DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF LIPID BASED NANO CARRIER SYSTEM CONTAINING ABIRATERONE ACETATE FOR PROSTATE CANCER THERAPY

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By

Sahajit Mallick

B. PHARM

Class roll no: 001711402007
Examination roll no: M4PHA19009
Registration no: 140833 of 2017-18

Under the guidance of

Prof. (Dr.) Biswajit Mukherjee

Division of Pharmaceutics
Department of Pharmaceutical Technology
Faculty of Engineering and Technology
Jadavpur University
Kolkata -700032

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DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY
FACULTY OF ENGINEERING AND TECHNOLOGY
JADAVPUR UNIVERSITY
KOLKATA -700032

Certificate of Approval

This is to certify that **Sahajit Mallick** (Class Roll No: 001711402007, Examination Roll No: M4PHA19009 and Registration No: 140833 of 2017-2018) has carried out the research work entitled "**DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF LIPID BASED NANO CARRIER SYSTEM CONTAINING ABIRATERONE ACETATE FOR PROSTATE CANCER THERAPY**" independently with proper care and attention under my supervision and guidance in the Pharmaceutics Research Laboratory in the Department of Pharmaceutical Technology, Jadavpur University. He has incorporated his findings into this thesis of the same title, being submitted by him, in partial fulfilment of the requirements for the degree of **Master of Pharmacy** from Jadavpur University. I appreciate his endeavour to do the project and his work has reached my gratification.

Prof. (Dr.) Pulok Kumar Mukherjee
Head of the Department
Department of Pharmaceutical Technology
Faculty of Engineering and Technology
Jadavpur University
Kolkata -700032

Prof. (Dr.) Biswajit Mukherjee
Project Guide
Division of Pharmaceutics
Faculty of Engineering and Technology
Jadavpur University
Kolkata -700032

Prof. (Dr.) Chiranjib Bhattacharjee
Dean
Faculty of Engineering and Technology
Jadavpur University
Kolkata- 700032, India

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 his 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: SAHAJIT MALLICK
Examination roll number: M4PHA19009
Registration number: 140833 of 2017-2018
Thesis title: DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF LIPID BASED NANO CARRIER SYSTEM CONTAINING ABIRATERONE ACETATE FOR PROSTATE CANCER THERAPY.

Signature with Date

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Date:

Place: Jadavpur University

SAHAJIT MALLICK

Dedicated to my family and my guide

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Chapter-1

INTRODUCTION

1. INTRODUCTION

1.1. Brief overview of Prostate Cancer

Cancer is a group of diseases which involves abnormal cell growth with the potential to invade or spread to different parts of the body. The term cancer originates from Greek word "karkinos" (latin: carcinus) a giant crab in Greek mythology. Cancer is characterized by uncontrolled proliferation of the cells and the ability of these cells to migrate from the original site and spread to distant sites. Cancer can lead to death if the spread is not controlled.

Prostate is a gland in the male reproductive system, development of cancerous tissue in the prostate is known as Prostate cancer (Ahmed et al., 2014). Prostate cancer is heterogeneous disease and the most commonly diagnosed non-cutaneous malignancy in men. Advanced-stage prostate cancer is often treated with androgen-deprivation therapy, which is associated with decreased bone mineral density and an increased risk of osteoporotic fragility fractures. In the past 5 year many new agents for targeting to the metastatic setting have been approved, and the remit of bone-targeted agents for the management of advanced-stage prostate cancer has gathered momentum. This collection will critically discuss the role of surgery, radiotherapy, and also hormonal therapy in men with high-risk disease based on the available trial data. In patients with low-risk disease, the role of active surveillance and focal therapy is covered together with the challenges of over diagnosis and under diagnosis in men with small-volume unpredictable disease. Other topics include multiparametric MRI for detection, staging and treatment planning, as well as the challenges of managing elderly men with prostate cancer.

Prostate cancer is the most frequently diagnosed common cancer and second leading cause of cancer-related death in males (Jemal et al., 2011); and leads to a depressing burden on Society, and rates are 2–5 times higher in developed countries compared with developing countries (Li et al., 2012). Most of the prostate cancers are growing slowly; however, some of it grow relatively quickly (Al Ekish et al., 2013). The spreading of cancer cells from the prostate to other parts of the body, particularly the bones and also lymph nodes. It may initially cause no symptoms. In later stages, it can lead to difficulty in urination, blood with urine or pain in the pelvis, back, or when urinating. Benign prostatic hyperplasia is a disease which may produce similar symptoms. Other late symptoms including tiredness due to low levels of red blood cells.

Factors that increase the risk of prostate cancer including older age, a family history of the disease, and race. About 99% of cases occur in males above 50 years of age. In the United States, it is more common in the African American people than the White American people. Other factors that may be involved such as diet high consumption of processed meat and red meat or milk products or low in certain vegetables.

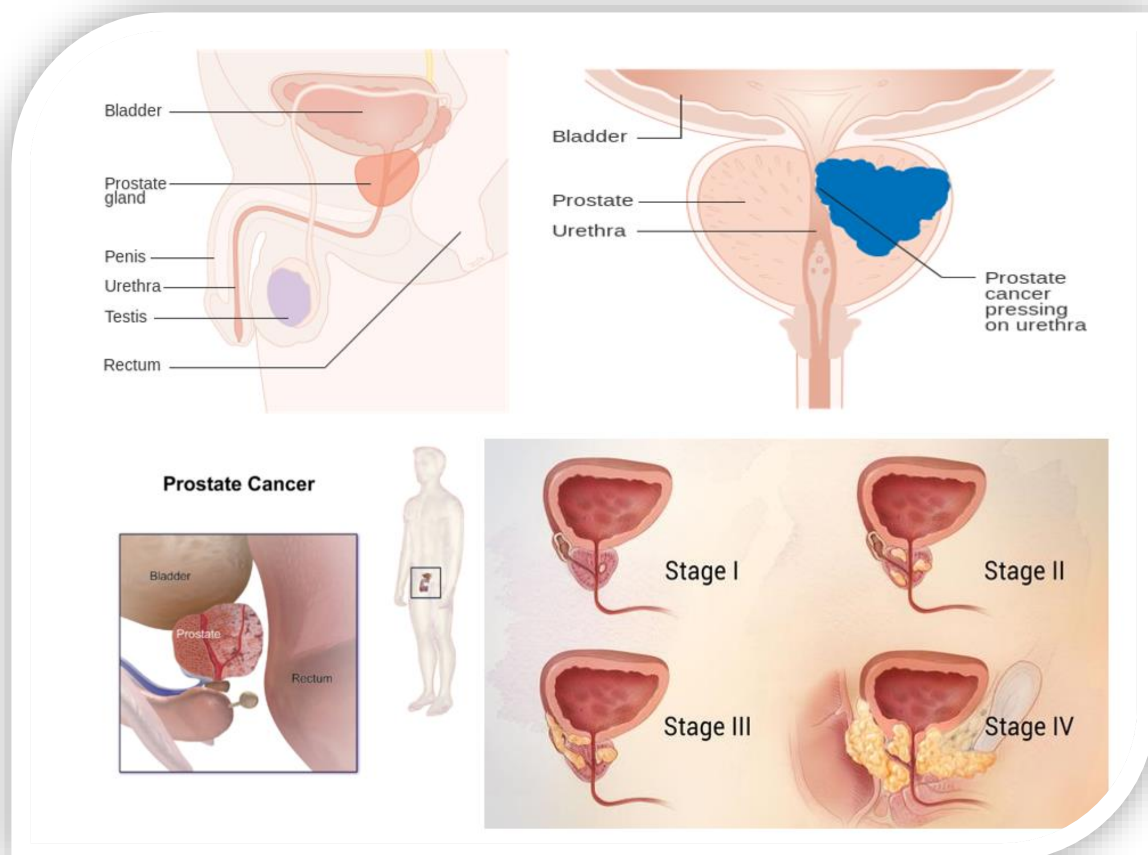

Figure 1: Prostate gland and prostate cancer. (https://en.wikipedia.org/wiki/Prostate_cancer)

Chemotherapy, often combined with surgery, radiation and hormone therapy, is an exclusive treatment for prostate cancer but most of the time; this is not very beneficial because together with the cytotoxicity offered by chemotherapeutic agent, several adverse effects are also associated with such treatment because of the non-specificity of chemotherapeutic agents. In order to achieve more specificity of such agents, targeted drug delivery systems were developed that target the drug to a particular affected cancerous tissue thereby reducing accumulation in other tissues and consequent ill effects (Chen et al., 2013). Therefore, there is a high and unmet demand for effective therapeutics for the metastatic prostate cancer (Luo et al., 2002). Improvement of formulation development has emerged as a potential way for augmenting the efficacy and safety of chemotherapy (Tomii et al., 2002). The development of new vehicles as well as drug formulations could enhance targeted drug delivery to the tissue while minimizing side effects and increasing patient compliance (Ghate et al., 2008).

1.2. Abiraterone acetate (AA) and effectiveness against prostate cancer

In 1993 Abiraterone acetate was first described and after Given the extension of survival with AA following prior docetaxel demonstrated in a large phase 3 trial, regulatory approval was granted by the Unites States FDA (Food and Drug Administration) in April 2011, it was introduced for medical uses (Guru et al., 2011).

1.2.1. Mechanism of action:

It is an androgen synthesis inhibitor – specifically, a CYP17A1 inhibitor – and thereby inhibits the production of androgens like testosterone and dihydrotestosterone in the body.

Targeting CYP17 as a strategy to inhibit androgen production, the key enzyme that mediates androgen synthesis in the testes and adrenal glands is CYP17, which bears components of 17a-hydroxylase and 17,20-lyase (Figure 2). Notwithstanding the suppression of cortisol synthesis by inhibition of the 17a-hydroxylase component, it was observed that congenital CYP17 deficiency manifested as hypogonadism and mineralocorticoid excess without symptoms of hypocortisolism (Rovner et al., 1979). It was hypothesised that the reciprocal increase in pituitary adrenocorticotrophic hormone (ACTH) production as a result of low cortisol leads to elevated corticosterone, which has glucocorticoid activity that mitigates the development of hypocortisolism (Costa-Santos et al., 2004). Therefore, AA was rationally designed to inhibit CYP17.

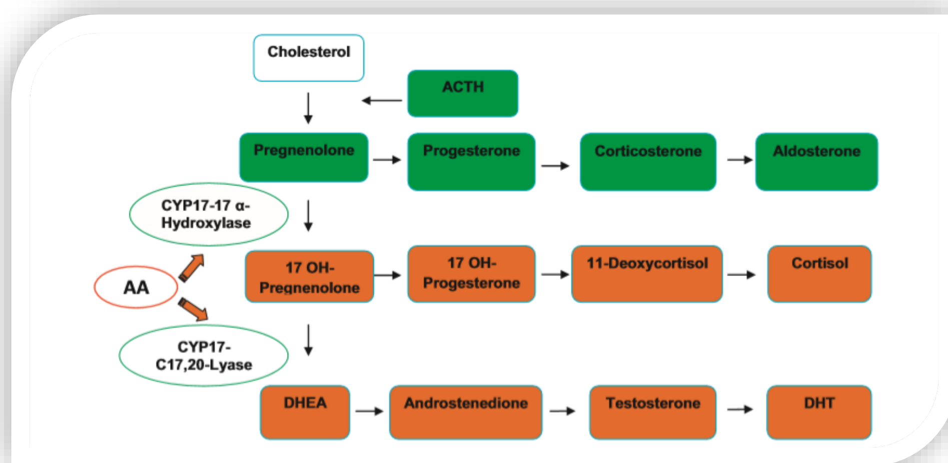

Figure 2: Steroid synthesis and the critically important enzymes and pathways. (Guru et al., 2011)

1.2.2. Chemistry of Abiraterone acetate:

Abiraterone, a pregnenolone-derived 3-pyridyl steroidal compound, was developed at the Institute of Cancer Research, United Kingdom (Barrie et al., 1994). The 16,17-double bond is necessary for its irreversible binding to CYP17, and the 3-pyridyl substitution results in its being 10–30 times as potent in the selective inhibition of CYP17 compared to ketoconazole (Jarman et al., 1998; Rowlands et al., 1995).

1.2.3. Pharmacokinetics of Abiraterone acetate:

Bioavailability of AA is 50% on most on empty stomach. The drug is highly protein bound (>99%) mainly to α1-acid glycoprotein, albumin, and is metabolised in the liver by CYP3A4 and SULT2A1 to inactive metabolites. The drug is excreted in feces (~88%) and urine (~5%), and has a terminal half-life of 12 ± 5 hours.

1.2.4. Contraindication:

Contraindications include hypersensitivity to abiraterone acetate. Although documents state that it should not be taken by women who are or who may become pregnant, there is no medical reason that any woman should take it. Women who are pregnant should not even touch the pills unless they are wearing gloves. Other cautions include severe baseline hepatic impairment, mineralocorticoid excess, cardiovascular disease including heart failure and hypertension, uncorrected hypokalemia, and adrenocorticoid insufficiency.

1.2.5. Interaction:

Spironolactone generally exerts anti-androgenic effects, but experimental evidence exists that it acts as an androgen receptor agonist in an androgen-depleted environment, capable of inducing prostate cancer proliferation (Luthy et al., 1988). This is supported by the observations described in several case reports (Dhondt et al., 2018). Therefore, spironolactone should be avoided in prostate cancer patients suffering from treatment-associated mineralocorticoid side effects of Abiraterone acetate.

1.2.6. Adverse effects:

Some common Side effect of Abirateron acetate include fatigue, arthralgia, hypertension, nausea, peripheral edema, hypokalemia, hot flashes, diarrhoea, vomiting, cough, headache, cardiac failure, angina pectoris, arrhythmia, atrial fibrillation, dyspepsia, hematuria, glucocorticoid deficiency, mineralocorticoid excess, and hepatotoxicity among others.

1.3. Targeted Drug Delivery System (TDDS)

The concept of targeted drugs is not new, but dates back to 1906 when Ehrlich (Allen, 1997) first postulated the 'magic bullet'. The challenge has been on three fronts: finding the proper target for a particular disease state; finding a drug that effectively treats this disease; and finding a means of carrying the drug in a stable form to specific sites while avoiding the immunogenic and nonspecific interactions that efficiently clear foreign material from the body.

❖ **Advantages of TDDS:**

1. Drug administration protocols may be simplified.
2. Toxicity is reduced by delivering a drug to its targeted tissues
3. Avoidance of hepatic first pass metabolism
4. Smaller amount of drug required to produce the desired effect
5. Enhancement of absorption of the targeted molecules such as peptides and particulates
6. No peak and valley plasma concentration
7. Selective targeting to infectious cells (Vyas & Khar, 2008)

❖ **Disadvantages of TDDS:**

1. Rapid clearance from the targeted systems
2. Immune reactions against IV administered carrier systems
3. Insufficient localization of targeted systems into tumor cells
4. Diffusion and redistribution of released drugs
5. Requires skill for manufacturing storage and administration
6. Difficult to maintain stability of dosage form
7. Requires highly sophisticated technology for formulation (Allen, 1997).

1.3.1. Types of Targeted Drug Delivery:

Targeting drug to a specific area not only increases the therapeutic efficacy of drugs but also aims to decrease the toxicity associated with drug to allow lower doses of the drug to be used in therapy. For the fulfilment of such conditions, two approaches are used extensively:

a) Passive targeting:

In passive drug targeting, the drug’s success is directly related to circulation time (Sagnella and Drummond, 2012). This is achieved by cloaking the nanoparticle with some sort of coating. Several substances can achieve this, with one of them being polyethylene glycol (PEG). By adding PEG to the surface of the nanoparticle, it is rendered hydrophilic, thus allowing water molecules to bind to the oxygen molecules on PEG via hydrogen bonding. The result of the bonding is a film of hydration around the nanoparticle which makes the substance antiphagocytic. The particles obtain this property due to the hydrophobic interactions that are natural to the reticuloendothelial system (RES), thus the drugloaded nanoparticle is able to stay in circulation for a longer period of time (Vlerken et al., 2007). To work in conjunction with this mechanism of passive targeting, nanoparticles that are between 10 to 100 nanometers in size have been found to circulate systemically for longer periods of time (Gullotti and Yeo, 2009).

