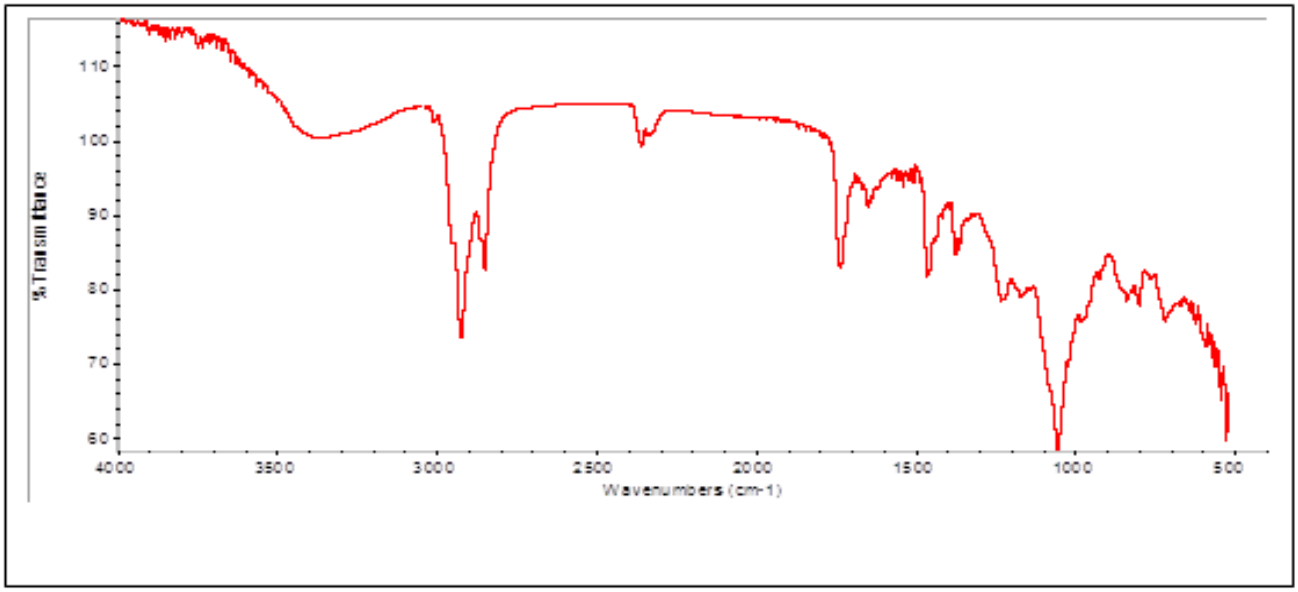


Figure 22(H) : FTIR spectra of blank NL formulation.

5.5. Preparation and characterization of nanoliposomes

Nanoliposomes were prepared by thin film hydration techniques. Different ratios of soyalecithin, cholesterol, BHT and drug (paclitaxel) were taken in order to develop optimized formulations based on physico-chemical characterizations. Drug (PTX) and excipients for different PTX-NL formulations were taken according to the ratios mentioned in Table 6.

Table 6 : Practical and theoretical drug loading % along with entrapment efficiency of different PTX-NL formulations

PTX-NL Formulation Code	Composition by weight (PTX:SPC: CHL)	Theoretical loading (%)	Practical loading (%)	Entrapment efficiency (%)
L0	1:8:5	7.14	3.56±1.16	49.85
L1	1:11:7	5.26	4.04±1.51	83.65
L2	1:12:7	5	4.16±1.76	83.20
L3	1:13:7	4.76	4.44±1.02	93.27

6.4.1. Characterization of nanoliposomes

As per the results of drug loading and entrapment efficiency, it was found that the formulation L3 was the optimized formulation. Therefore morphological characterizations such Cryo-TEM, FESEM, AFM and particle size were carried out for L-3.

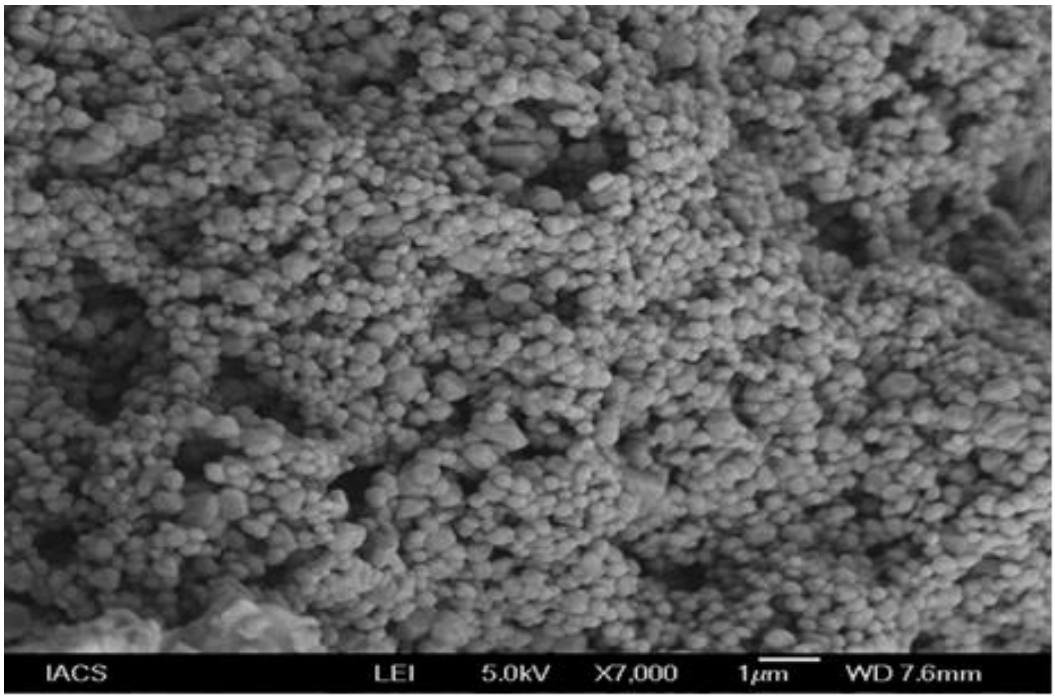


Figure 23 : FESEM image of L3

FESEM images (**Figure 23**) of the freshly prepared optimized formulation revealed that prepared nanoliposome had smooth surface and average sizes of the liposome was found to be around 100 nm and with a thickly densed distribution . However, smaller liposomal structures (25-30 nm) were also present.

Mean vesicle diameter (Z-average), polydispersity index (PDI) and zeta potential of the prepared formulations were determined by dynamic light scattering (DLS). The average size of the PTX-NLs formulation- L3 was 173.3 nm and PDI data (0.366) suggests that the formulations had a narrow size distribution pattern **(Figure 24).**

The zeta potential of formulation L3 was -60.7 mV **(Figure 25).** Higher values of zeta potential for the selected formulations signify their higher stability in a suspended state (*Dey et al, 2016).*

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 173.3	Peak 1:	156.9	100.0	42.97
PdI: 0.366	Peak 2:	0.000	0.0	0.000
Intercept: 0.966	Peak 3:	0.000	0.0	0.000

Result quality : Good

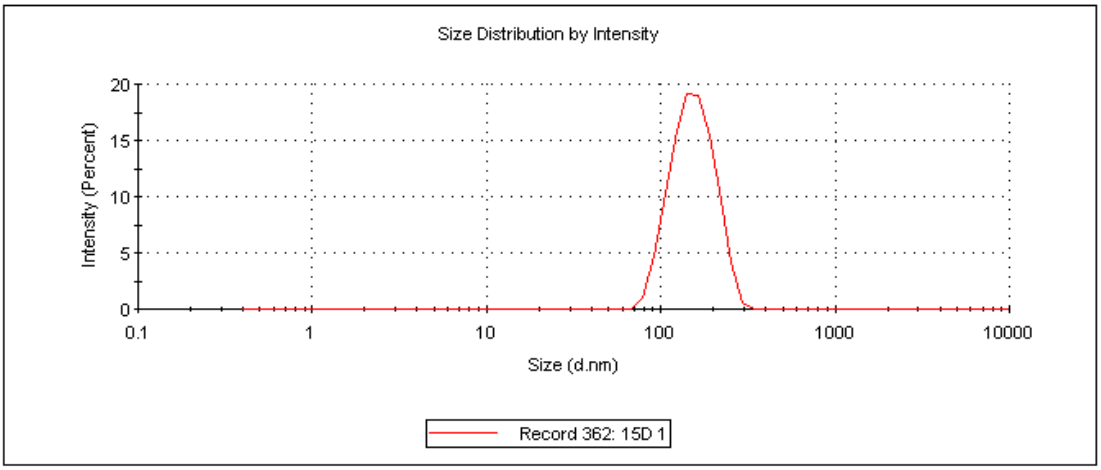


Figure 24 : Particlre size distribution pattern of lyophilized formulation L3.

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -60.7	Peak 1:	-60.7	100.0	8.92
Zeta Deviation (mV): 8.92	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.968	Peak 3:	0.00	0.0	0.00

Result quality : Good

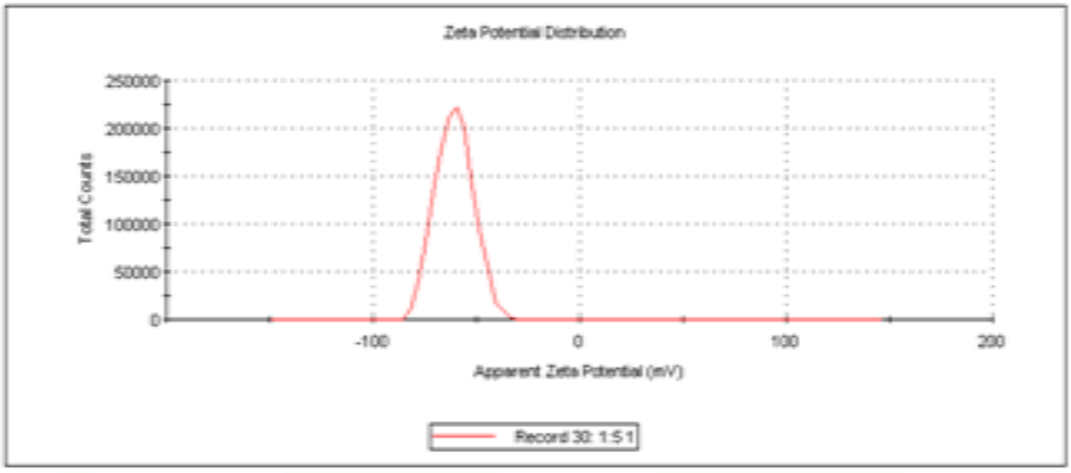


Figure 25 : Zeta potential of lyophilized formulation L3.

Cryo-TEM study (**Figure 26**) was conducted for the selected nanoliposomes to provide details on internalstructure, lamellarity etc. The Cryo-TEM images showed formation of unilamellar lipid vesicles along with very few bilamellar vesicles as depicted in Figure 26. The formulation showed a mixture of smaller (20-30 nm) and little larger vesicles (around 100 nm). The nanoliposoms were spherical showing darker outer lipid layer enclosing the inner core and without any perforation or leakage on the outer membrane justifying formation of stable structure.

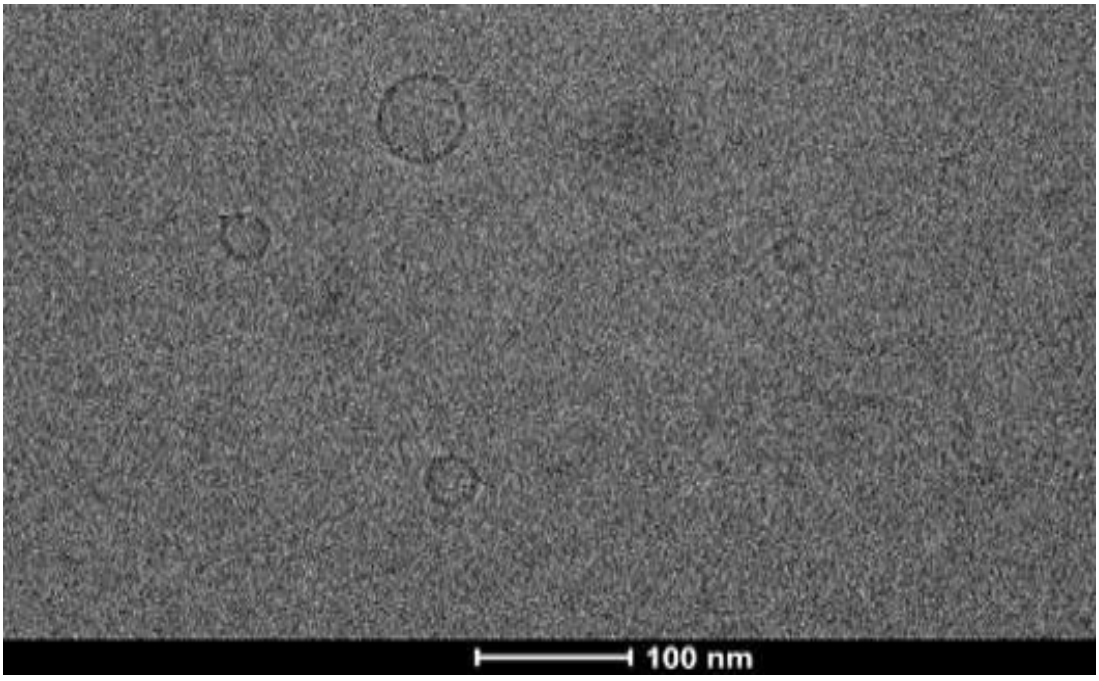


Figure 26 : Cryo-TEM image of L3

AFM data revealed that the average dimension of nanoliposomal formulations. The mean height of liposomal formulation was found to be 2.23 nm and their width varied from 25.0 nm to 38.3 nm (**Figure 27 A,B**).