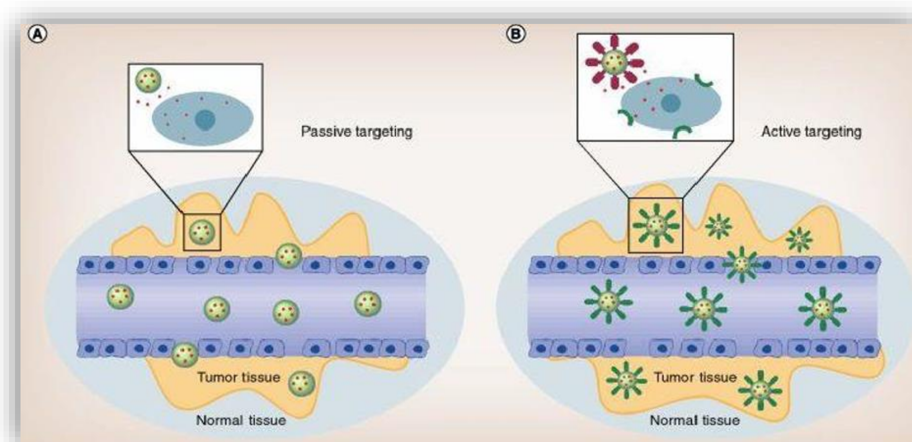

Figure 3: Active and passive targeting. (http://www.medscape.com/viewarticle/770397)

b) Active targeting:

Active targeting means a specific ligand-receptor type interaction for intracellular localization which occurs only after blood circulation and extravasations. This active targeting approach can be further classified into three different levels of targeting which are 1) First order targeting refers to restricted distribution of the drug carrier systems to the capillary bed of a predetermined target site, organ or tissue e.g. compartmental targeting in lymphatics, peritoneal cavity, plural cavity, cerebral ventricles and eyes, joints. 2) Second order targeting refers to selective delivery of drugs to specific cell types such as tumour cells and not to the normal cells e.g. selective drug delivery to kupffer cells in the liver. 3) Third order targeting refers to drug delivery specifically to the intracellular site of targeted cells e.g. receptor-based ligand mediated entry of a drug complex into a cell by endocytosis (Kannagi et al., 2004). By utilizing both passive and active targeting, a drug-loaded nanoparticle has a heightened advantage over a conventional drug. It is able to circulate throughout the body for an extended period of time until it is successfully attracted to its target through the use of cell-specific ligands, magnetic positioning, or pH responsive materials. Because of these advantages, side effects from conventional drugs will be largely reduced as a result of the drug-loaded nanoparticles affecting only diseased tissue (Mitra et al., 2015).

1.3.2. Monoclonal anti PSMA antibody: A Novel Approaches of Prostate Targeting

Monoclonal antibodies (mAb or moAb) are made by identical immune cells that are all clones of a unique parent cell. Monoclonal antibodies can have monovalent affinity, in that they bind to the same epitope (the part of an antigen that is recognized by the antibody). In contrast, polyclonal antibodies bind to multiple epitopes and are usually made by several different plasma cells (antibody secreting immune cell) lineages.

An Antigen found in the membrane of prostate gland is prostate-specific membrane antigen (PSMA). Prostate-specific membrane antigen (PSMA), also known as gamma-seminoprotein or kallikrein-3 (KLK3), is a glycoprotein enzyme encoded in humans by the KLK3 gene. PSMA is a member of the kallikrein-related peptidase family and is secreted by the epithelial cells of the prostate gland (Aparicio et al., 2013).

Originally developed with a type of prostate cancer cell line known as LNCaP cells, the mAb 7E11 was the first anti-PSMA antibody. It recognizes and binds a PSMA intracellular or cytoplasmic epitope. New mAbs, such as J591, however, continue to be discovered and developed (Troyer et al., 1995). Lately, novel targeting agents such as aptamers (Levy-Nisenbaum et al., 2008), short peptides and other small molecules (Brown, 2010) have become the targeting molecules of the new generation.

1.4. Nanotechnology: an overview

Nanotechnology involves the development of material at atomic, molecular and macromolecular level. The Greek word ―nano‖ means dwarf. Applying this technology scientists are able to comprehend materials by the scale of atoms and molecules having properties such as at least 1 to 100 nm dimension, can be designed using methodologies, which control the physical and chemical attributes of molecular-scale structures, and able to combine to make large structures (Safari et al., 2014). 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. 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 increase 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, 2006).

1.4.1. Nanomaterials in nanomedicine

a) Liposomes

Liposomes are vesicular structure with an aqueous core surrounded by a hydrophobic bilayer created by the extrusion of phospholipids. The advantages of liposomes are they are nontoxic, flexible, biocompatible, biodegradable and non-immunogenic for systemic and non-systemic administration. Liposomes are also surface modified in order to target drugs to diseased tissue or organs. Surface modification is done by coating liposomes with inert and biocompatible polyethylene glycol which prevents recognition by opsonins thereby preventing recognition by phagocytic cells.

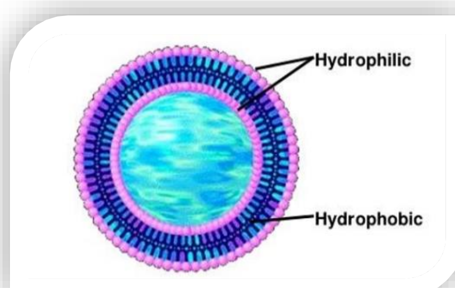

Figure 4: Schematic diagram of liposome. (Mukherjee et al., 2009)

b) Polymeric nanoparticles:

Nanoparticles are vesicular systems in which the drug is confined to a cavity surrounded by unique polymer membranes. The advantages of using nanoparticles for drug delivery result from their two main basic properties. Firstly, nanoparticles because of their small size, can penetrate through smaller capillaries and are taken up by cells, which allow efficient drug accumulation at the target sites. Secondly, the use of biodegradable materials for nanoparticle preparation allows sustained drug release within the target site over a period of days or even weeks.

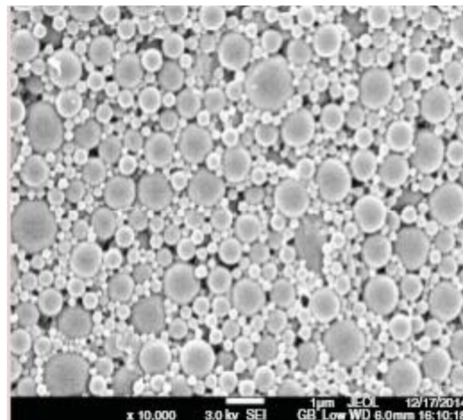

Figure 5: Pictorial view of nanoparticles. (Paul et al., 2017)

c) 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).

d) Nanoemulsion

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).

e) Nanogels

Nanogels are cross linked polymeric particles that can be considered as hydrogels if they are composed of water soluble or swellable 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).

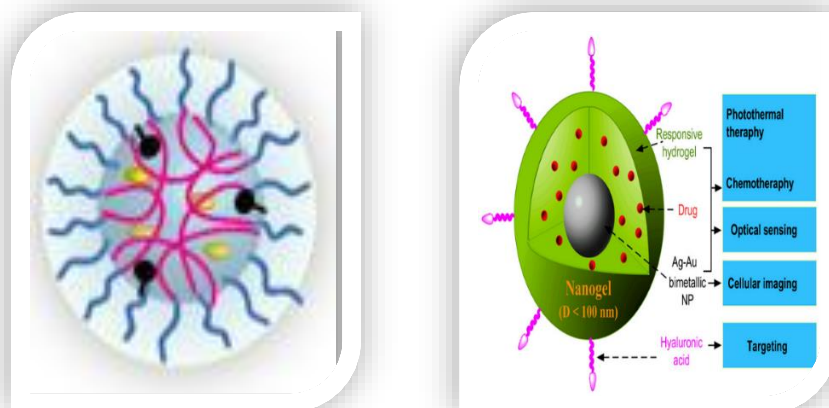

Figure 6: Schematic diagram of nanogels.
(https://elements.chem.umass.edu ; http://www.mdpi.com/2310-2861/3/2/16)

f) Solid lipid nanoparticles (SLNs)

Solid lipid nanoparticle is a type of carrier system based on solid lipid matrix, i.e. lipids solid at the body temperature. SLNs are particles made of solid lipids i.e. highly purified triglycerides, complex glyceride mixture or waxes stabilized by various surfactants. The main characteristics of SLN include a good physical stability, protection of incorporated drug from degradation, controlled drug release and good tolerability. They have been exploited for the dermal, peroral, parenteral, ocular, pulmonary and rectal delivery (Mukherjee et al., 2009).

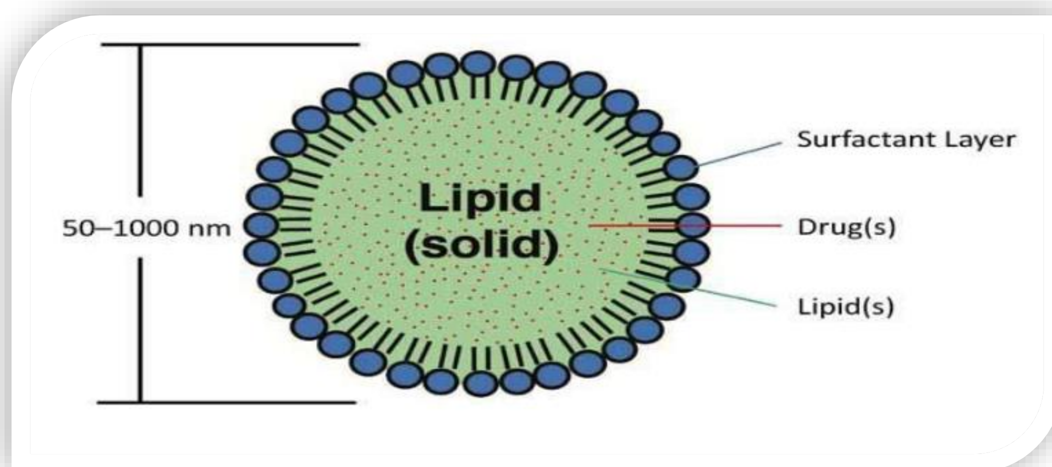

Figure 7: Schematic diagram of solid lipid nanoparticles.

(http://www.eurekaselect.com/137264/article)

g) Dendrimers

Dendrimers are branched, synthetic polymeric macromolecular nanocarriers having tree like architecture. 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).

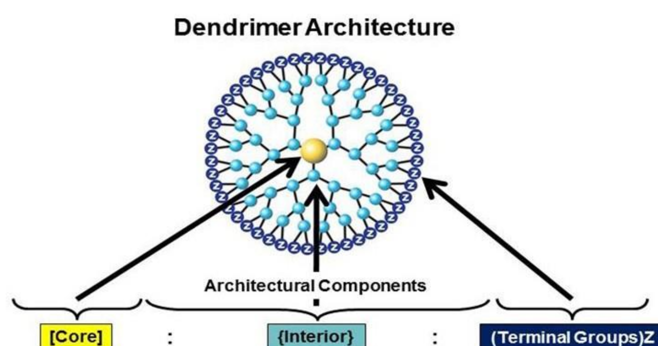

Figure 8: Architectural diagram of dendrimers. (Singh et al., 2014)

h) 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., 2013).

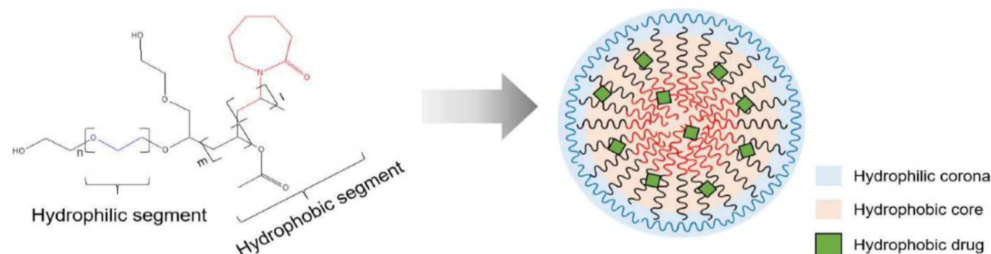

Figure 9: Schematic diagram of polymeric micelles.

(https://www.mdpi.com/1999-4923/10/4/208)

i) Quantum dots

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).

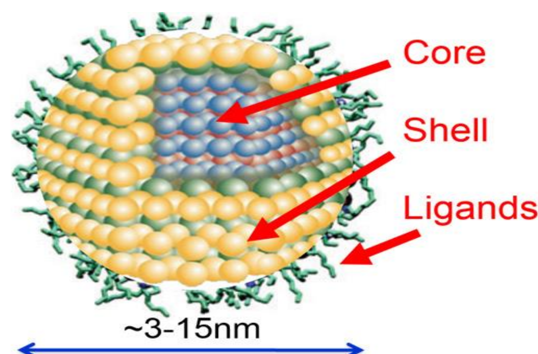

Figure 10: Schematic diagram of quantum dots. (Mukherjee et al., 2015)

j) Nanoshells

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. With the advancement in technology it has become possible to synthesize nanoshells in desired shape, size and morphology. For obtaining desired morphology core particles having different morphologies such as rod, wire, tube, ring etc are coated with thin shell in core shell structure. These shells are economical as costly and precious materials can be deposited on inexpensive cores. The targeting of nanoshells to desired location can be achieved by immunological means and their application include various fields such as providing chemical stability to colloids, biosensors, drug delivery such as in diabetis, cancer chemotherapy etc (West and Halas, 2000).

k) Fullerenes

Fullerenes are a family of carbon molecules with a cage like structure with hollow spheres (Bakry et al., 2007). In September 1985, Robert F. Curl, Jr., Richard E. Smalley, and Harold W. Kroto discovered fullerene C60 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). It has been reported that a metal moiety can be easily entangled into the inner hollow sphere of the fullerene molecule which have been investigated to be used for MRI technology (Bakry et al., 2007). The unique chemical structure of C60 nanoparticles rendered them to acquire the photophysical properties to use them in photodynamic therapy for biological application and the production of reactive oxygen species when they are irradiated with light in the visible wavelength is the main mechanism behind their use (Pikhurov et al., 2013).

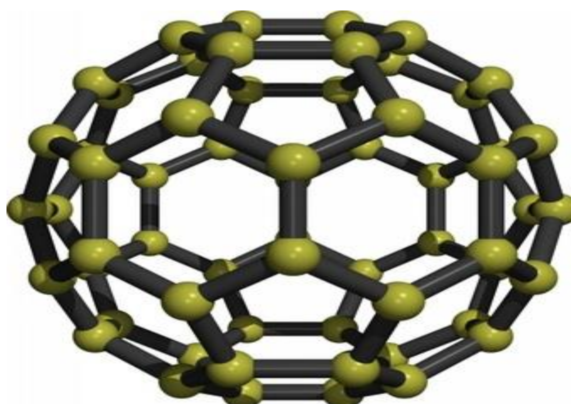

Figure 11: Schematic diagram of fullerene. (Mukherjee et al., 2015)

l) Carbon nanomaterials

Carbon nanocarriers used in drug delivery system are differentiated into nanohorns (CNH) and nanotubes (CNT). Nanohorns (CNH) are a type of single wall nanotubes and they exhibit similar properties to that of nanotubes. The formation of nanohorns does not require the presence of a metal catalyst thereby they can be prepared at a very low cost and are of high purity. The immobilization of drug depends on adsorption on nanohorn walls or nanoprecipitation of drug on nanohorns (Ajima et al., 2008).

Carbon nanotubes are hexagonal network of carbon atoms about 1 nm in diameter and 100 nm in length composed of a layer of graphite rolled up into a cylinder. Carbon nanotubes are of two types: single walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) (Kohler et al., 2004). Nanotubes are characterised by an enormous surface area and an excellent electrical and thermal conductivity (Beg et al., 2011). There are three ways of drug immobilization in the carbon nanocarrier which are: encapsulation of the drug in the carbon nanotube (Tripisciano et al., 2010), chemical adsorption on the surface or in the spaces between the nanotubes (Chen et al., 2011) and attachment of active agents to functionalized carbon nanotubes (Perry et al., 2011).

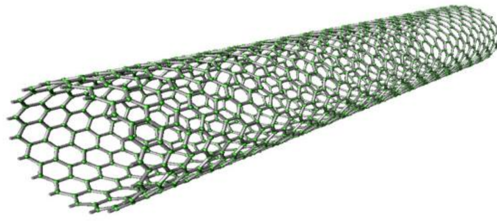

Figure 12: Schematic diagram of Carbon nanotubes. (https://www.gaia3d.co.uk/3d-models/3d-chemistry/carbon-nanotube/)

m) Nanofibers

Nanofibers 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 nanofibers such as template synthesis, drawing, self-assembly, electrospinning, phase separation, melt-blown etc. A wide range of polymers such as gelatine, collagen, polyvinyl alcohol, chitosan and carboxymethyl cellulose can be subjected to electro-spinning techniques to produce nanofibers. Nanofibers 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).