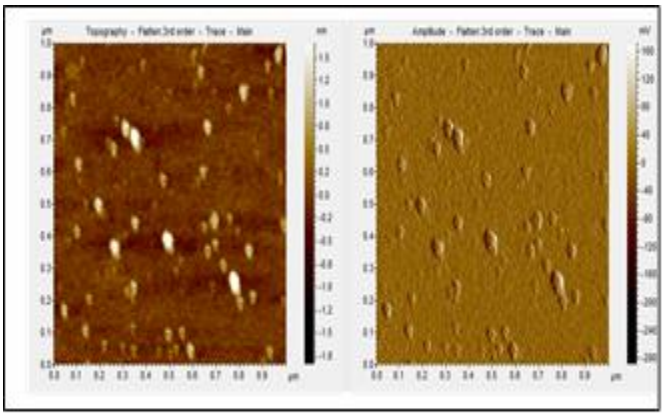


Figure 27 (A) : 2D AFM images of L3

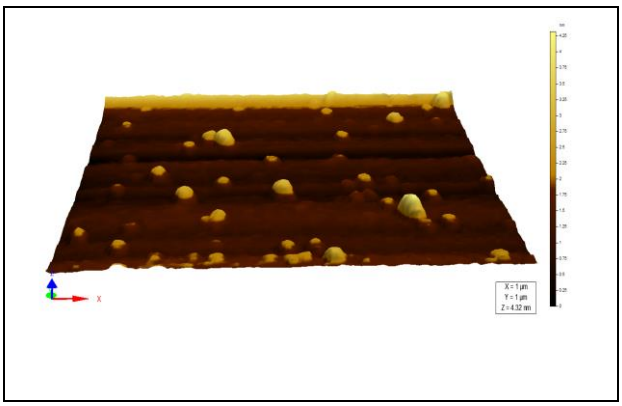
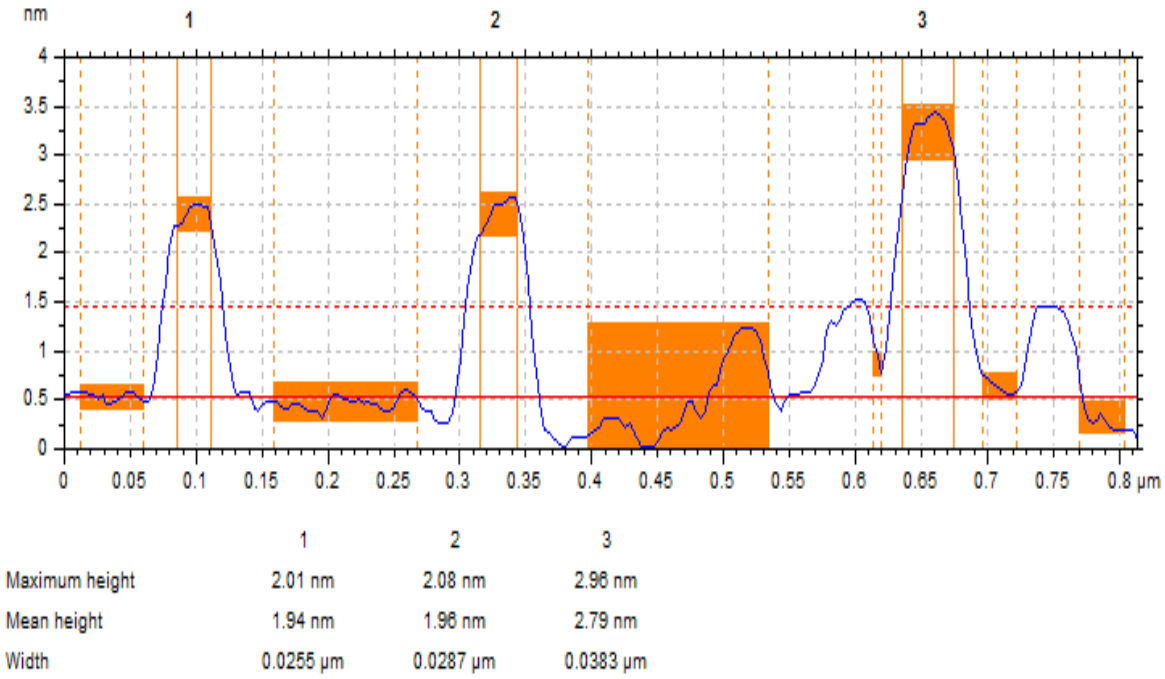


Figure 27 (B) : 3D AFM images of L3



	1	2	3
Maximum height	2.01 nm	2.08 nm	2.96 nm
Mean height	1.94 nm	1.96 nm	2.79 nm
Width	0.0255 µm	0.0287 µm	0.0383 µm

Figure 27 (C) : 3D view; Step height measurement.

Release characteristics of paclitaxel

In-vitro drug release of the optimised formulation (L3) was studied in phosphate buffer saline (PBS) (pH 7.4) with 1% tween 80 medium using dialysis method . The dissolution profile showed paclitaxel released in a sustained manner from its nano-liposomal carrier in 72 hrs of study. The maximum drug permeates through the dialysis membrane in to the media was 79.88±0.68% after 72 h (**Figure 28 A**).

Further the drug release data was analyzed by different release kinetic models such as zero-order, first-order, Higuchi kinetics, Korsmeyer–Peppas model and Hixon–Crowell model for the highest regression coefficient value ($R^{2.}$). From the analysis it was found that the release profile has maximum regression coefficient in Korsmeyer–Peppas model (R^2=0.9828) which signify that the drug release followed diffusion control release pattern (**Figure 28 B).**

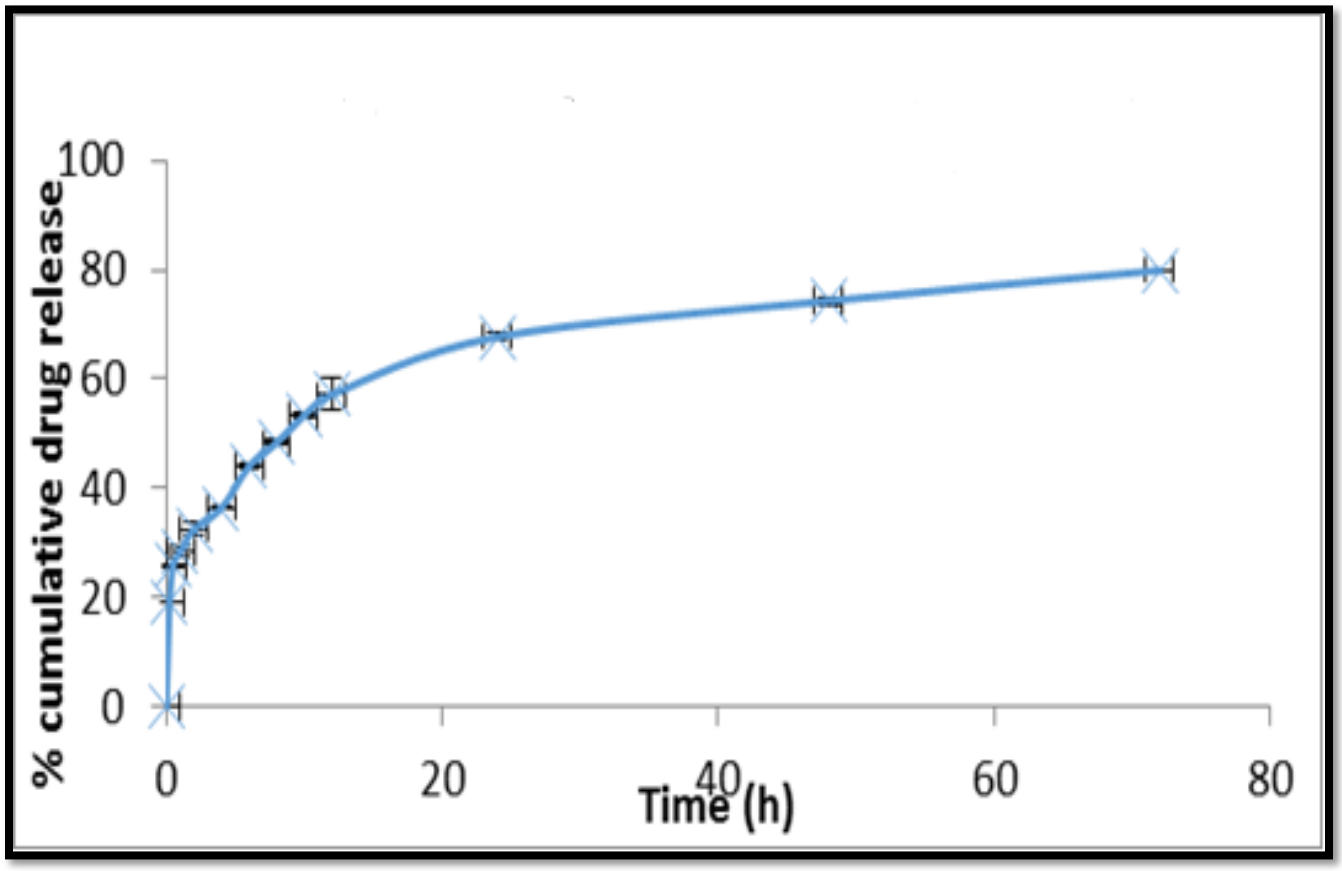


Figure 28 (A) : *In-Vitro* drug (paclitaxel) release profile of L3 in PBS and 1% Tween 80 medium (Data represented Mean ± SEM of 3 sets of experiments.)

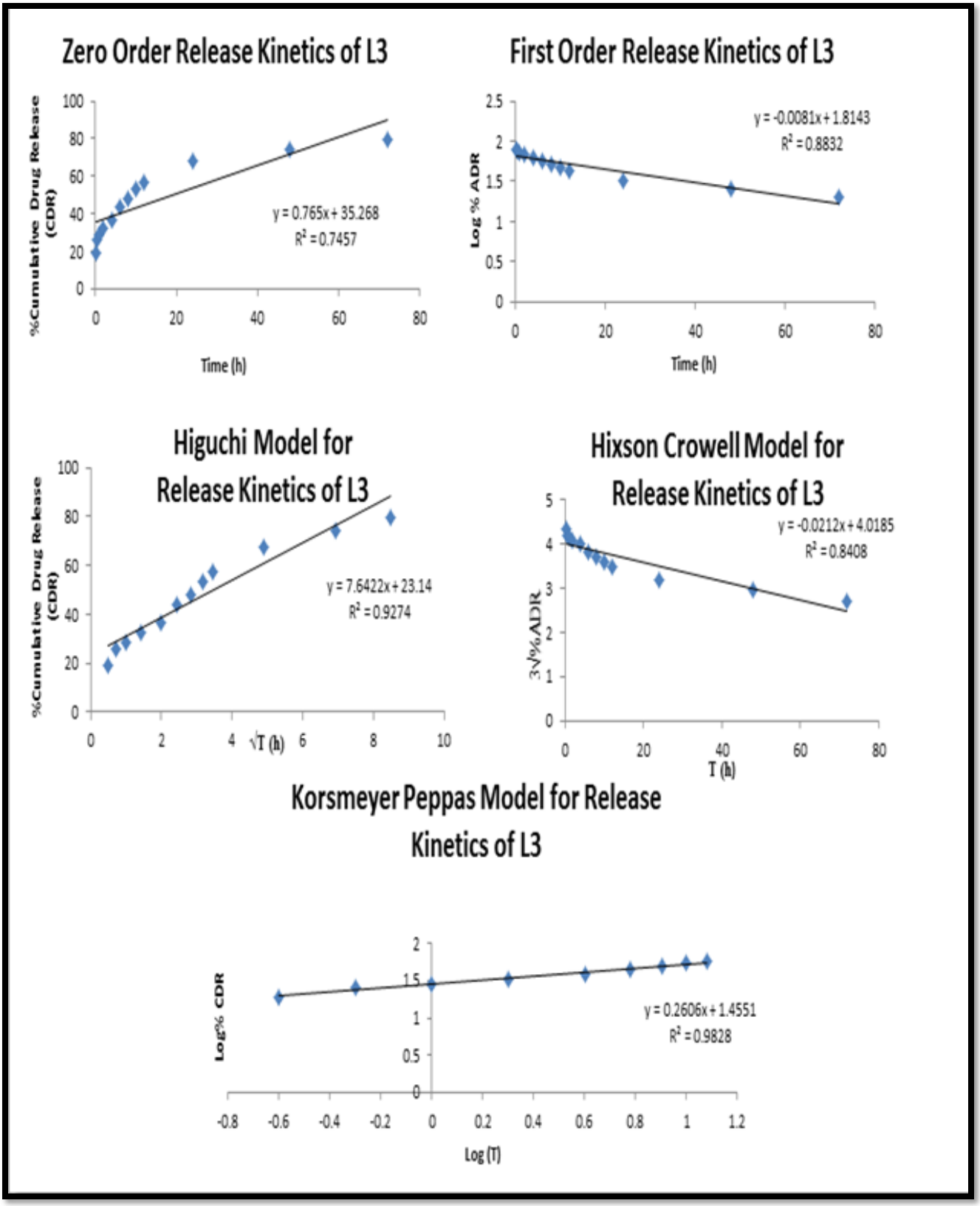


Figure 28 (B) : Different release kinetics of paclitaxel. (CDR=cumulative drug release, ADR= amount of drug remain, T=time)

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Table 7 : The regression values (R^2) of the optimized formulation L3 by using various release kinetic models.