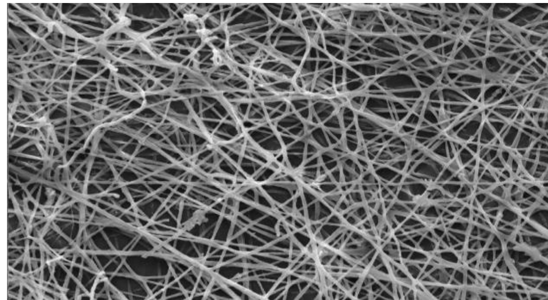

Figure 13: Schematic diagram of Nanofibers.
(https://www.revolutionfibres.com/learn/)

1.5. Liposome and Nanoliposome: an overview

1.5.1. Liposome

Liposomes are artificially constructed closed vesicles consist of one or more concentric bilayer membrane of discrete phospholipid molecules enclosing an equal number of aqueous compartments, which generally used to deliver both lipophilic and water-soluble microscopic substance to the body cells. The bilayer is composed of two lamellae in which polar head groups of phospholipids (or sphingolipids) are arranged towards the aqueous phase and the lipophilic acyl chains are arranged towards themselves and attached side by side to form a tight packing (Laouini et al., 2012; Mansoori et al., 2012).

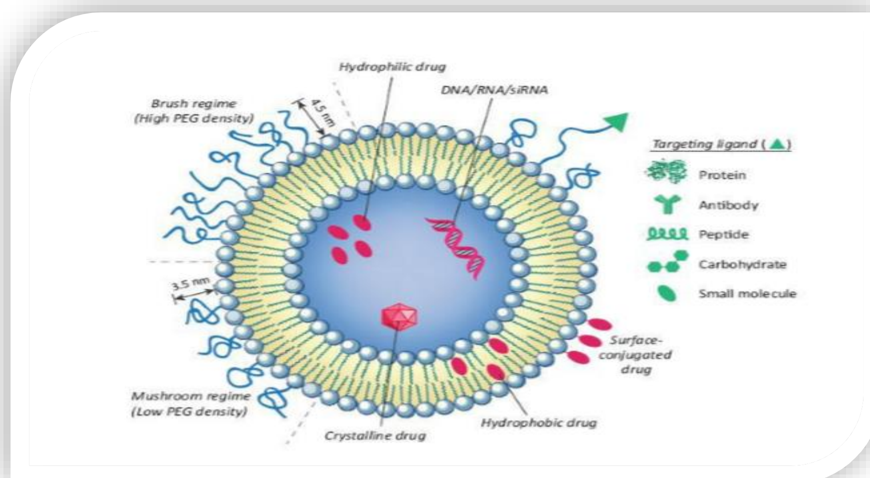

Figure 14: Schematic diagram of liposome with targeting ligand.

(https://www.intechopen.com/books/application-of-nanotechnology-in-drug-delivery)

- **Mechanism of Liposome Formation:**

Liposome forming materials like phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) etc. have a hydrophilic polar head group 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 sides 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. However, a number of vesicles may be enclosed in a large vesicle to form multivesicular vesicle (Albert et al., 2002).

- **Advantages of Liposomal Drug Delivery:**

1) 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.

2) Improved pharmacokinetic profile: Liposomal drug delivery increases circulation lifetime of therapeutic agents and reduces their elimination especially for PEGylated liposomes.

3) 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.

4) Targeted delivery: It provides passive targeting to the selected tissues.

5) Active targeting: It is possible to attach several targeting ligands such as antibody, aptamer with liposome to achieve active targeting.

6) 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.

7) Low drug requirement: Liposomal delivery requires relatively very low amount of drug which increases therapeutic index & reduces adverse effects.

8) 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 vesicular membrane with cellular membrane.

9) Site avoidance effect: Site avoidance effect can be achieved easily by liposomes (Dua et al., 2012).

- **Limitation of Liposomal Drug Delivery:**

1) Low drug entrapment: In most of the cases low entrapment of drug into liposome increases material consumption and labour.

2) Leakage and fusion: Leakage and fusion of encapsulated material requires special attention during formulation and also modification in method & material.

3) Toxic effect: Some liposomal ingredients exert toxic effect.

4) Oxidation and hydrolysis: Phospholipids are susceptible to oxidative and hydrolytic degradation which seeks attention in special storage condition for liposome.

5) Instability: Instability of liposomal carriers in biological system is another problem.

6) Cost: Large scale production of liposomal formulation is relatively costly.

1.5.2. Nanoliposome

Nanoliposomes are nanometric versions of liposomes which are one of the most applied encapsulation and controlled release system. In order to have a better understanding of Nanoliposome it is essential to understand the older technology from which Nanoliposomes have developed i.e. liposomes. The word liposome is derived from two Greek word lipos (fat) and soma (body or structure) meaning a structure in which a fatty envelope encapsulates internal aqueous compartment (Mozafari, 2011). Liposomes are simple microscopic vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (Bangham et al., 1965). Liposome can also be defined as a microstructure consisting of one or more concentric spheres of lipid bilayer separated by water or aqueous buffer compartment (Weiner

et al., 1989). Various amphiphatic molecules have been used to form liposomes. The drug molecules can either be encapsulated in aqueous space or intercalated into lipid bilayers. The exact location of drug will depend on its physicochemical characteristics and the composition of lipids (Fielding, 1991).

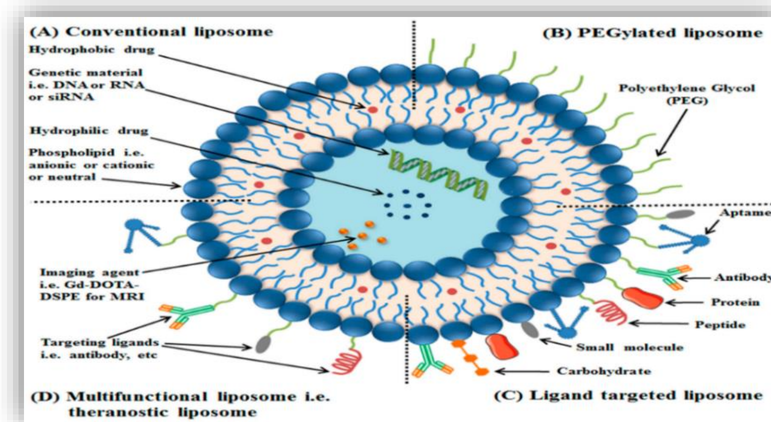

Figure 15: A schematic elastration of an ideal multifunctional liposomes with encapsulated drugs and gens, imaging agent, cell-penetrating agent and specific targeting moiety. (http://www.mdpi.com/1422-0067/19/1/195/htm)

➢ Advantages of Nanoliposome over Liposome:

1) The thermodynamic stability of nanoliposome is higher compared to liposome due to its smaller size.

2) It allows easier penetration into the cells.

3) In case of nanoliposome relatively lower amount of drug is required per equal volume of liposome which reduces drug toxicity and also gives economic advantage.

4) Nanoliposomes provide more surface area compared to liposomes and have the potential to increase solubility, enhance bioavailability, improve controlled release and enable precision targeting of the encapsulated material to a greater extent compared to liposomes (Mozafari, 2011).

➢ Limitation of Nanoliposome over Liposome:

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.

- **Mechanism of Drug Release from Nanoliposome:**

1) Due to swelling of the nanoliposome followed by hydration and then release through diffusion.

2) Due to enzymatic reaction causing cleavage or degradation of the polymer at the site of delivery and subsequent release of the drug from the entrapped inner core.

3) Due to dissociation of the drug from the polymer and its release from the swelled nanoliposome (Ghosh et al., 2000).

- **PEGylation of liposomes:**

When drugs or proteins are administered parenterally, they are either quickly cleared off from the circulation by glomerular filtration or by reticulo endothelial system (RES), with the consequence of a rapid loss of their biological properties as well as therapeutic efficacy, so more frequent administrations are needed. Many studies are done to overcome this problem by enhancing the stability. The most successful approach is modification by linking one or more PEG molecule to the surface of the drug carrier e.g. liposomes. The polymer most widely used for protein PEGylation is linear mPEG, end-capped on one side with a methoxyl group and terminated with a hydroxyl group. The most used method for preparation of clinically suitable liposomes is the anchoring of the PEG moiety to the liposomal membrane via a cross-linked lipid like PEG-distearoylphosphatidylethanolamine (PEG-DSPE).

Rationale for using PEGylation of liposomes:

- PEGylation Improves Pharmacodynamic and Pharmacokinetic properties of drug by increasing water solubility.
- PEGylation shows increased half-life and decreased plasma clearance.
- PEGylation enhances circulation time in blood as well as increases the stability.
- PEGylation decreases immunogenic, pyrogenic and antigenic reactions.
- PEGylation acts as steric barrier for inhibiting liposome fusion (Nag et al., 2013; Milla et al., 2012; Bru et al., 2002).

1.5.3. Materials Used for Preparation of Liposome

Liposomes can be prepared from a variety of lipids and mixtures. Phospholipids are most commonly used for preparation of liposomes. Examples of phospholipid include

- Phosphatidyl choline
- Phosphatidyl ethanolamine
- Phosphatidyl glycerol
- Phosphatidyl serine
- Sphingo myelin
- Lyso phospholipid (Dua et al., 2012)

1.5.4. Classification of Liposomes

➢ **Classification of liposome based on vesical size and lamellarity**

- Multilamellar large vesicles (**MLV**)**:** (>0.5µm)
- Oligolamellar vesicles (**OLV**)**:** (0.1-1.0 µm)
- Unilamellar vesicles (**UV**)**:** All sizes
- Small Unilamellar vesicles (**SUV**)**:** (20-100nm)
- Medium sized unilamellar vesicles (**MUV**)**:** (250-500nm)
- Large unilamellar vesicles (**LUV**)**:** >100nm
- Giant unilamellar vesicles (**GUV**)**:** >1µm
- Multivesicular vesicles (**MVV**)**:** usually >1µm

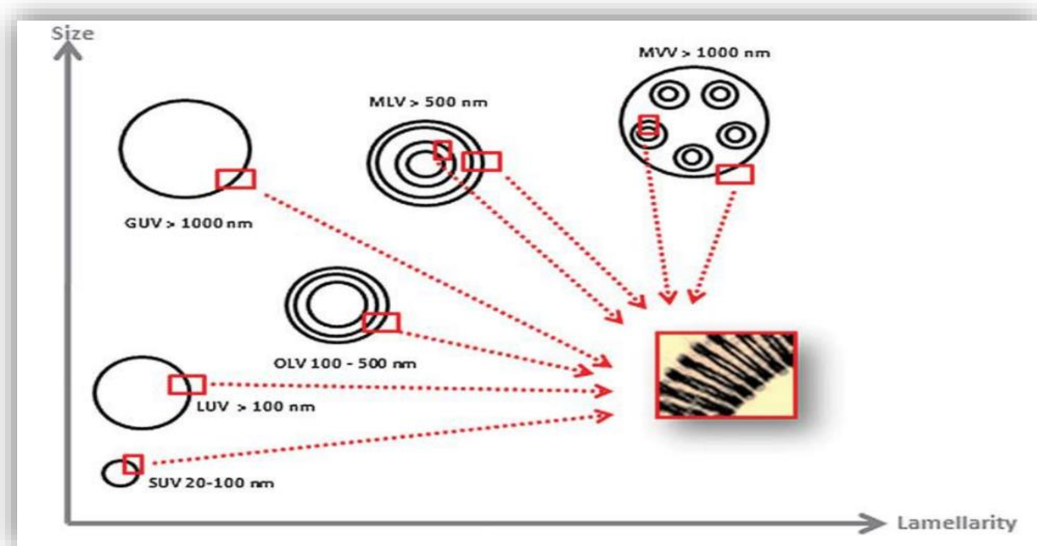

Figure 16: Liposomes classification based on size and lamellarity. (Bordi et al., 2006; Laouini et al., 2012)

➢ **Liposome classification based on preparation methods**

- **REV:** Single or oligolamellar vesicle made by reverse phase evaporation method.
- **MLV / REV:** Multilamellar vesicles made by reverse phase evaporation method.
- **SPLV:** Stable plurilamellar vesicles.
- **FATMLV:** Frozen and thawed multilamellar vesicles.
- **VET:** Vesicles prepared by extrusion method.
- **FUV:** Vesicles prepared by fusion.
- **FPV:** Vesicles prepared by french press method.
- **DRV:** Dehydration- rehydration vesicles.
- **BSV:** Bubblesomes (Kulkarni et al., 2012).

- **Liposome classification based on composition**
  - **Conventional liposome:** Made of neutral or negatively charged phospholipid.
  - **Fusogenic liposome:** Reconstitute ultraviolet inactivated sendai virus envelop.
  - **Cationic liposome:** Cationic lipids make up the membrane of these liposomes.
  - **Long circulatory liposome:** The lipids used for this type of formulation are neutral lipids with a high transition temperature.
  - **pH sensitive liposomes:** These liposomes fuse with cells when the pH is low, thus releasing its content into the cell cytoplasm.
  - **Immuno liposome:** Long circulatory liposome with attached monoclonal antibody. (mansoori et al., 2012).

1.5.5. Types of liposome

I. Conventional liposomes:

These are neutral or negatively charged liposomes typically composed of only phospholipids, glycolipids and/or cholesterol without derivatization to increase the circulation time. These liposomes are generally used for passive targeting to the phagocytic cells of mononuclear phagocytic system, localizing predominantly in the liver and spleen. Conventional liposomes have also been used for antigen delivery.

II. Sterically stabilized liposomes / Long-circulating Liposomes:

The development of these represented a milestone in liposomal drug delivery research. Long-circulating liposomes are obtained by modulating the lipid composition, size and charge of the vesicle. At present the most popular way to produce long-circulating liposomes is to attach hydrophilic polymer polyethylene glycol (PEG) covalently to the outer surface. Such PEG coated liposomes are also called “stealth” or “sterically stabilized” liposomes.

Since conventional liposomes are recognised as foreign body by the immune system polyethylene glycol (PEG)-lipid (commonly known as pegylated lipid) such as PEG-phosphatidylethanolamine (PEG-PE) is often included in the formation of liposomes. Pegylated liposomes reduces the uptake of liposomes by the mononuclear phagocytic system(MPS) or the reticuloendothelial system resulting in prolonged circulation half life. By persisting in the bloodstream pegylated liposomes can localize into tumours and most sites of inflammatiion.

III. Targeted liposomes:

In addition to PEG coating, most stealth liposomes have some sort of biological species attached as a ligand to the liposome to enable binding via a specific expression on the targeted delivery site. These targeting ligands could be monoclonal antibodies (known as immunoliposome),vitamins or specific antigens. Targeted liposomes can target nearly any cell type in the body and deliver drugs that would naturally be systemically delivered.

IV. Cationic liposomes:

Cationic liposomes are positively charged liposome and used for nucleic acid delivery. Cationic liposomes interact with negatively charged phosphate backbone of DNA or RNA, leading to the neutralization of charge. Cationic liposomes are prepared using a cationic lipid and a colipid, such as dope or cholesterol. The cationic amphiphiles differ markedly and may contain single multiple charges. The three basic components of cationic lipids include i)hydrophobic lipid anchor group which helps in forming liposomes and can interact with cell membranes ii) a linker group and a iii) positively charged headgroup which interacts with nucleic acid leading to nucleic acid condensation and charge neutralisation (Mahato et al., 2007).

V. Niosomes:

Niosomes are non-ionic surfactant-based liposomes. Niosomes are formed mostly by cholesterol incorporation as an excipient. The particle size ranges from 10nm-100nm. Niosomes are biodegradable, relatively nontoxic, more stable and inexpensive, an alternative to liposomes, the current deepening and widening of interest of niosomes in many scientific disciplines and, particularly are applied in medicine (Khandare et al., 1994; Baillie et al., 1985).

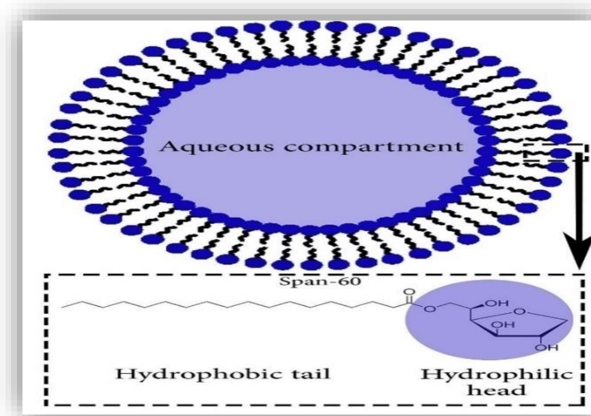

Figure 17: Schematic diagram of niosomes. (https://en.wikipedia.org/wiki/Niosome)

VI. Transferosomes:

Transferosomes are ultra-deformable hydrophilic lipid vesicles that purportedly cross the skin under the influence of a trans epidermal water activity gradient (Ceve and Blume, 2001). These vesicles are up to 105 times more deformable than unmodified liposomes. This characteristic allows transferosomes up to 200-300 nm in size to squeeze through pores in stratum corneum. These pores are less than one tenth of liposome diameter (Ceve and Blume, 2001; Ceve et al., 1997). Nonoccluded condition is best for transferosome drug delivery, as this type of delivery requires a hydration gradient for maximal penetration (Paul and Ceve, 1995). Transferosomes have markedly enhanced the delivery of macromolecules, such as insulin and gap junction proteins by transdermal route (Ceve et al., 1998). The entrapment efficiency of liposomes and niosomes was almost equal; however, slightly less entrapment in case of niosomes estimated may be accounted to the pore formation characteristic of the span 85 in bilayers of the niosomes (Hofer et al., 2000).