	Zero order	First order	Higuchi	Krosmeyer-Peppas	Hixson Crowell
R^2	0.7457	0.8832	0.9274	0.9828	0.8408

6.6. In vitro cytotoxicity study on cancer cells

In vitro cytotoxicity study in cancerous cells revealed that the PTX-NL formulation L3 was more cytotoxic than free-drug (PTX alone) as IC_{50} concentration for the formulations in A549 cells and NCI H23 were found as 2 µg/ml and 4 µg/ml.

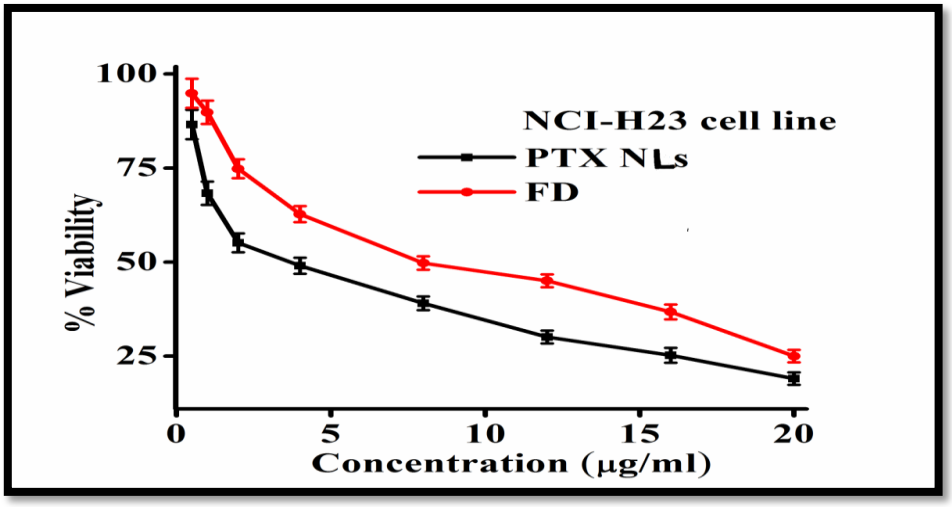


Figure 29 (A) : Cytotoxicity study in 549 cells.

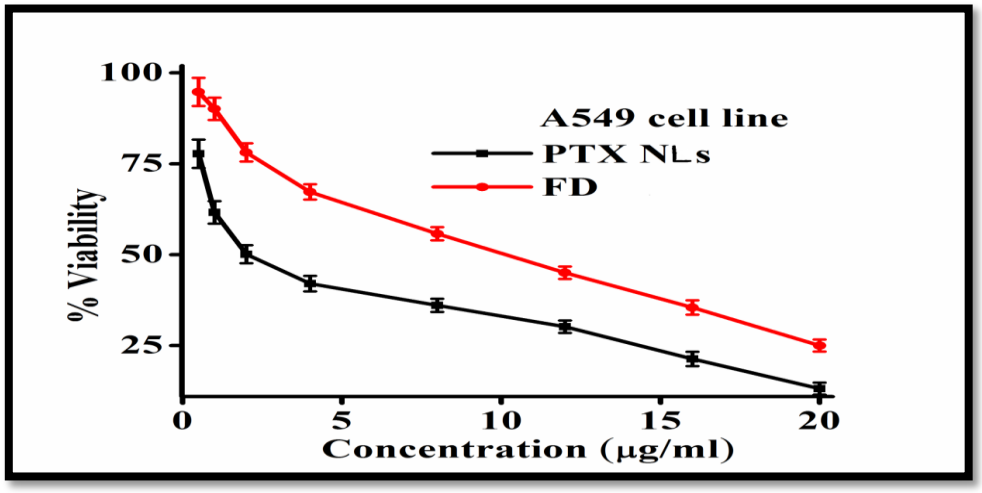
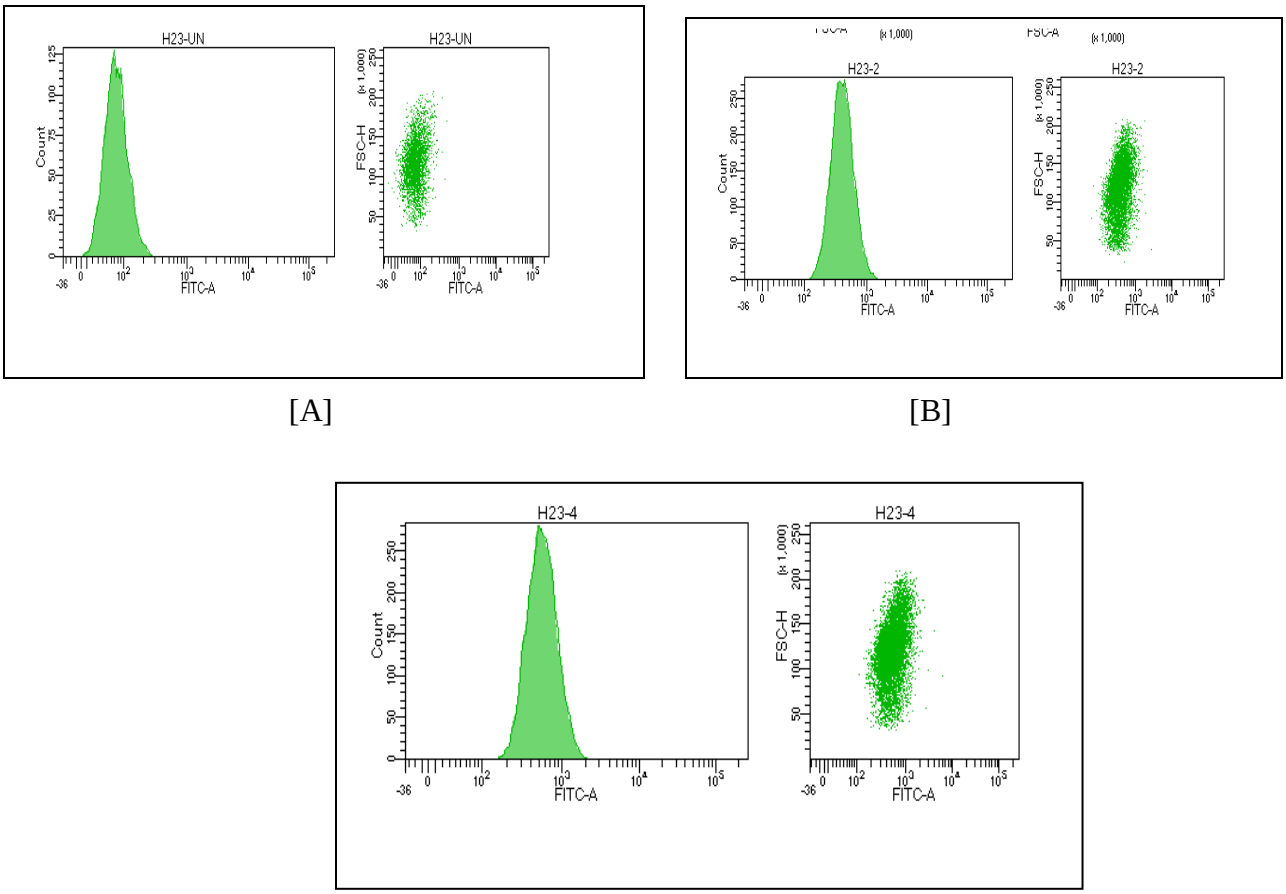


Figure 29 (B) : Cytotoxicity study in NH-23 cells

6.7. In vitro cellular uptake

In vitro cellular uptake with FITC-tagged nanoliposomal formulations revealed that nanoliposomal formulations were internalized more in time-dependent manner as evidenced from FITC-median values of control cells and cells treated with L3 **(Figure 30 and Table 8).**



[A] [B]

[C]

Figure 30 : In vitro cellular uptake of FITC-tagged PTX-NL formulation L3 in NCI-H23 cells and in control cells (cells without treatment) [A], and cells treated with L3 at 1 h [B] and 4 h [C].

Table 8 : FITC-median values of control cells and cells treated with FITC-tagged L3 PTX-NL formulation

Control cells	Treated cells at 1 h	Treated cells at 4h
68	367	511

6.8. Nuclear Condensation Study

Different levels of nuclear condensations such as chromatin condensation, DNA fragmentations and plasma membrane blebbing are the characteristics of apoptosis. Therefore they were visualized by fluorescence microscopy upon staining with DAPI. Fragmentations were found to be more intensive in cancerous cells treated with paclitaxel loaded nanoliposomal (PTX NL) formulation L3.

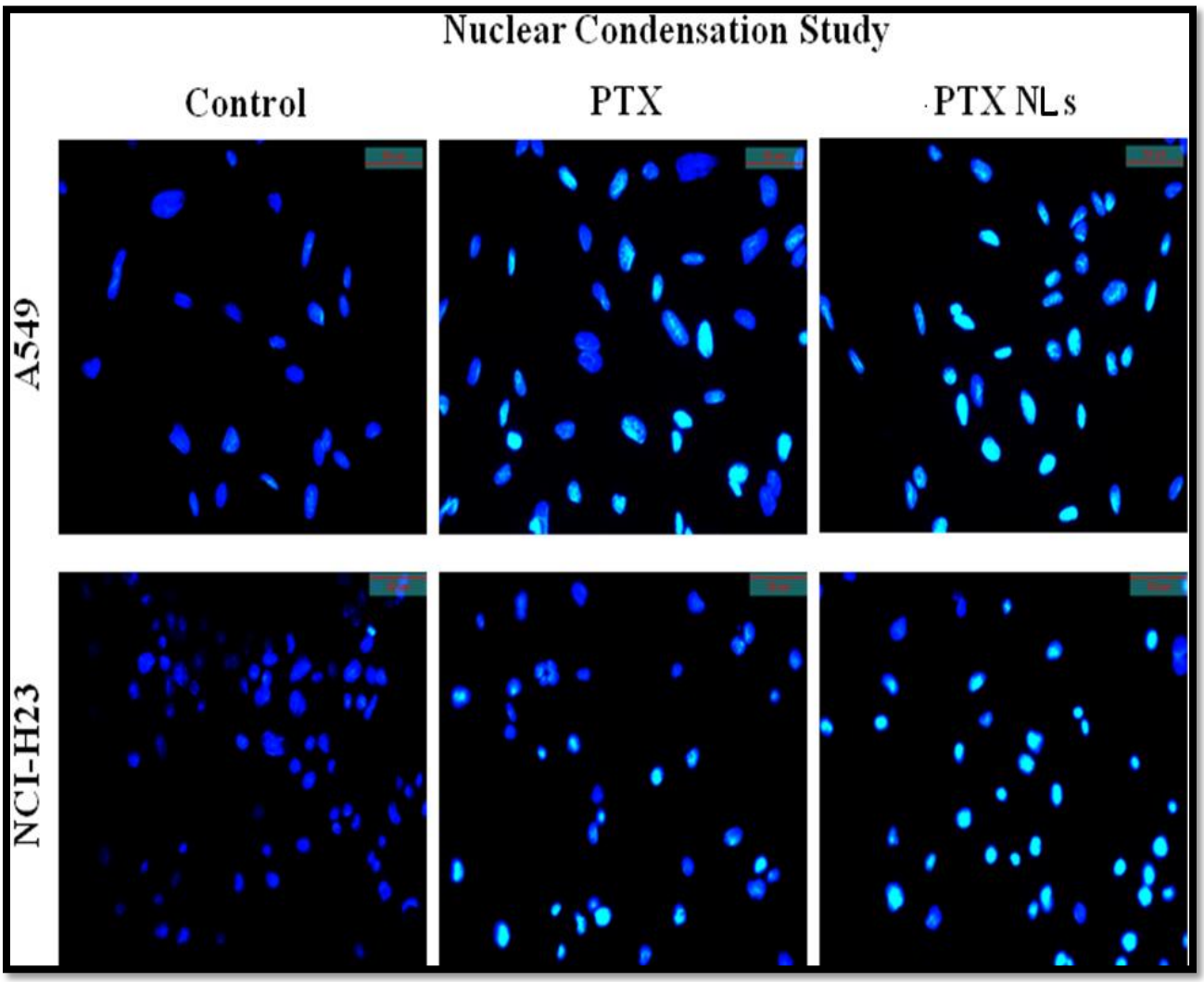


Figure 31 : Nuclear fragmentation study in different cancer cells (A549 and NCI-H23) upon treatment with free-drug (PTX alone) suspension and with PTX- NL (L3).