VII. Ethosomes:

Ethosomes are multilamellar vesicles composed of phospholipids (soyphosphotidyl choline), ethanol and water. Ethosomes is known to be an efficient enhancer of permeability (Valiakka-koskela et al., 1998; Kirjavainen et al., 1999) experiments using fluorescent probes and ultracentrifugation have shown that ethosomal system have a higher entrapment capacity for molecules of various lipophilicities, e.g. Acyclovir, minoxidil and testosterone (Horwitz et al., 1999; Touitou et al., 2001).

VIII. Proliposomes:

Proliposomes are defined as dry free flowing particles that immediately form liposomal dispersion on contact with water body. Proliposomes are composed of water-soluble porous powder as carrier upon which one may load phospholipids and drugs dissolved in organic solvent. The drugs and phospholipids are deposited in micro porous structure of the carrier materials, thus maintaining the free-flowing surface characteristics of the carrier materials. Then they are free flowing particulate properties permit the fabrication of proliposomes into solid dosage forms such as tablets and capsules, which are then converted to liposomes on contact with water or biological fluids. Proliposomes can be stored and sterilized in dry state and dispersed/dissolved to form an isotonic multilamellar Liposomal suspension by addition of water as needed (song et al.,2002; Hu and Rodes, 2000).

1.5.6. General Methods of Liposome Preparation

There are several methods (Figure 18) for preparation of different types of liposomes. The choice of method depends on the following parameters:

1) The physicochemical characteristics of the material to be entrapped and those of the liposomal ingredients.

2) The nature of the medium in which the lipid vesicles are dispersed.

3) The effective concentration of the entrapped substance and its potential toxicity.

4) Additional processes involved during application/ delivery of the vesicles.

5) Optimum size, poly-dispersity and shelf-life of the vesicles for the intended application.

6) Batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products (Dua et al., 2012).

Depending upon the mode of dispersion liposome preparation methods are classified as

1) Passive loading method: The drug is encapsulated during liposome formation (Akbarzadeh et al., 2013).

a) Mechanical dispersion method

b) Solvent dispersion method

c) Detergent removal method

Passive loading is the process, in which the drug(s) are encapsulated during the formation of liposome. The hydrophilic drugs are loaded within the internal aqueous core of liposome 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 un-entrapped drug molecules can be removed from liposome 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).

2) Active loading method: The drug is encapsulated after liposome formation.

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 (Haran et al., 1993). 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 liposome (Qiu et al., 2008).

All the methods of preparing liposomes involves four basic stages (Akbarzadeh et al., 2013)

- Drying down lipids from organic solvent.
- Dispersing the lipid in aqueous media
- Purifying the resultant liposome
- Analyzing the final product

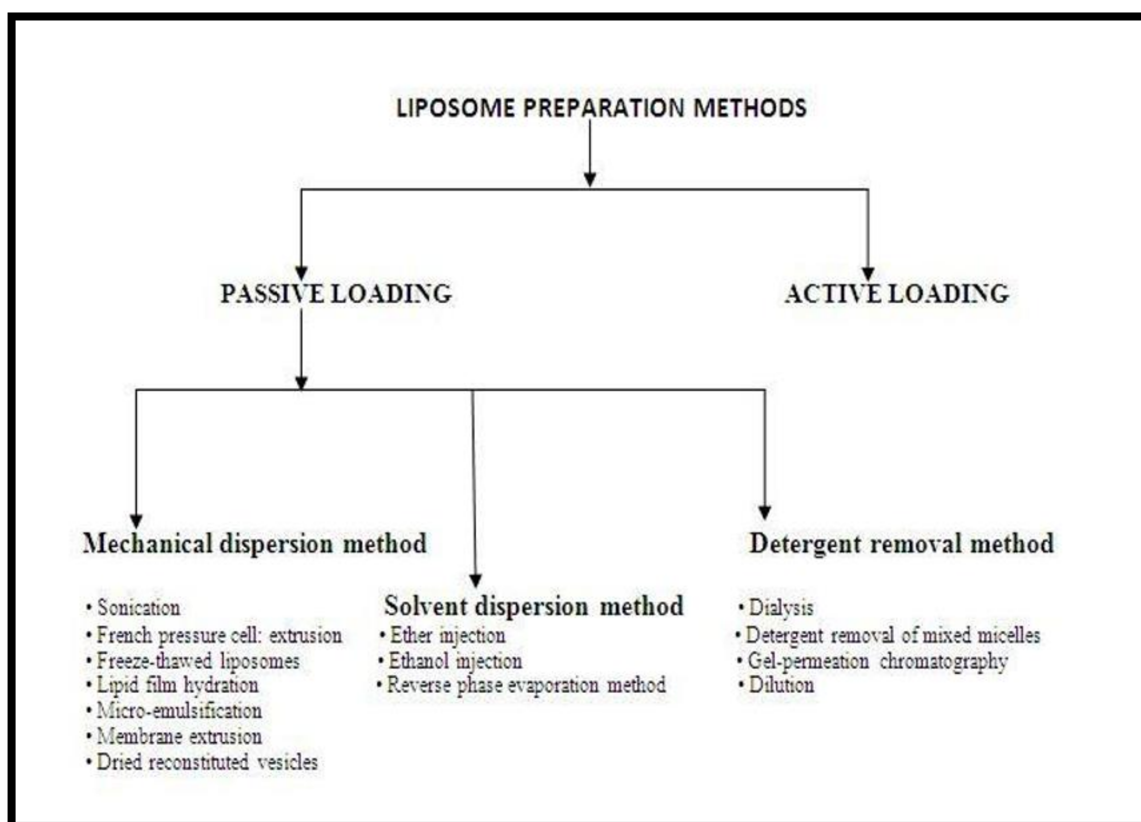

Figure 18: General methods Liposome preparation. (Akbarzadeh et al.,2013)

❖ Mechanical Dispersion methods

Lipid hydration method

In lipid hydration method the lipids are first dissolved and mixed in an organic solvent in a round bottom flask to ensure homogeneous mixture of lipids. The lipids are generally dissolved in chloroform. Once the lipids have been thoroughly mixed the organic solvent is removed to obtain a lipid film. The organic solvent is generally removed by rotary vacuum evaporation to yield a lipid film on the sides of the round bottom flask. The lipid film is thoroughly dried to remove residual organic solvent by placing the round bottom flask in a vacuum dessicator overnight. Then the film is hydrated by adding aqueous buffer and vortexing the dispersion for some time. The hydration step is done above the gel-liquid crystalline transition temperature Tc of the lipid or above the Tc of the highest melting component in the lipid mixture. The components to be encapsulated are added either to organic solvent containing lipids or to aqueous buffer depending on their solubilities. This method is very suitable for preparation of MLVs. The disadvantages of this method are low internal volume, low encapsulation efficiency and the size distribution is heterogeneous (Sipai et al., 2012).

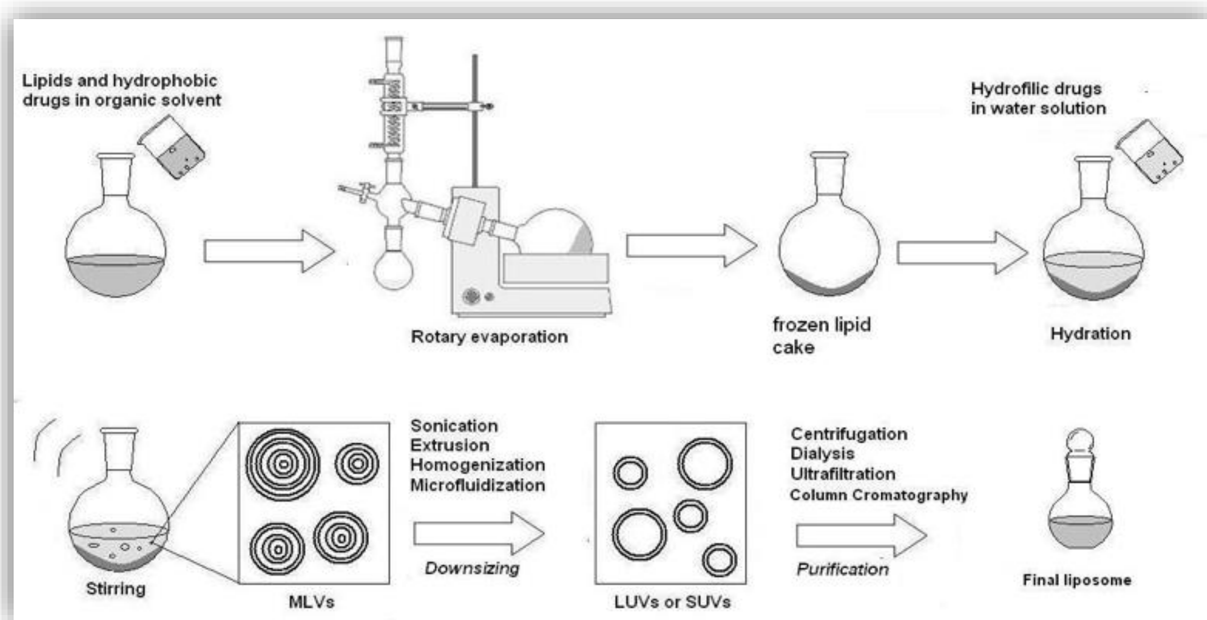

Figure 19: Preparation of liposome by lipid hydration method.
(https://www.intechopen.com/source/html/44386/media/image7.png)

Handshaking method

In order to produce liposomes, lipid molecules must be introduced into an aqueous environment. At the time of hydration of lipid film, the lamella swells and grows into myelin figures. Only mechanical agitation provided by vortexing, shaking, swering or pipetting causes myelin figures to break and reseal the exposed hydrophobic edges resulting in the formation of liposomes made by handshaking method.

Sonication

Sonication is perhaps the most extensively used method for the preparation of SUV. Here, MLVs are sonicated either with a bath type sonicator or a probe sonicator under a passive atmosphere. The main disadvantages of this method are very low internal volume/ encapsulation efficacy, possible degradation of phospholipids and compounds to be encapsulated, elimination of large molecules, metal pollution from probe tip, and presence of MLV along with SUV (Riaz, 1996; Abolfazl et al., 2013).

French pressure cell method

One of the first and very useful method for the preparation of liposome developed is the extrusion of preformed large liposomes in a French press under very high pressure. The method involves extrusion of MLVs at 20,000 psi at 4 degree centigrade through a small orifice. The method is simple, rapid, reproducible and involves gentle handling of unstable materials. The resting liposomes are somewhat larger than sonicated SUVs. The drawbacks of the method are the temperature is difficult to achieve and the working volume is relatively small (Dua et al., 2012; Hamilton et al., 1984).

Micro-emulsification

In this method small vesicles are formed from concentrate lipid solution using micro fluidizer. The lipids are introduced into the fluidizer as a suspension of large MLV. Microfluidizer then pump the suspension at a very high speed through 5 micrometre screen. After that, it is forced to long micro channels where two streams collide with each other at a right angle. Then the fluid is collected and may be recycled to obtain the vesicle with optimum dimension.

Dried-reconstituted vesicles (DRVs)

In this method solid lipids are dispersed in finely divided form using freeze drying technique before contact with the aqueous fluid which will medium for the final suspension. A suspension of empty SUVs is frozen and lyophilized instead of drying the lipids from an organic solution. Thus, the SUVs dried lipid is already highly organized into membrane structure, which on addition of water can rehydrate, fuse and reseal to form vesicles with a high capture efficiency (Jain et al., 2005).

Freeze thaw method

In this method, SUVs are rapidly frozen and followed by slow thawing. The brief sonication disperses aggregated materials to LUV. The formation of unilamellar vesicles is due to fusion of SUV during the process of freezing or thawing. This type of fusion is strongly inhibited by increasing the ionic strength of the medium and by increasing the phospholipid concentration. The encapsulation efficiencies from 20-30% can be obtained. The disadvantages with the method are divalent metal ions, sucrose and high ionic strength salt solutions cannot be entrapped efficiently (Pick, 1981).

❖ Solvent Dispersion method

Ethanol Injection method

An ethanol solution of lipids is injected rapidly into an excess of saline or another aqueous medium through a fine needle. The force of injection is unusually sufficient to achieve complete mixing, so that ethanol is diluted almost instantaneously in water and phospholipid molecules are dispersed evenly throughout the medium. This procedure yields a high proportion of SUVs (diameter 25 nm), although lipid aggregates and larger vesicles may form if the mixing is not thorough enough. The advantage of this method is that the procedure is simple and rapid and avoids exposure to harsh conditions of both lipids and the material to be encapsulated. The drawbacks of the method the liposomes are very dilute, it is difficult to remove all ethanol as it forms azeotrope with water, volume of ethanol that can be introduced into the medium (7.5% V/V maximum), the population is heterogeneous and the possibility of various biologically active macromolecules to inactivation in the presence of even low amounts of ethanol (Batzri & Korn, 1973).

Ether Injection method

A solution of lipids dissolved in diethyl ether or ether/methanol mixture is slowly injected to an aqueous solution of the material to be encapsulated at 55-65 degree centigrade or under reduced pressure. The subsequent removal of ether under vacuum leads to the formation of liposomes. This method also treats sensitive lipids very gently and very little risk of causing oxidative degradation ether is free from peroxides. The main drawbacks of the method are that the population is heterogeneous(70-190nm) and the exposure of compounds to be encapsulated to organic solvents or high temperature. First water in oil emulsion is formed by brief sonication of a two-phase system containing phospholipids in organic solvent (diethyl ether or isopropyl ether or mixture of isopropyl ether and chloroform) and aqueous buffer. The organic solvents are removed under reduced pressure, resulting in the formation of a viscous gel. The liposomes are formed when residual solvent is removed by continued rotary evaporation under reduced pressure resulting in the formation of viscous gel. This method has been used to encapsulate small and large macromolecules. The main disadvantage of this method is the exposure of the materials to be encapsulated to organic solvents and to brief periods of sonication (Deamer & Bangham, 1976).

Double emulsion vesicles

In this method the outer of the liposome membrane is created at a second interface between two phases by an emulsification of an organic solution in water. If the organic solution which already contains water droplets is introduced into excess aqueous medium followed by mechanical dispersion a multi compartment vesicle is obtained which may be described as double emulsion. These vesicles are suspended in aqueous medium and have an aqueous core, the two compartments being separated from each other across a thin film of organic solvent. Removal of this solvent clearly results in an intermediate size unilamellar vesicles. The theoretical entrapment yield is 100% (Matsumoto et al., 1977).

Reverse phase evaporation method

In this method first water in oil emulsion is formed by brief sonication of a two-phase system containing phospholipids in organic solvent (diethyl ether or isopropyl ether or mixture of isopropyl ether and chloroform) and aqueous buffer. The organic solvents are removed under reduced pressure, resulting in the formation of a viscous gel. The liposomes are formed when residual solvent is removed by continued rotary evaporation under reduced pressure. This method has been used to encapsulate small and large macromolecules. The main disadvantage of this method is the exposure of the materials to be encapsulated to organic solvents and to brief periods of sonication. The vesicles formed are unilamellar and have a diameter of 0.5µm. The encapsulation percentage is found to be not greater than 50% (Szoka et al., 1978).

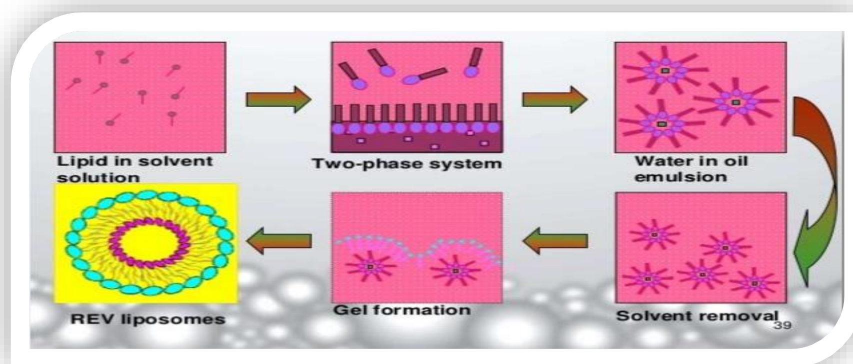

Figure 20: Schematic diagram of liposome preparation by reverse phase evaporation method.

(http://www.slideshare.net)

❖ Detergent removal method

The detergents at their critical micelles concentration have been used to solubilise lipids. As the detergent is removed the micelles become progressively richer in phospholipid and finally combine to form LUVs. The detergent can be removed by dialysis. The advantages of detergent dialysis method are excellent reproducibility and production of liposome populations which are homogeneous in size. The main drawback of the method is the retention of traces of detergent within the liposome (Deepthi et al., 2014).

❖ Industrial Production of Liposomes

(i) Detergent Dialysis

A pilot plant under the trade name of LIPOPREPR II-CIS is available from Diachema, AG, and Switzerland. The production capacity at higher lipid concentration (80mg/ml) is 30 ml liposomes/minute. But when lipid concentration is 10-20 mg/ml 100mg/ml then up to many liters of liposomes can be produced. In USA, LIPOPREPR is marketed by Dianorm Geraete (Maierhofer, 1985).

(ii) Micro fluidization

Mayhe et al., (1984) suggested a technique of microfluidization / microemulsification / homogenization for the large-scale manufacture of liposomes. The reduction in the size range can be achieved by recycling of the sample. The process is reproducible and yields liposomes with good aqueous phase encapsulation. Riaz and Weiner (1995) prepared liposomes consisting of egg yolk, cholesterol and brain phosphatidyl serindiasodium salt (57:33:10) by this method. First MLV were prepared by these were passed through a Microfluidizer (Microfluidics Corporation, Newton, MA, USA) at 40 psi inlet air pressure. The size range was 150-160 nm after 25 recycles. In the Microfluidizer, the interaction of fluid streams takes place at high velocities (pressures) in a precisely defined micro channel, which are present in an interaction

chamber. In the chamber pressure reaches up to 10,000 psi this can be cause partial degradation of lipids.

(iii) Proliposomes

In proliposomes, lipid and drug are coated onto a soluble carrier to form free flowing granular material which on hydration forms an isotonic liposomal suspension. The proliposome approach may provide an opportunity for cost-effective large-scale manufacture of liposomes containing particularly lipophilic drugs (Payne et al., 1986).

Lyophilization

Lyophilization is done in order to improve the physicochemical stability of the nanoparticles to achieve a pharmaceutically acceptable product. Freeze-drying (lyophilization) involves the removal of water from formulation in the frozen state by the process of sublimation at extremely reduced pressures. The process is generally used to dry products that are thermolabile and would be destroyed by heat-drying. The technique has a great potential as a method to solve long term stability problems with respect to liposomal stability. It is exposed that leakage of entrapped materials may take place during the process of freeze- drying and on reconstitution. Recently, it was shown that liposome when freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate commonly found at high concentrations in organism) retained as much as 100% of their original contents. It shows that trehalose is an excellent cryoprotectant (freeze-protectant) for liposomes (Crowe et al., 1987). Cryoprotectants that are added include glucose, mannitol or sorbitol to ensure re-dispersibility and to prevent crystallization of the liquid suspension (Esquisabel et al., 1997). Freeze-driers ranges in size from small laboratory models to large industrial units are available from Pharmaceutical Equipment Suppliers. Recently Schrier et al. (1994) have studied the *in vitro* performance of formulations prepared from lyophilized liposomes.

1.5.7. Factors affecting on liposome formulation

Formulation factors affecting the degree of drug entrapment: The extent of drug entrapment and retention as well as factors influencing them are important considerations in the design of liposome mediated drug delivery system. Drugs may be entrapped in the aqueous and/or lipid phase in the liposome.

a) **Aqueous entrapment:** This relates to the aqueous volume in the liposome. The larger the aqueous volume the greater the amount of polar drug that can be incorporated in the liposome. Multiple component liposomes encapsulate higher percentage of water-soluble drugs than single compartment vesicles because of the larger volume of the encapsulated aqueous phase in the former. Formulations that promote formation of MLVs are thus associated with higher aqueous entrapment. Osmotic swelling and or incorporation of charged lipids increases the aqueous volume of liposomes. Aqueous solubility of the drug is another factor. Cholesterol modifies the fluidity of the lipid membrane thereby

influencing the degree of retention of drugs by vesicles as well as stabilising the system against enzyme degradation.

b) **Lipid entrapment:** Lipid soluble drugs are entrapped in the lipid layers of liposome. Here the entrapment efficiency can be as high as 100% irrespective of liposomal type and composition. An example of a drug that is hydrophobic in nature is camptothecin. The retention such hydrophobic drug is also high when the liposomes are placed in aqueous biological environment because of their high lipid water partition coefficient.

Factors affecting the formulation stability of liposome: The stability of the liposomes refers to their ability to retain entrapped solutes, chemical stability of both the entrapped solutes and the lipid membrane. Solute leakage depends on membrane permeability and on the interaction with components of biological fluid. Membrane fluidity can be controlled to reduce leakage by supplementing the lipid bilayer with cholesterol. The rate of solute leakage also depends on the lamellar structure of liposome for example MLVs are less prone to leakage than ULVs. In order to minimise leakage liposomes are stored in freeze dried form (Sipai et al., 2012).

1.5.8. Characterisation of Liposomes

liposomes are normally characterised by their visual appearance, particle size, morphology, entrapment efficiency and surface charge with the help of advanced micro processing techniques like scanning electron microscopy (SEM), field emission scanning electron microscope (FESEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The average particle diameter, their size distribution and charge affect the physical stability and also the in-vivo distribution. The surface charge of the liposomes affects the physical stability and redispersibility of the polymer dispersion as well as their *in vivo* performance.

Particle size and size distribution

Particle size, distribution and morphology are the most important parameters of characterisation of liposome. Particle size has been found to affect drug release with smaller particles providing larger surface area. As a result of that most of the drug loaded onto them will be exposed to the particle surface leading to fast drug release. On the other hand, drugs slowly diffuse inside larger liposome. Also, smaller particles have a tendency to aggregate during storage and transportation of liposome dispersion (Redhead et al., 2001). Particle size and morphology are measured by using different electron microscopy techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The several tools for determining particle size are as follows:

➢ **Dynamic light scattering**

Currently the most popular and fastest method of determining particle size is by dynamic light scattering. DLS is widely used to determine the size of Brownian liposomes in colloidal suspension in the nanometre and submicron range (De Assis et al., 2008). The size distribution pattern of the vesicles can be obtained from the polydispersity index (PDI) data.

➢ **Scanning electron microscopy (SEM)**

SEM is a surface imaging method in which the incident electron beam scans across the sample surface and interacts with the sample to generate signals reflecting the topographical detail of the specimen surface (Johal, 2011; Ratner et al., 2004). For SEM characterization, liposomes should be first converted into a dry powder which is then mounted on a sample holder and then it is coated with a conductive metal such as gold, platinum using a sputter coater. The sample is then scanned using a focussed beam of electrons. The incident electron causes emission of elastic scattering of electrons referred to as backscattered electrons, inelastic scattering of electrons named low energy secondary electrons and characteristic X-ray light called cathodoluminescence from the atoms on the sample surface. Among these emissions detection of secondary electrons is the most common mode in SEM (Johal, 2011). The size, size distribution and shape of nanomaterials are directly acquired from SEM but the process of drying and contrasting samples may lead to shrinkage of the specimen and alter the characteristics of the nanomaterial (Hall et al., 2007). Also many biomolecules that are nonconductive specimens when scanned by an electron beam tends to acquire charge and insufficiently deflect the electron beam leading to imaging defects. During sample preparation coating an ultrathin layer of electrically conducting material onto the biomolecules is often required (Suzuki, 2002; Hall et al., 2007).

➢ **Transmission electron microscopy (TEM)**

TEM works on the same principle as SEM but in TEM a focussed monochromatic beam of electrons is transmitted through a very thin foil of sample. In TEM the liposome dispersion is deposited onto support grids or films. In order to make the liposome withstand the instrument vacuum and facilitate handling they are fixed by either using a negative staining material such as phosphotungstic acid and derivatives or by plastic embedding. The surface characteristics of the sample are obtained when a beam of electron gets transmitted through an ultra-thin sample. Both SEM and TEM reveal the size and shape heterogenicity of nanomaterials as well as degree of aggregation and dispersion but TEM has the advantage over SEM in providing better spatial resolution and capability for additional analytical assessments (Hall e al., 2007).

➢ **Atomic force microscopy (AFM)**

Atomic force microscopy offers ultra-high resolution in particle size measurement and is based on the phenomenon of physical scanning of samples at submicron level by using a probe tip of atomic scale (Muhlen et al., 1996). In AFM samples are usually scanned in contact or non-contact mode depending on their property. In contact mode topographical map is generated by tapping the probe on to the surface across the liposome sample and in case of non-contact mode

the probe hovers over the conducting surface. The main advantage of AFM is its ability to image non conducting samples without any specific treatment thereby allowing imaging of delicate biological and polymeric micro and nano structure (Shi and Farber, 2003). Also, AFM provides the most accurate description of particle size, size distribution and real picture which helps in understanding the effects of various biological conditions (Polakovic et al., 1999).

Surface charge of liposome (Zeta potential)

The intensity of surface charge of liposome is very important as it determines the interaction of liposome with the biological environment. The colloidal stability of liposome is analysed through measurement of zeta potential and this potential provides an indirect measure of the surface charge. The measurement of zeta potential values provides an estimate of the storage stability of liposome and high zeta potential value either positive or negative is preferred in order to maintain optimum stability and prevent aggregation of the particles. Zeta potential is the overall charge a lipid vesicle acquires in a particular medium. It is a measure of the magnitude of attraction or repulsion between particles in general and lipid vesicles in particular. Evaluation of the zeta potential of liposome helps to predict the stability and *in vivo* fate of liposome (Pangi et al., 2003).

Vesicle Shape

In addition to size and surface properties, the shape of nanomaterial also plays an important role in drug delivery, degradation, transport, targeting and internalization (Champion et al., 2007). It has been observed that efficiency of drug delivery carrier is highly influenced by controlling the shape of the carriers, while phagocytosis of drug delivery carrier through macrophages is also dependent on carrier shape (Champion and Mitragotri, 2009).

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.

Lamellarity

The average number of bilayers present in liposome i.e., lamellarity can be determined by freeze fracture electron microscopy or cryo-transmission electron microscopy (cryo-TEM) and by P-NMR. In later technique the signals are recorded before and after the addition of a broadening agent such as manganese ions which react with the outer leaflet of the outermost bilayers.

Now a day's freeze fracture electron microscopy has become a very popular method to study structural details of aqueous lipid dispersions (Jain et al., 2005; Laouini et al., 2012).

Drug loading

A high loading capacity is the measure of successful nanoliposomal system because it reduces the amount of matrix material for administration. Drug loading and entrapment efficiency depends on the solid-state drug solubility in polymer which in turn is related to the polymer composition, the molecular weight, drug-polymer interaction and the presence of end functional group (Govender et al., 1999).

Entrapped volume

The entrapped volume is defined as the aqueous entrapped volume per unit quantity of the lipid. This entrapped volume is a crucial parameter that governs the morphology of liposomes. It is determined by measuring the quantity of water by replacing the external medium with spectrophotometrically inert fluid (i.e. deuterium oxide) and then measuring water signal by NMR.

Entrapment (Percent capture)

It is essential to measure the quantity of material entrapped inside liposomes before the study of behaviour of this entrapped material in physical and biological systems, since the effects observed experimentally will usually be dose related. In general, two methods may be used i.e. nini column centrifugation and protamine aggregation.

Drug release

A number of methods are applied in order to determine the in-vitro release of drug from the liposome: 1) Reverse dialysis bag technique 2) Dialysis bag diffusion technique

3) Shaking incubator technique 4) Centrifugal ultra-filtration technique

1.5.9. Applications of liposomes

Major applications of liposome are seen in the field of Pharmacology and Medicine and they are divided basically into two major parts –

- Diagnostic applications
- Therapeutic applications

Liposomes generally contain drugs, markers and they are used as a model, tool or reagent in the basic studies of cell interaction, cell recognition, mode of action etc.

1) Cancer Chemotherapy: There are different types liposomal formulation of anticancer drugs is available which show less toxicity when compared to free drugs (Fidler et al., 1989). Anticancer drugs are less selective which results in toxicity of the healthy cells, entrapment of drug in liposomal carrier shows better results (Jain et al., 1997). Liposomal entrapment of the

drugs e.g. Doxorubicin, Danorubicin along with Vincristine showed reduced cardiotoxicity, dermal toxicity and better survival compared to free drug. Encapsulation of etoposide in liposomes significantly delayed tumor growth compared to non-liposomal etoposide. Liposomal formulation of adriamycin resulted in an increased drug concentration in the liver and lungs and a decreased concentration in the heart. Encapsulation of c-myb antisense oligodeoxynucleotides into immunoliposomes showed enhancement of their toxicity toward targeted cells while avoiding non-targeted cells from antisense effects and may be efficacious for the delivery of drugs with broad therapeutic applications to tumor cells (Goyal et al., 2005)

2) Parasitic disease and infection: Conventional liposomes are digested by phagocytic cells in the body after intravenous administration so they are ideal vehicles for the targeting of drug molecules into these macrophages. The best results show in human therapy are probably liposomal Amphotericin B in antifungal therapies. Liposome encapsulated antiviral drugs such as acyclovir, ribavirin, or azide thymidines have also shown reduced toxicity compared to free drugs (Lasic et al.).

3) Liposomes as carrier of vaccines:

a) Immunological adjuvants: There are several advantages of liposomes used as carrier of vaccines:

- Conversion of non- immunogenic substance to immunogenic one.
- Hydrophobic antigen may be reconstituted.
- Small amount of antigen can be used as immunogens.
- During liposomal delivery adjuvants may be incorporated with antigen.
- By inclusion in liposomes toxic or allergic reaction of antibody can be minimized. Liposomised diphtheria toxoid is equally effective and shows same immune response.

b) Carrier of antigens: Liposomes are widely used as an efficient system for the delivery of many biological substances. When liposomes are administered, a major fraction is taken up by the liver and spleen. Some methods can be adopted for delivery of liposomes to target cells are-

- The uptake of liposomes by Reticular Endothelial System (RES) can be reduced using small, neutral, unilamellar liposomes having cholesterol.
- By modifying the surface of liposomes which converts the liposome less recognizable by RES.
- Attaching specific ligand to the surface of liposome which can specifically bind to the receptors.

When cholera toxin has been formulated with DMPC, CH liposomes, the toxicity was completely eliminated and antigenicity is enhanced.

c) Antimicrobial therapy: The liposomes are able to localize in the liver and spleen, especially the RES component, where many pathogenic microorganisms reside; they can be therefore used for targeting of antibiotics.

d) Gene therapy: Cationic liposome formulation like lipofectin, lipofectamine, transfectace, transfectam can be easily used as non-viral human gene delivery system. Cationic liposomes formulated with dioleyloxypropyl trimethylammonium chloride (DOTMA) such as lipofectin is successful for in-vitro and in-vivo gene delivery. For transfecting genes plasmid liposome shows more efficiency than micelles of same composition (Goyal et al., 2005).

e) Radiochemotherapeutics: Indium-111 [(111)In] labeled oxine when delivered by liposomes with a higher concentration of PEG (6 mol%) shows longer circulation time due to reduced phagocytic activity and enhanced tumor targeting efficiency (Chow et al., 2009). Rhenium-188 [(188)Re] labeled PEGylated liposomes loaded with anticancer drugs showed the potential advantages of tumor specific drug delivery and inhibition of cancer growth (Jain et al., 2013).

f) Oral delivery: Liposomal oral delivery of drugs shows higher therapeutic efficiency compared to the other forms as it increases the solubility and absorption in gut. According to Segal et al., 1979, liposomal delivery of anti-arthritic drugs is very effective as it cannot be destroyed easily by peripheral effects. Liposome encapsulated insulin is a very potential system for oral delivery in diabetes.

g) Pulmonary delivery: Many bronchodilators can be formulated as liposome for controlled release to the respiratory tract.

h) Ophthalmic delivery: Many drugs such as iodoxuridine, adrenaline, triamcinolone acetonide, benzyl penicillin etc. can be formulated as liposome for their ocular delivery. The effectiveness sometime depends on size of liposome, drug distribution, and stability of liposomes.

i) Food technology: Liposomes and nanoliposomes have been now extensively used in the food industry to deliver flavours, nutrients and recently, for their ability to incorporate antimicrobials that helps in the protection of food products against microbial contamination.

j) Cosmetics: The lipids in liposome are well hydrated and able to reduce the dryness of the skin, which is a major cause of aging so liposomes are widely used in cosmetics (Jain et al., 1997).

1.5.10. Liposomal formulations available in market

Table 1: Liposomal formulations available in market.