CHAPTER-7
DISCUSSION

7. DISCUSSION

7.1. HPLC spectra of paclitaxel and calibration curve

Paclitaxel produced sharp peak at 227 nm in spectrophotometric scan, which revealed that the drug was pure in form. Further, calibration curve of the drug had high regression value (R^2=0.9), suggesting the accuracy of the experimental data.

7.2. Factors to be considered for selection of materials and preparation of liposome formulation

7.2.1. Selection of materials

Among the different excipients used for nanoliposomal formulations, soya-lecithin was taken as a natural phospholipid. The bilayer of nanoliposomes is predominantly made up of phospholipid molecules. The hydrophilic section of the phospholipids is oriented towards the internal and external aqueous phases and the hydrophobic groups associate with their counterparts on the other phospholipid molecules.In addition to phospholipid molecules, nanoliposomes can incorporate other ingredients such as sterols in their structure. Sterols are important constituent of most natural membranes and their incorporation into nanoliposome bilayer can bring about major changes in the characteristics of the formulation. The most commonly employed sterol in the structure of the lipid vesicles is cholesterol. Cholesterol does not by itself form bilayer structures, however, it can be incorporated into phospholipid membranes in very high concentrations, e.g. up to 1:1 molar ratios of cholesterol to a phospholipid molecule *(Maherani B and Wattraint O ,2017 ; Mozafari ,2010).* Cholesterol orients into the membrane with its hydroxyl group oriented towards the aqueous surface and aliphatic chained aligned parallel to acyl chains in the center of the bilayer. Cholesterol fills in the gaps created by imperfect packing of lipid species and modulates membrane fluidity, elasticity, and permeability. Inclusion of cholesterol stabilizes liposomal phospholipid membranes against disruption by plasma proteins and results in decreased binding of plasma opsonisation responsible for rapid clearance of liposomes from circulation. It modifies membrane fluidity by preventing crystallization of the acyl chains of the phospholipid molecules and providing steric hindrance to their movement. This phenomenon contributes to the stability of nanoliposome formulation and reduces the permeability of their bilayer membrane to solutes.

There are also scientific reports on the effect of cholesterol in increasing the size homogeneity and improving the polydispersity index of phospholipid vesicles *(Sebaaly C et al.,2016)*. BHT was used as an antioxidant. This antioxidant acts as a scavenger of free radicals and there by protects the susceptible ingredients and extends the stability and shelf life of the lipid vesicles *(,Danaei M et al., 2018;Mozafari et al.,2010).*

7.2.2 Pre-formulation study

Drug-excipients interaction study is an important pre-formulation study to develop a stable formulation. This study can be done using various methods such as Differential Scanning Calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FTIR) etc.

In the study, FTIR analysis was conducted as the pre-formulation method to assess any possible drug-excipients interaction. When the FTIR spectrum of the physical mixture of Paclitaxel and excipients were compared, no shifting of the predominant peaks of the drug and excipients was found demonstrating that there was no chemical interaction between the drug and the selected excipients, thus suggesting that no chemical interaction had taken place.

However, minor shifting of some peaks might be due to the physical interactions by formation of weak physical bonds such as H-bonding, van der Waals force of attraction or dipole-dipole interaction etc. among the various functional groups of SLE, CHL and PTX molecules. These physical interactions might have helped to form NLs structure. Further, in the lyophilized formulations (with and without drug), absence of any peak of the drug suggests that the drug was encapsulated completely.

7.2.3. Preparation of Paclitaxel loaded nanoliposomes (PXT-NL)

In **lipid layer/thin film hydration** method, soya lecithin and cholesterol were used to produce thin layer of lipid along the inner wall of a round bottom flask. An antioxidant, butylated hydroxyl toluene (BHT) was used to reduce the chance of oxidation as phospholipids and cholesterol that are prone to oxidation. Minimum amount of chloroform was used to solubilize the drug and lipid mixture to minimize existence of residual chloroform after evaporation of organic solvent.

The formulation should be prepared in completely dried flask as the presence of moisture it will emulsify the chloroform and may cause bubbling of lipid solution during evaporation which ultimately hampers smooth thin lipid layer formation. Shaking of the flask well for proper mixing of lipids in the chloroform solution is absolute necessary for the formation nanoliposome of good morphology. The organic solvent was evaporated in a rotary vacuum evaporator rapidly with optimum rotation of flask. Temperature and speed of rotation of the flask should be controlled in such a way that depending upon the amount of solvent present, it can help in formation of good layer of lipid without bubble or crystal formation *(Mukherjee et al, 2007).*

The vacuum in a rotary evaporator is commonly insufficient for complete removal of chloroform. So, the flask was kept overnight in a vacuum desiccator for removal of residual chloroform. Hydration of lipid layer was carried out in an inert atmosphere. Hydration swells the lipid layer and helps to disperse it in the aqueous media. The lipid layer, upon dispersion in aqueous media, forms bilayer structure as well as large multilamellar vesicles (LMVs). After hydration the lipid dispersion was sonicated in a bath sonicator.

Sonication is the crucial step which controls size reduction and size distribution during preparation of liposome. Ice cooling of sonicator bath may be used to control overheating during sonication. Sonication breaks the bilayer and LMVs in small fragments *(Satapathy et al, 2016).* After sonication the dispersion was allowed to stand for minimum one hour without any agitation. During this time small bilayer fragments rejoin to form small unilamellar vesicles (SUVs).

Entrapment of drug molecules into the liposome depends upon the physicochemical characteristics of drug, concentration of drug, drug to lipid ratio, and temperature at which formulations are prepared *(Rudra et al, 2010).* The liposome dispersion was then kept in a refrigerator at 4-8°C for overnight. After that it was centrifuged to separate liposomes as a precipitated pellet. The rotational speed and timing of centrifugation affect the nature and amount of sediment. The liposomal pellet was collected in a Petridish then it was pre-freezed and lyophilized to obtain dry liposome powder or flake which helps in long time storage of liposomes without affecting efficacy.

7.3. Physiochemical characterization and evaluation of developed nanoliposomes.

7.3.1. The morphological characterization.

Drug delivery systems with a size below 100 nm have now emerged as a frontier research area in many recent works. It has been observed that nanosize drug delivery systems of size more than 200nm are frequently attacked by RES followed by clearance from plasma, where as the size below 10nm also suffers from a problem of frequent blood clearance *(Bae et al, 2011).* Nano-drug delivery systems of sizes between 10-100 nm are actually smartly escaped from RES, showing improved circulation half-lives and thus are now widely investigated for targeted tumor therapy (*Alexis et al, 2008; Davis et al, 2008)*. Therefore, the study was intended to keep the size of the final selected formulation within 100 nm which we met successfully.