Liposomal Product	Trade name and manufacturer	Therapeutic indication
Liposomal Doxorubicin	**Doxil,** JNJ	ovarian cancer, AIDS related Kaposi's sarcoma, multiple myeloma.
Liposomal Amphoteracin B	**AmBisome,** Astellas Pharma U.S.	Used against fatal fungal infections with fever and low white blood cells.
Liposomal Daunorubicin	**DaunoXome,** NeXstar Pharmaceuticals	Used for treatment of Kaposi’s sarcoma.
Liposomal verteporfin	**Visudyne,** Bausch+Lomb	Used for treatment of agerelated macular degeneration, pathologic myopia and ocular histoplasmosis.
Liposomal cytarabine	**DepoCyt,** SigmaTau Pharmaceuticals	Used by intrathecal administration for treatment of neoplastic meningitis and lymphomatous meningitis.
Liposomal morphine sulfate	**DepoDur,** Pacira Pharmaceuticals	Used by epidural administration for treatment of postoperative pain following major surgery.
Liposomal cisplatin	**Lipoplatin,** Regulon Inc.	Used for treatment of epithelial malignancies such as lung, head and neck, ovarian, bladder and testicular cancers.
Hepatitis A vaccine	**Epaxal,** Crucell Spain S.A.	Used as a vaccine adjuvant in this formulation.
Influenza vaccine	**Inflexal V,** Berna Biotech Ltd.	Used as a vaccine adjuvant.

Chapter-2

LITERATURE REVIEW

2. LITERATURE REVIEW

Banerjee et al., (2004) reported that polyethylene glycol phospholipid was derivatized with an anisamide ligand, which was then incorporated into the DOX (Doxorubicin) loaded liposome and the resulting anisamide-conjugated liposomal DOX showed significantly higher toxicity to DU145 cells than non-targeted liposomal DOX. The cytotoxicity of the targeted liposomal DOX, however, was significantly blocked by haloperidol, suggesting that the increased cytotoxicity was specifically mediated by the sigma receptors. Fluorescence imaging studies after intravenous (i.v.) administration showed that incorporation of anisamide into liposomes significantly improved their accumulation into the tumor. Targeted liposomal DOX injection showed significant growth inhibition of established DU-145 tumor in nude mice with minimal toxicity. Free DOX was effective, but associated with significant toxicities.

Narayanan et al., (2009) reported the preparation of liposome and shown the efficacy of liposome encapsulated curcumin, and resveratrol individually and in combination in male B6C3F1/ J mice. Further, we examined the chemopreventive effect of liposome encapsulated curcumin and resveratrol in combination in prostate-specific PTEN knockout mice. *In vitro* assays using PTEN-CaP8 cancer cells were performed to investigate the combined effects curcumin with resveratrol on (i) cell growth, apoptosis and cell cycle (ii) impact on activated p-Akt, cyclin D1, m-TOR and androgen receptor (AR) proteins involved in tumor progression. Combination of liposomal forms of curcumin and resveratrol significantly decreased prostatic adenocarcinoma *in vivo* ($p < 0.001$). *In vitro* studies revealed that curcumin plus resveratrol effectively inhibit cell growth and induced apoptosis. Molecular targets activated due to the loss of phosphatase and tensin homolog (PTEN) including p-Akt, cyclin D1, mammalian target of rapamycin and AR were downregulated by these agents in combination. Findings clearly suggest that phytochemicals in combination may reduce prostate cancer incidence due to the loss of the tumor suppressor gene PTEN.

In a different study **Wang et al., (2014)** developed a liposomal drug delivery system conjugated with cyclic arginine-glycine-aspartic acid-tyrosine-lysine peptide (cRGDyk) as αvβ3 integrin ligand was thus developed to improve therapeutic efficacy in a mice model of bone metastasis from prostate cancer. The resultant liposomes were characterized in terms of size, morphology, zeta potential, stability, drug encapsulation percentage and loading efficiency, and drug release. Compared with free cisplatin and cRGDyk-free liposomes, cRGDyk conjugated liposomes showed significantly higher cellular uptake and higher cytotoxicity of loaded cisplatin, as evidenced by *in vitro* cell experiments. *In vivo* results revealed that free cisplatin and free cRGDyk could relieve tumor-induced pain but had no contributions to tumor regression and overall survival improvement. cRGDyk-free liposomal drug system with prolonged blood circulation time could accumulated in the tumor sites in the bone through enhanced permeability and retention (EPR) effects and however, did not exhibit desirable therapeutic efficacy superior to free cisplatin and free cRGDyk. This strongly suggested that ERP effects were not effective in treating metastases. By taking advantages of targeted drug delivery and synergistic antitumor activity of cRGDyk and loaded cisplatin, cRGDyk conjugated liposomal drug system could inhibit osteoclastic and osteoblastic bone lesions, relieve pain, and improve overall survival.

Stuart et al., (2016) described the synthesis and efficacy of a novel-targeted liposome which can selectively deliver N,N,N',N'tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) to PSMA+ cells using the SZTI01 DNA aptamer. Once internalized by targeted PCa cells, liposomes release TPEN, which chelates Zn2+ and results in enlarged ROS levels leading to cell death. The targeted, TPEN-loaded liposomes enable killing of targeted PCa cells under treatment conditions where free TPEN is ineffective. Targeted liposomes are also nontoxic to nontargeted cells under these same conditions. Additionally, they demonstrated that targeted liposomes loaded with a fluorescent dye preferentially localize to C4–2 tumors, consistent with active targeting. Targeting of TPEN via aptamer-targeted liposomes (Ap-Lips) provides an opportunity to use Zn2+ chelation as a treatment for advanced CRPC, where traditional chemotherapy is ineffective.

Tian et al., (2018) reported about the synthesis of a multifunctional liposomal system containing celecoxib and genistein drugs. The combinatorial effect of these drugs leads to the specifically induce the apoptosis of prostate cancer cells than normal fibroblast cells. The mechanistic study suggests that increased reactive oxygen species (ROS) formation and a diminish in cellular GSH concentration, along with inhibition of COX-2 synthesis and Glut-1 receptors are the key processes behind the inhibition of prostate cancer cells and overall these results provide strong evidence for the role of COX-2 and Glut-1 proteins for the progression of prostate cancer and highlighting the potential of celecoxib and genistein as a useful and combinatorial pharmacological agent for chemotherapeutic purposes in prostate cancer.

Singh et al., (2018) in a study, prepared a multifunctional antioxidant nanoliposome containing PTEN plasmid and cerium oxide nanoparticles (CeNPs). The efficient delivery of PTEN plasmid to human prostate cancer cells (PC-3) leads to restoration of the expression of lost PTEN protein in the cell cytoplasm. The delivered superoxide dismutase (SOD)-mimetic CeNPs were also found to decrease the cytoplasmic free radical levels in prostate cancer cells. The above two activities induced DNA fragmentation and micronucleus formation in prostate cancer cells. and it was also found that these multifunctional antioxidant nanoliposomes inhibit the PI3K/AKT signaling pathway to negatively regulate the cell viability of prostate cancer cells. The mRNA expression pattern of other relevant proteins predominantly involved in cancer cell proliferation and apoptosis suggested that the high PTEN expression could control the synthesis of oncogenic proteins.

Nassir et al., (2019) reported the effect of oleuropein (OL) loaded surface functionalized folate – PEG liposomes (OLFML) to inhibit the 22Rv1 prostate cancer cells and compared with plain oleuropein solution. Cell viability assay and various apoptosis studies including phosphatidylserine externalization assay, TUNEL assay, and analysis of mitochondrial membrane potential and caspase-3 activation assay were performed. *In vivo* pharmacokinetic study revealed that OL-FML was able to maintain a longer and increased the concentration of oleuropein in blood. The comparative effect of the developed system and plain oleuropein was studied in tumor-induced mice model in terms of change in tumor volume, body weight, and the survival probability. In each study, a noticeable improvement in the induced condition was observed with OL-FML treatment. The effects were majorly attributed to passive targeting mechanisms including long circulation, EPR, and liposomal endocytosis as well as active targeting through overexpressed folate receptor binding.

Chapter-3

AIM OF THE RESEARCH WORK

3. AIM OF THE RESEARCH WORK

As the potential of nanocarriers in different types of cancer has been well established, but in case of metastatic castration-resistant prostate cancer (mCRPC) and metastatic high-risk castration-sensitive prostate cancer (mCSPC), there was no nano formulation yet. Abiraterone acetate tablet formulation available in U.S market but it is not a suitable dosage form for terminally ill patients. So, we are trying to develop an intra venous (I.V.) dosage form. Abiraterone acetate, if supplied through the nanoliposome delivery system then the targeting of drug to the prostate cancer cells can be achieved successfully. Now the focus has been shifted towards the development of targeted nanocarriers by decorating surface of the nanoliposome. Additionally, if abiraterone acetate is given by the nanoliposome drug delivery system then sustained release action can be achieved, systemic toxicity of drug can be reduced and bioavailability can be increased.

Considering the above-mentioned hypothesis, main aim in this research work is to develop a formulation of ligand conjugated nanoliposome of abiraterone acetate and to characterize them *in vitro.*

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

- **(i)** Pre-formulation study to check drug-excipient interaction.
- **(ii)** Preparation and characterization of nanoliposomal formulation containing abiraterone acetate in terms of morphology, drug loading, *in vitro* drug release and stability study.
- **(iii)** Conjugation of ligand to the optimized nanoliposomes and characterization by agarose gel electrophoresis in order to confirm conjugation of ligand to nanoliposome successfully.
- **(iv)** *In vitro* cytotoxicity study and biodistribution study in order to explore their potential against prostate cancer.

Chapter-4

MATERIALS

4. MATERIALS

4.1. Chemicals: cholesterol, soya-L-α-lecithin (SLE), butylated hydroxy tolune (BHT), sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, chloroform, ethanol are used for the preparation of abiraterone acetate nanoliposome.

The following chemicals were used for the study, depicted in Table 2.

Table 2: List of chemicals with their source.

Name	Source
Abiraterone acetate	SUN Pharmaceutical (Mumbai, India)
Cholesterol	HiMedia Laboratories Pvt. Ltd. (Mumbai, India)
Soya-L-α-lecithin	HiMedia Laboratories Pvt. Ltd. (Mumbai, India)
Butylated Hydroxy Tolune (BHT)	Qualigens Fine Chemicals (Mumbai, India)
Fluorescein isothiocyanate	Sigma-Aldrich Co. (Bangalore, India)
Dialysis Membrane-60	HiMedia Laboratories Pvt. Ltd. (Mumbai, India)
Sodium chloride	Merck LifeScience Pvt. Ltd. (Mumbai, India)
Potassium chloride	Merck LifeScience Pvt. Ltd. (Mumbai, India)
Disodium hydrogen phosphate	E Merck Ltd. (Mumbai, India)
Potassium dihydrogen phosphate	Merck Specialities Pvt. Ltd. (Mumbai, India)
Chloroform	Merck LifeScience Pvt. Ltd. (Mumbai, India)
2-Hydroxypropyl-β-cyclodextrin (HP-β-CD)	HiMedia Laboratories Pvt. Ltd. (Mumbai, India)
Acetonitrile	Merck LifeScience Pvt. Ltd. (Mumbai, India)
Dichloromethane	Merck Specialities Pvt. Ltd. (Mumbai, India)
Ethanol	E Merck Ltd (Mumbai, India)
Acetone	Merck LifeScience Pvt. Ltd. (Mumbai, India)
Sodium hydroxide pellets	Merck Specialities Pvt. Ltd. (Mumbai, India)
Hydrochloric acid (35%) (HCL)	Merck LifeScience Pvt. Ltd. (Mumbai, India)
HPLC water	Merck LifeScience Pvt. Ltd. (Mumbai, India)

4.2. Equipments: Various instruments used in the preparation of abiraterone acetate nanoliposome and the company from which they are obtained are given below Table 3.

Table 3: List of equipments used in the liposome preparation with their source.

Name	Source
Rotary Vacuum Evaporator	Rotavap Superfit Model-PBU-6, Mumbai, India
Aspirator A3S	Eyela, Tokyo Rikakikai Co Ltd., Tokyo, Japan
Low temperature circulating bath	Instrumentation India, Kolkata, India
Vacuum desiccators	Tarson, Kolkata, India
Bath type sonicator	Trans-o-Sonic, Mumbai, India
Advanced Microprocessor UV-Vis single beam spectrophotometer (Model Intech-295) Software- UV Professional V1.39.0	Model Intech-295, Gentaur GmbH, Aachen, Germany
Lyophilizer	Instrumentation India, Kolkata, India
Digital Balance	Sartorius, Goetingen, Germany
High speed Ultra cold Centrifuge (Model Z 32 HK)	Hermle Labortechnik GmbH, Wehingen, Germany
FTIR (ECO-ATR, Model ALPHA) Software- OPUS 7.5	Bruker Optik, Germany
Deep freezer (-80 ºC)	New Brunswick Scientific, Freshwater Boulevard Enfield, USA
Magnetic stirrer	Remi Equipments, Mumbai, India
pH meter	Eutech Instruments, Haridwar, India
Vortex mixture (Model CM100)	Remi Equipments, Mumbai, India
Distillation Plant	Sicco, Kolkata, India
All glass Apparatus	Borosil, Mumbai, India
Pipette & Micro Tips, Centrifuge tube	Tarsons Products Pvt. Ltd. Kolkata, India
Micro centrifuge (SPINWIN)	Lab equipments and chemicals, Kolkata, India
Incubator shaker	Indian Instruments and chemicals, Kolkata, India
Scanning electron microscope	Zeiss instruments, Switzerland
Zeta potential & particle size analyser	Zetasizer, Nano zs 90, Malvern Instrument Ltd. UK
Field emission scanning electron microscope	JSM, JEOL, Tokyo, Japan
Atomic force microscope	Di CP II, Veeco Instruments, USA
Transmission electron microscope	Jeol JEM 2100F; JEOL-FRANCE, Paris, France

4.3. Description about some Special used Materials

4.3.1. Abiraterone acetate (AA)

IUPAC name: [(3*S*,8*R*,9*S*,10*R*,13*S*,14*S*)-10,13-dimethyl-17-pyridin-3-yl-2,3,4,7,8,9,11,12,14,15-decahydro-1*H*-cyclopenta[a]phenanthren-3-yl] acetate

Formal name: (3β)-17-(3-pyridinyl)-androsta-5,16-dien-3-ol, acetate ester

Molecular formula: $C_{26}H_{33}NO_2$

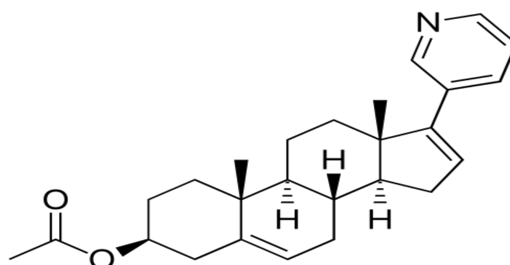

Figure 21: Structure of Abiraterone acetate. (https://en.wikipedia.org/wiki/Abiraterone_acetate#/media/File:Abiraterone_acetate.svg)

Molar Mass: 391.555 g/mol

Formula Weight: 391.6

Melting Point: 144 to 145 °C (291 to 293 °F)

λ max: 254 nm

Stability: ≥ 2 years at -20°C

Bioavailability: 50% at most on empty stomach. (Benoist et al., 2016)

Protein binding: 99% protein bound, mainly to α1-acid glycoprotein and albumin.

Metabolism: metabolised in the liver by CYP3A4 and SULT2A1 to inactive metabolites.

Excretion: The drug is excreted in faces (~88%) and urine (~5%).

Elimination Half-life: Abiraterone: 12 ± 5 hours. (Benoist et al., 2016)

Brand names: Zytiga (Janssen Biotech a subsidiary of Johnson & Johnson) (www.fda.gov)

Yonsa (Sun Pharmaceutical)

Description: It is a solid white crystalline drug.

Solubility: AA is soluble in organic solvents such as ethanol, DMSO, and dimethyl formamide (DMF), which should be purged with an inert gas. The solubility of AA in ethanol and DMF is approximately 16 mg/ml and approximately 2 mg/ml in DMSO.

AA is sparingly soluble in aqueous buffers. For maximum solubility in aqueous buffers, AA should first be dissolved in ethanol and then diluted with aqueous buffer solution. It has a solubility of approximate 0.3 mg/ml in a 1:2 solution of ethanol: PBS (pH 7.2) using this method.

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.

Chemical name: 1, 2-Diacyl- sn-ue-glycero-3-phospholcholine

Molecular formula: $C_{42}H_{80}NO_8P$

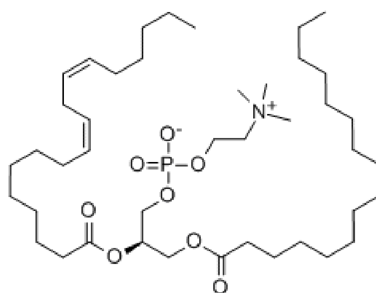

Figure 22: Structure of Lecithin. (https://www.chemicalbook.com)

Source: Lecithin containing phosphatidylcholine are produced from vegetable, animal and microbial sources. Soybean, sunflower and rapeseed are the major plant sources of commercial lecithin. Soybean is the most common source.

Molar Mass: 772-787 g/mol

Solubility: Lecithin is soluble in benzene, hexane, and chloroform, partially soluble in alcohol and practically insoluble in acetone.

4.3.3. Cholesterol (CHL)

Chemical name: Cholest-5-en-3β-ol

Molecular formula: $C_{27}H_{46}O$

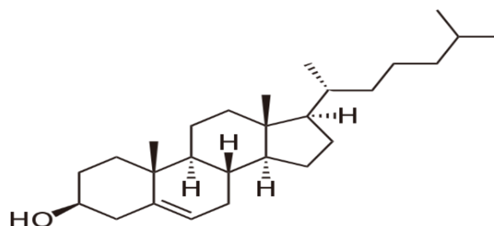

Figure 23: Structure of Cholesterol. (https://en.wikipedia.org/wiki/File:Cholesterol.svg)

Molar Mass: 386.65 g/mol

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

Melting point: 147-150 °C

Boiling point: 360°C

Solubility: Insoluble in water, slowly soluble in alcohol (About 1 gram in 100 ml). It is soluble in acetone, hot alcohol, chloroform, dioxane, ether, ethyl acetate, hexane or vegetable oil.

4.3.4. Butylated hydroxy 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-tertbutyl-4-hydroxytoluene

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

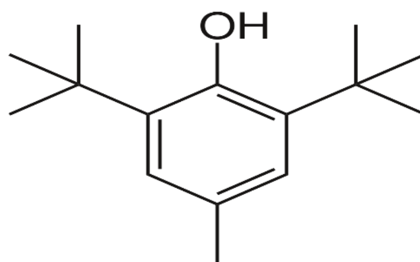

Figure 24: Structure of BHT. (https://en.wikipedia.org/wiki/Butylated_hydroxytoluene)

Molar Mass: 220.35 g./mol

Appearance: White to yellow powder.

Density: 1.048 g/cm3

Melting point: 70°C

Boiling point: 265°C

BHT is primarily used as a food additive that exploits its antioxidant properties. BHT is also used as an antioxidant additive in diverse products such 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. Development of Calibration Curves

5.1.1. Preparation of phosphate buffer saline at pH 7.4: Phosphate buffer saline pH 7.4 was prepared according to I.P protocol. 2.38 g disodium hydrogen phosphate (Na_2HPO_4), 0.19 g of potassium dihydrogen phosphate (KH_2PO_4) and 8 g of sodium chloride (NaCl) were dissolved in 1000 ml double distilled water and pH of the solution was adjusted to 7.4 using a pre-calibrated pH meter (Eutech Instruments).

5.1.2. Determination of absorption maxima of abiraterone acetate: For the determination of absorption maxima, about 1 mg of abiraterone acetate was weighed accurately and dissolved in acetonitrile and dichloromethane (ACN: DCM) solvent, prepared at a ratio 3:1 and in ethanol-PBS (pH 7.4) mixture (1:3) separately. The solution was then scanned in the wavelength range of 190 nm to 400 nm with the help of an Advanced Microprocessor UV-VIS single beam spectrophotometer (Model Intech-295, Gentaur GmbH, Aachen, Germany) using ACN-DCM mixture and ethanol-PBS mixture as blank respectively. A single characteristic peak at 253.0 nm in acetonitrile: dichloromethane (3:1) was obtained and a single characteristic peak was obtained at 253.0 nm in ethanol-PBS (pH 7.4) which was close to the published lambda max of abiraterone acetate at 254.0 nm.

5.1.3. Preparation of standard curve: Two separate calibration curves were prepared- one in acetonitrile and dichloromethane (3:1 v/v) (for calculation of drug loading into nanoliposome) and other in ethanol-PBS (1:3 v/v) for calculating drug release from nanoliposome. Two stock solution of abiraterone acetate was prepared by weighing 2 mg of drug accurately on a single pan weighing balance and dissolved in 2 ml of both volumetric flasks containing acetonitrile and dichloromethane (3:1 v/v) and ethanol-PBS (1:3 v/v) respectively, after successive dilutions. The final concentration of both the stock solution was made to 50µg/ml. From that six different concentrations were prepared- 5 µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml and 30µg/ml for both the cases. The respective absorbance was read with triplicates using acetonitrile and dichloromethane (3:1 v/v) and ethanol-PBS (1:3 v/v) at 253.0 nm respectively. Each calibration curve was plotted by drawing absorbance against concentration.

5.2. Pre-formulation study through FT-IR spectroscopy

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. Abiraterone acetate, SLE, CHL, BHT, physical mixture of excipients, physical mixture of drug and the excipients, blank nanoliposome and drug loaded nanoliposome were prepared separately using ATR technique and scanned over a wave number range of 4000-525 cm^{-1} using ECO-ATR (Bruker, Germany). The obtained spectrophotograms were then interpreted for the functional groups present.

5.3. Procedure for the development of nanosized liposomes

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 nanosize liposomes (NLs) with some required modifications of critical formulation and process parameters (Satapathy et al., 2016; Dutta et al., 2018). In short, weighed amounts of AA, SLE, CHL were taken in a 250 ml round bottom flask and were dissolved in chloroform by vigorous shaking. Butylated hydroxyl toluene (BHT) [1 % (w/v)] was added to the above mixture as an anti-oxidant. The mixture was placed in a rotary vacuum evaporator (Rotavap superfit, PBU-6) fitted with an aspirator A3S (Eyela, Rikakikai Co. Ltd., Tokyo, Japan) and a circulating water bath (Instrumentation India, Kolkata, India) and was rotated at 130 rpm at 40°C. The solvent was evaporated leaving behind a thin film of lipids around the wall inside the round bottom flask. The flask was kept in a vacuum desiccator overnight for complete removal of residual organic solvent. On the day 2, the preparation was hydrated with phosphate buffer, pH 7.4, in a rotary vacuum evaporator fitted with a water bath and rotated at 150 rpm at 60°C for 1 hour for lipid film reasonably dispersed in the aqueous phase. During hydration, multilamellar lipid vesicles are formed, which are then converted to smaller unilamellar lipid vesicles by sonication in a bath type sonicator (Trans-o-sonic, Mumbai, India) for about one hour. After sonication, the preparation was kept at room temperature for two hours to regain the vesicle structure, followed by overnight storage at 4°C. On the day 3, the preparation was centrifuged at 16 000 rpm for one hour. The product was collected, stored overnight at -20°C for pre-cooling followed by lyophilization (Instrumentation India, Kolkata, India) for 12 h for fully drying.

5.3.1. Development of fluorescent NLs

For the preparation of fluorescent NLs, FITC was used as the fluorescent material. About 0.4 g FITC was dissolved in 100 ml of chloroform and ethanol mixture at 3:1 ratio to prepare a stock solution of 0.4 % (w/v). From the stock, about 50 µl solution was taken and dissolved in the organic phase (chloroform) during the first step of preparation (Satapathy et al., 2016). The rest of the procedure was same as mentioned above.

5.4. *In vitro* characterization of drug loaded NLs

We prepared several formulations by varying different critical formulation parameters such as drug to lipid ratio, concentration of cholesterol, concentration of lecithin etc. and process variables such as time of hydration, hydration temperature, duration of sonication, speed and time of centrifugation etc. All the prepared formulations were evaluated by different *in vitro* techniques such as drug loading efficiency, size analysis, zeta potential, and surface morphology study. Based on these data, the best formulations were selected which were taken for further *in vitro* and *in vivo* studies.

Drug loading and entrapment efficiency study

For *in vitro* drug loading study, weighed amount (5 mg) of NLs was taken with 2 ml mixture of ACN and DCM (3:1). The mixture was vortexed for 5 min followed by centrifugation at 16, 000 rpm for 15 min, at 4°C. The resultant sediment was discarded and the absorbance of the supernatant was measured at 253.0 nm using advanced microprocessor UV-VIS single beam spectrophotometer (Model Intech-295), keeping CAN-DCM (3:1) as blank (Dey et al., 2016). The percentage drug loading and the drug loading efficiency were calculated using the following formula.

- Theoretical drug loading (%) = [Amount of drug taken to prepare NLs / (Amount of SLE+CHL+BHT) + drug taken] X 100

- Practical drug loading (%) = (Amount of drug in NLs/Amount of NLs obtained) X 100

- Drug loading efficiency (%) = (Practical drug loading/Theoretical drug loading) X 100.

Determination of size distribution and zeta potential

Mean vesicle diameter (Z-average), polydispersity index (PDI) and zeta potential of the prepared formulations were determined by dynamic light scattering (DLS), sometimes referred to as photon correlation spectroscopy method. About 1 mg of lyophilized 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 (Malvern Instrument Ltd, Malvern, UK) and the fluctuations of the scattered light are detected at a known scattering angle θ by a fast photon detector. The data were interpreted by the instrument software (DTS software version 4.0).

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

The surface morphology of experimental NLs was analysed by FESEM. Lyophilized samples were spread on a carbon tape over a stub, vacuum dried, platinum coated and were examined using FESEM (JSM 6100; JEOL, Tokyo, Japan).

Energy dispersive X-ray (EDX) analysis by Scanning electron microscopy (SEM)

The elemental composition of the experimental NLs was investigated by EDX technique which works as an integrated feature of the scanning electron microscope (Zeiss, Switzerland).

Cryo-Transmission electron microscopy (TEM) and Atomic force microscopy (AFM) study

On the basis of particle size, drug loading and SEM study, out of the three given formulation one formulation was selected as the best one and considered for further analysis. For analysis of morphology by transmission electron microscopy the selected Abiraterone acetate loaded nanoliposome sample was dispersed in milli-Q water and sonicated for 10 minutes and a small drop of this suspension was pipetted onto a carbon coated copper grid and

allowed to dry overnight at room temperature. The grid can then be directly observed in a TEM (JEOL, JEM, 2100F) with an accelerating voltage of 200kV once the medium has evaporated.

For analysis of surface morphology of nanoliposome by Atomic Force Microscopy a small drop of optimized formulation suspension was spread on a cover slip and then it is allowed to dry overnight in a desiccator and then it is observed under the instrument (di CP II, Vecco Instruments).

***In vitro* drug release study**

- **In PBS (pH 7.4)**

For *in vitro* drug release study, 5 mg of lyophilized NLs was reconstituted in 1 ml PBS (pH 7.4) and was taken into a dialysis bag (Himedia dialysis membrane-60, Mumbai, India). Prior to the experiment, the dialysis bags (spectra/Por® 2) were soaked before use in PBS, pH 7.4 at room temperature for 12 hours to remove the preservative, followed by rinsing thoroughly in distilled water. The two ends of the dialysis bag were tightly bound with cotton thread. The whole assembly was then put inside a beaker (100 ml capacity), containing 50 ml of PBS as drug release medium. The entire set up was then kept on a magnetic stirrer and stirred with a magnetic bead at 100 rpm at room temperature (Rudra et al., 2011). At predetermined time intervals, 1 ml sample was withdrawn from the drug release medium and add with an equivalent volume of fresh medium. The samples were analysed using a spectrophotometer at 253.0 nm against PBS as blank. The concentration was calculated from the calibration curve.

- **In PBS (pH 7.4) with 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD)**

The above-mentioned method is same only a solution was prepared as a release media by mixing of 2-Hydroxypropyl-β-cyclodextrin (1% w/v) with PBS (pH 7.4).

Drug release kinetics study

To predict the mechanism of drug release from the experimental NLs, data obtained from the *in vitro* drug release studies were plotted in various kinetic models such as zero order (cumulative amount of drug released versus time), first order (logarithmic value of cumulative amount of drug remained versus time), Higuchi model (cumulative amount of drug released versus square root of time), Korsmeyer–Peppas model (logarithmic value of cumulative amount of drug released versus logarithmic value of time), and Hixson–Crowell model (cube root of percentage drug remained versus time) (Rudra et al., 2011). The linearity of the plots was assessed from the calculated R^2 values.

5.5. Stability study

Stability study was done to know that how long time the formulation will remains stable. It was done by keeping the formulation in stability chamber with 75% RH (relative humidity) in different temperature, i.e. at 4°C, 25°C and 40°C respectively for three months. After three months all formulation were further analysed by FTIR and DLS study.

Chapter-6

RESULTS

6. RESULTS

6.1. The UV absorption spectrum of Abiraterone acetate (AA)

For the determination of maximum absorption spectrum of Abiraterone acetate (AA) a dilute solution of AA in acetonitrile-dichloromethane (3:1) and ethanol-PBS (pH 7.4) mixture (1:3), respectively were prepared and scanned between 190-400 nm in a UV-Vis spectrometer with acetonitrile-dichloromethane and ethanol-PBS as blank reference. The spectrum showed distinct peak at 253.0 nm in both the cases.

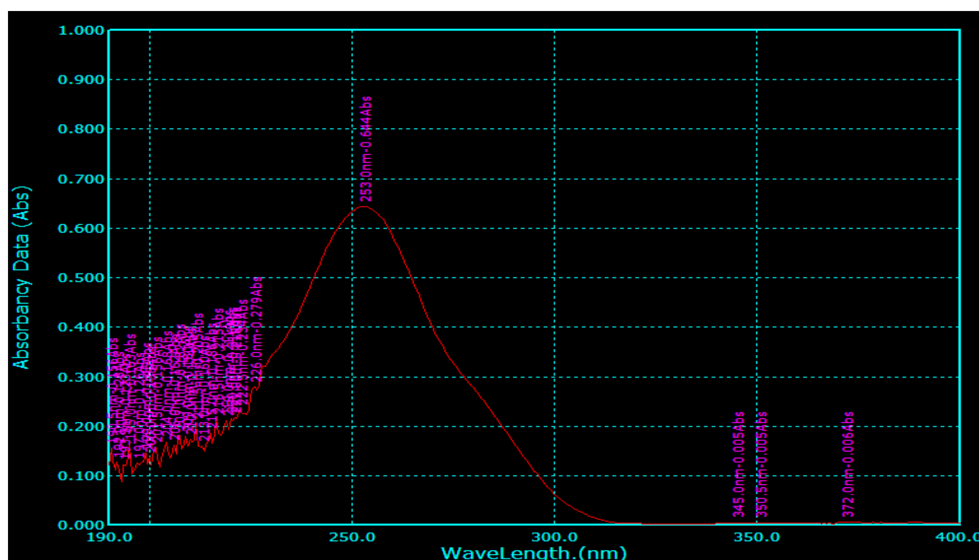

Figure 25: The UV absorption maxima of AA in acetonitrile: dichloromethane (3:1) showing λ max at 253.0 nm.

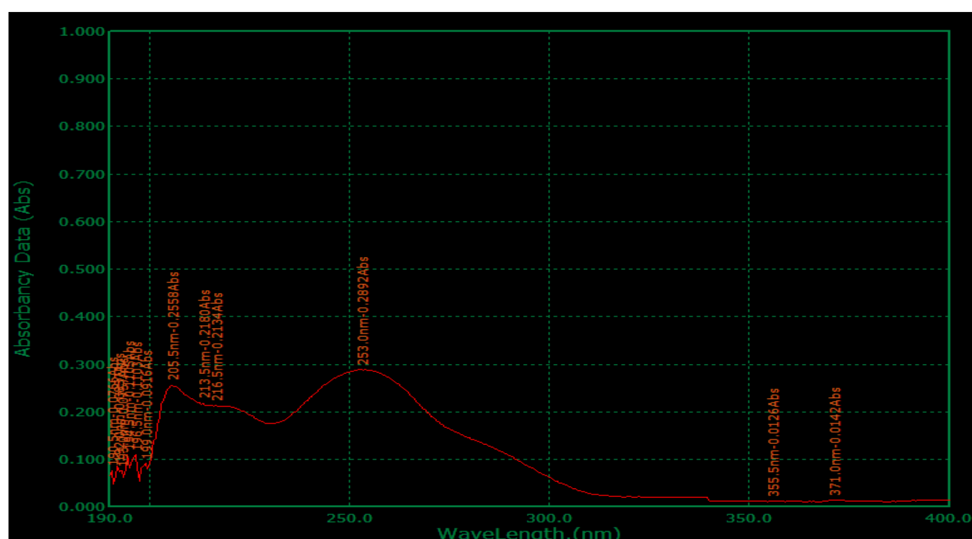

Figure 26: The UV absorption maxima of AA in ethanol: PBS (pH 7.4) mixture (1:3) showing λ max at 253.0 nm.