The average particle size of the optimized PTX-NL formulation (L3) was found to be 173 nm (as per detected in DLS) which falls in the range of ideal size to achieve therapeutic benefit. Very small size (less than 10 nm) nanocarriers are very much prone to rapid renal clearance and size more than 400 nm is very much prone to phagocytosis *(Mukherjee et al, 2014).*

FESEM image of the optimized nanoformulation (L3) of PTX-NL, revealed more homogenous distribution of the liposomal particles and the size of the liposomal formulation was found to be around 100 nm which is smaller than those detected in dynamic light scattering (DLS). The difference in size may be due to the DLS method that measures the hydrodynamic diameter and while preparing the sample in milli-Q water, liposomal formulation may swell resulting in increase in the size.The sedimentation rate of the colloidal carriers is directly proportional to their size. Thus, larger size drug carriers tend to precipitate out more easily than the smaller drug carriers or nanosize drug carriers, as the precipitation phenomenon is governed by Stokes' law *(Gupta, 2006).*

Cryo-TEM analysis is now becoming an essential tool for the evaluation of lipid based nano constructs. Due to their delicate nature, the lipid based membrane structures are more prone to get damaged under high vacuum in case of normal TEM (freeze fractured TEM). The cryo-TEM analysis allows for direct imaging of nanosize lipid based membrane structures in their native state at a very low temperature condition. The Cryo-TEM images showed formation of unilamellar lipid vesicles along with very few bilamellar and formulation showed a mixture of smaller (20-30 nm) and little larger vesicles (around 100 nm).

The nanoliposoms were spherical showing darker outer lipid layer enclosing the inner core and without any perforation or leakage on the outer membrane justifying formation of stable structure.

Zeta potential is a measure of net surface charge on the particle and potential distribution at the interface. The charge density of nanoliposomal surface and the binding affinity of various ions to the lipid vesicles can be determined by measuring zeta potential (ZP).

The ZP of a nanoliposome is the overall charge that the nanovesicle acquires in a particular environment or suspension medium *(Mozafari.,2010).* Depending on the composition, zeta potential of liposome can be positive, neutral or negative. It affects the physical stability (aggregation) and *in-vivo* behaviour of formulation.

Generally zeta potential of liposomes is negative due to the presence of terminal carboxylic group in lipids *(Satapathy et al, 2016).* Zeta potential less than -30 mV or greater than +30 mV is considered to be stable at colloidal form for a prolong period and prevents settling down while in suspension *(Satapathy et al, 2016, Shaw et al. 2017).* Zeta potential value of the optimized nanoformulation was found to be -60.7 MV suggesting optimized formulation (L3) had good colloidal stability, which predicts its prolonged stability in a suspended form. Further, in our body, positively charged drug carriers are eliminated more quickly than the negative charge drug carriers, claiming more blood residence time of the experimental formulations *(Oh et al, 2014).*

7.3.2. Release characteristics of paclitaxel from the NLs

Among the four PTX-NL formulations we had prepared, the L3 showed highest amount of drug loading and entrapment efficiency as analyzed by HPLC method and it was used for *in-vitro* drug release study. An essential point in evaluation of liposomal drug delivery systems is the rate at which the drug is released from the carrier. *In vitro* drug release studies are used not only in quality control of drug formulations, but also to predict their *in vivo* behavior.

In vitro drug release data were collected over 72 hours and cumulative releases of drug from the optimized PTX-NL formulation- L3 was plotted against time in order to determine the drug release pattern. The release characteristics of drug (PTX) revealed that initial burst release followed by slow and sustained release of drug. Burst release was possibly due to the diffusion of drug associated with inner near surface region of nanoparticulate matrix upon contact with dissolution media.

Drug diffusion through the tortuous polymeric pathways from the deeper core of polymeric matrix resulted in sustainable drug release for a prolonged period.

These biphasic release patterns may be beneficial to achieve the therapeutic drug concentration within a short period of time and for maintaining the drug concentration within the therapeutic window for a prolonged duration of time.

Further, the drug release pattern was also correlated with established models of release kinetics such as zero order, first order, Higuchi model, Hixon-Crowell model and Krosmeyer-Peppas model, considering the regression coefficient (R^2) values respectively (0.7457, 0.8832, 0.9274, 0.8408 and 0.9828). Fitting of the release data of PTX into the different kinetic models revealed that drug release patterns were best fitted in Korsemeyar-Peppas kinetic model as evidenced from the values of regression coefficient (R^2) and release exponent (R^2 = 0.9828). Drug release from PTX-NLs might follow complex mechanisms of diffusion as well as erosion as the data was best fitted to Korsmeyer–Peppas kinetic model (*Sahana et al, 2010).*

7.4. In vitro study in lung cancer cells

Cytotoxicity study by MTT assay revealed nanliposomal formulation almost 3-6 times more cytotoxic than free-drug. This may be due to the strong hydrophobic nature of PTX resulting in their inefficient delivery inside the cells.

Further, in vitro cellular uptake revealed that nanoliposomal formulation was internalized more as the incubation time increases. This may due to the effective penetrations of nanoliposomal carrier through the fenestrations in blood capillary due to their improper formations by enhanced permeability retention (EPR) phenomenon.

Mutations in proto-oncogenes and tumor suppressor genes are responsible for malignant transformation of normal resulting in cancer. Therefore, induction of powerful apoptosis is only the key to eradicate malignancy. Therefore, potential of chemotherapeutics is judged by their ability to induce apoptosis. Apoptosis normally occurs by two pathways, namely extrinsic and intrinsic pathway. PTX exerts its apoptotic activity by stabilization of microtubules as well as by modulating the activity and expressions anti apoptotic proteins of Bcl-2.

Nuclear degradation is considered as one the signature characteristics of apoptosis. Therefore nuclear degradation study was conducted to observe the changes in cells upon induction of apoptosis by PTX. Data suggest that L3 caused more nuclear degradation than PTX alone.

CHAPTER-8
CONCLUSION

8. CONCLUSION

Lung cancer is one of the devastating malignancies due to high turnover rate of blood resulting in significant mortality. In the present study paclitaxel loaded nanoliposome with a smooth morphology along with good drug loading had been obtained to achieve prolonged drug release in lungs. In vitro study in cancerous cells revealed anticancer potential of paclitaxel loaded nanoliposomal formulation (L3). However, in vivo study in lung cancer model in rodents is necessary in order to explore its potential in full extent. Further their in vivo delivery is a real challenge. In our laboratory we developed an apparatus with which nanoformulations can be delivered to lungs without disturbing the normal breathing pattern. Therefore, our future objective is to deliver the paclitaxel nanoliposomal formulations of through this device to rodents with lung cancer and analyse various pharmacokinetic parameters.

CHAPTER-9
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9. REFERENCES

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