6.2. The calibration curves of AA

Two different calibration curves were prepared- one in ethanol: PBS (pH 7.4) mixture (1:3) for studying the invitro drug release and the other in acetonitrile: dichloromethane (3:1) for determining the drug loading of nanoliposome. Each reading was made in triplicate. The dilutions prepared for both the standard curves were 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml, 25µg/ml and 30µg/ml. The standard curve for AA in ethanol: PBS (pH 7.4) mixture (1:3) is depicted in figure 27(a) and standard curve for AA in acetonitrile: dichloromethane (3:1) is depicted in figure 27(b). Corresponding data of absorbance against concentration are given in Table 4 and 5 respectively.

Table 4: The mean absorbance of AA samples against the various concentrations of the drug in ethanol: PBS (pH 7.4)

Concentration (µg/ml)	Mean absorbance (n=3)
0	0
5	0.133 ± 0.003
10	0.271 ± 0.002
15	0.4 ± 0.005
20	0.542 ± 0.003
25	0.668 ± 0.007
30	0.793 ± 0.009

Mean absorbance is represented as Mean absorbance ± SD (n=3)

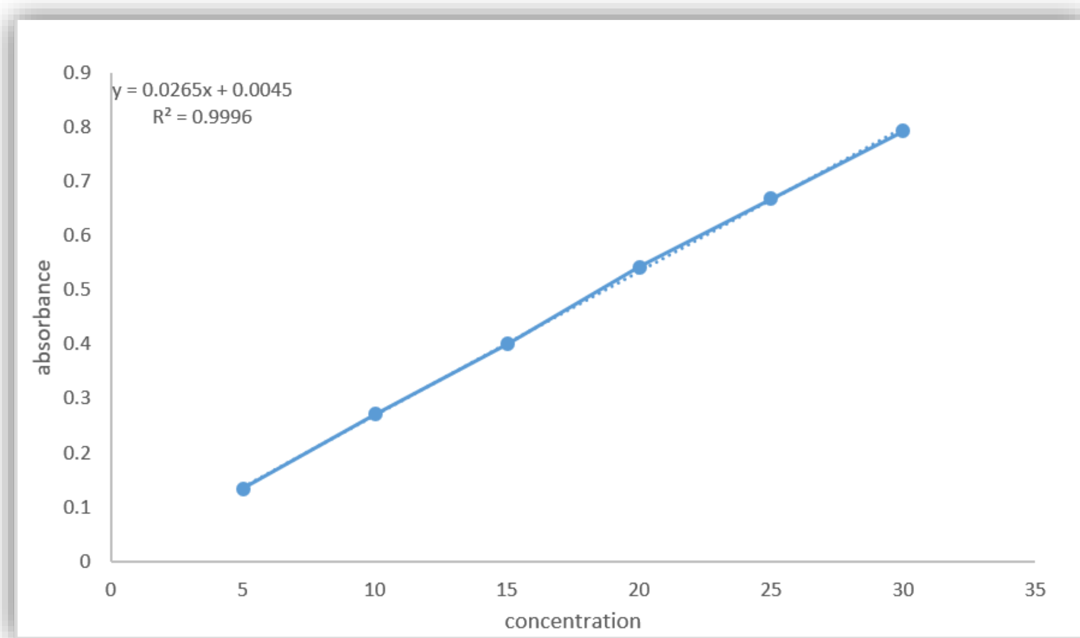

Figure 27(a): Calibration curve of AA in ethanol: PBS (pH 7.4)

Table 5: The mean absorbance of AA samples against the various concentrations of the drug in acetonitrile: dichloromethane (3:1)

Concentration (µg/ml)	Mean absorbance (n=3)
0	0
5	0.153 ± 0.002
10	0.313 ± 0.005
15	0.466 ± 0.003
20	0.615 ± 0.007
25	0.773 ± 0.005

Mean absorbance is represented as Mean absorbance ± SD (n=3)

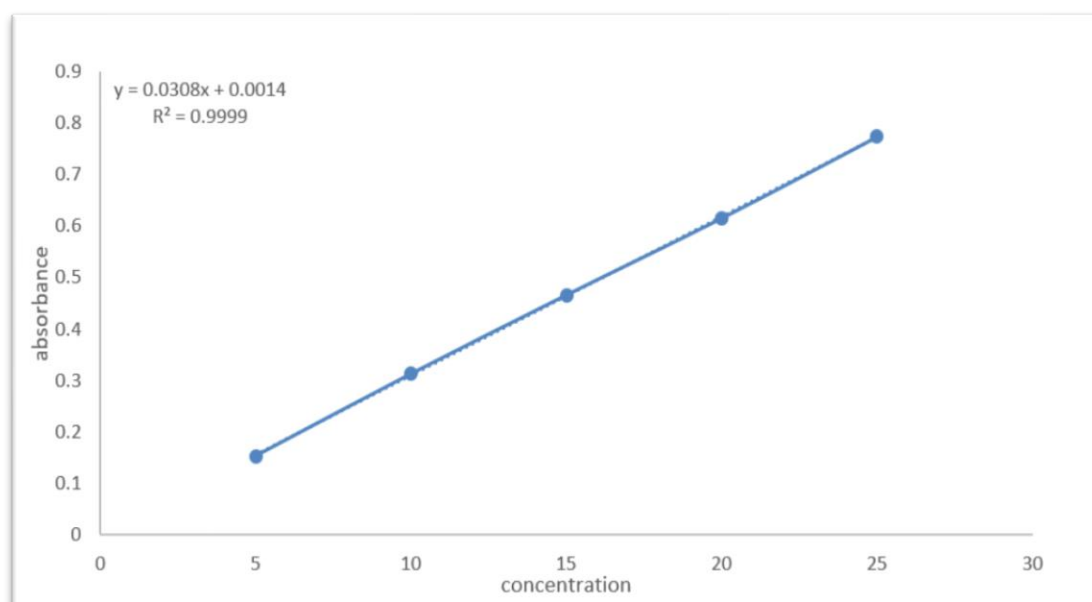

Figure 27(b): Calibration curve of AA in acetonitrile: dichloromethane (3:1)

6.3. The study of drug excipient interaction through FTIR spectroscopy

Drug-excipient interaction was investigated using FTIR (ATR) spectroscopy to detect any interactions between the drug and the excipients.

The following spectrum of abiraterone acetate (AA), SLE, CHL, BHT, physical mixture of excipients, physical mixture of drug and excipients, blank nanoliposome and drug loaded nanoliposome are given below in the following figure respectively.

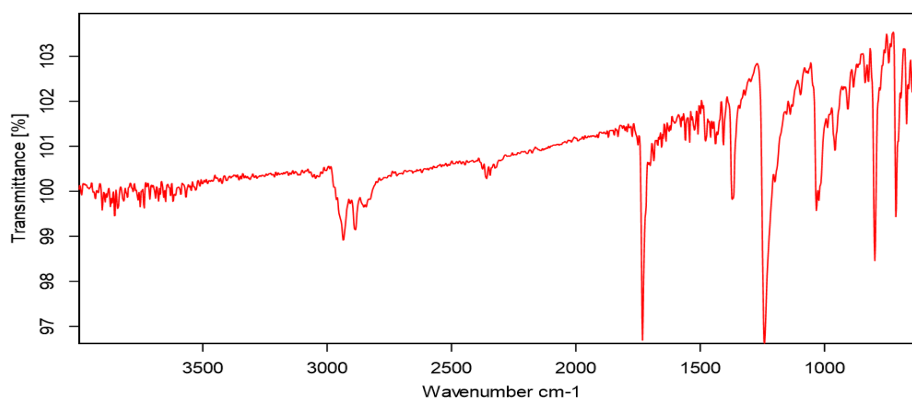

Figure 28: FTIR spectrum of AA.

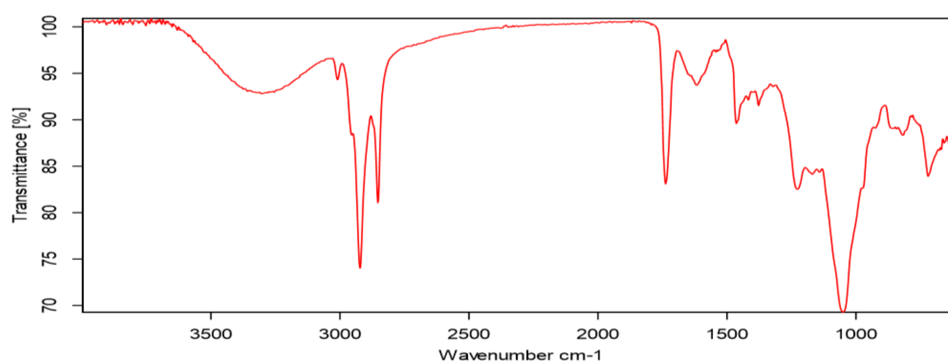

Figure 29: FTIR spectrum of SLE.

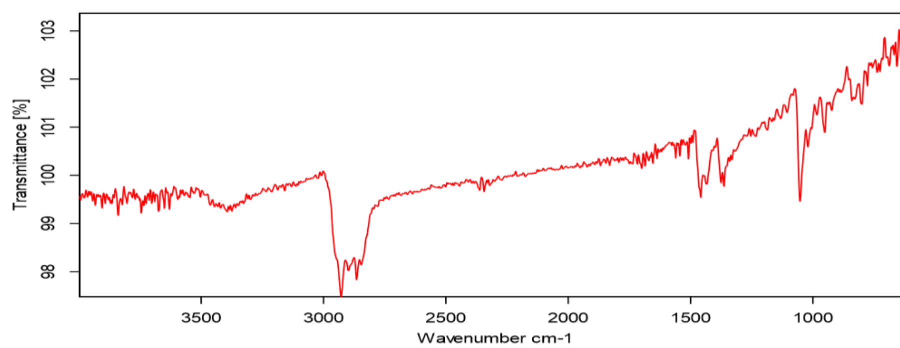

Figure 30: FTIR spectrum of CHL.

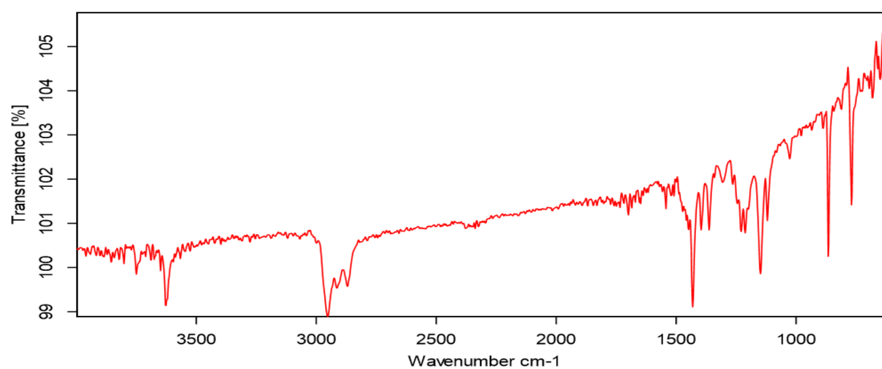

Figure 31: FTIR spectrum of BHT.

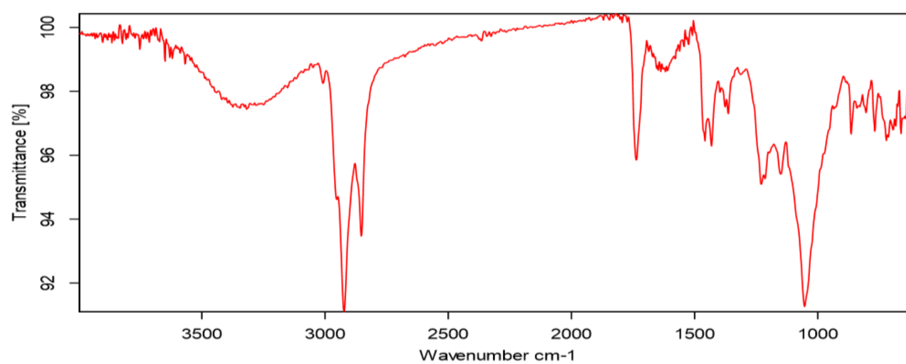

Figure 32: FTIR spectrum of physical mixture of excipients.

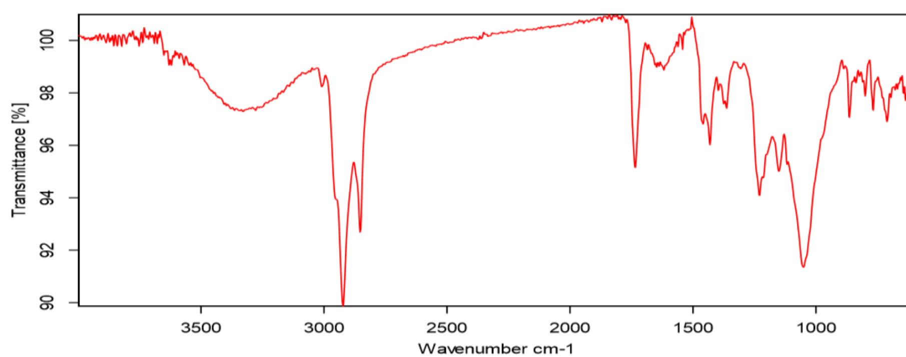

Figure 33: FTIR spectrum of physical mixture of drug and the excipients.

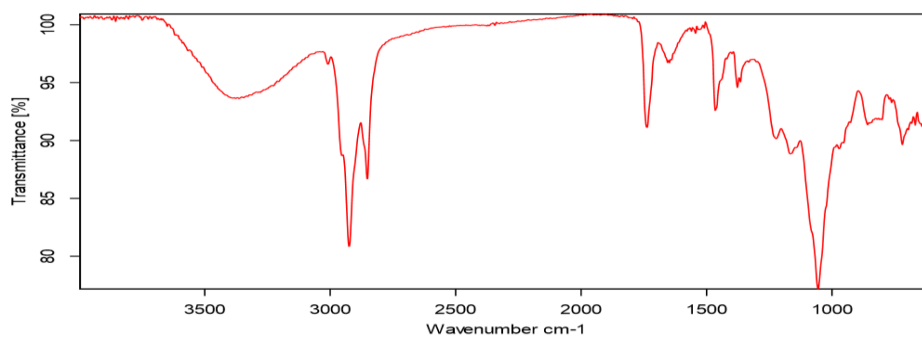

Figure 34: FTIR spectrum of blank nanoliposome (BNL 2).

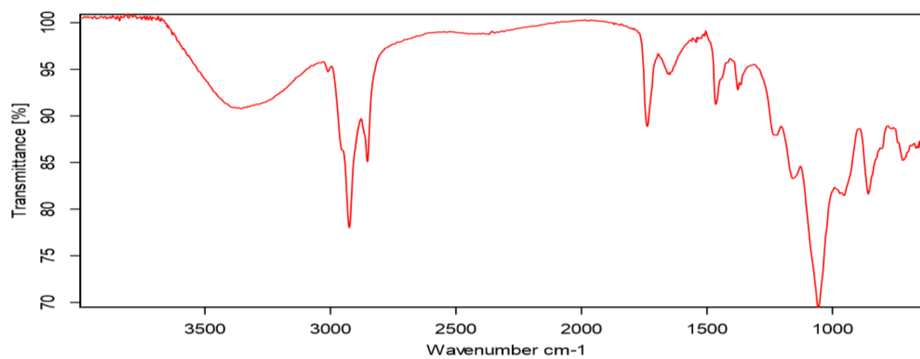

Figure 35: FTIR spectrum of drug-loaded nanoliposome (DNL 2).

6.4. Characterisation of nanoliposome

6.4.1. Drug loading and entrapment efficiency study:

Composition of the experimental formulations along with their respective drug loadings and entrapment efficiencies were given in the following table 6. Maximum drug loading was determined for NL-2 along with the highest entrapment efficiency value and yield percentage among the experimental formulations.

Table 6: Composition, practical drug loading %, entrapment efficiency % of the experimental nanoliposome formulation.

Formulation	Amount of drug taken (mg)	Amount of CHL taken (mg)	Amount of SLE taken (mg)	Theoretical drug loading (%)	Practical drug loading (%)	Entrapment efficiency(%)
NL-1	10	50	100	6.172	3.566 ± 0.065	57.777 ± 1.06
NL-2	10	50	150	4.716	4.166 ± 0.081	88.337 ± 1.71
NL-3	10	50	125	5.347	2.96 ± 0.186	55.358 ± 3.48
NL-4	10	70	125	4.830	3.412 ± 0.071	70.641 ± 1.48
NL-5	10	50	200	3.816	2.28 ± 0.126	59.748 ± 3.32

Values are represented as mean ± SD (n=3)

6.4.2. Particle (vesicle) size, polydispersity index and zeta potential study:

Different nanoliposomal formulations containing AA were prepared by thin film hydration method by altering the drug and SLE, CHL composition. All the formulation had particle size average (Z-average) less than 250 nm.

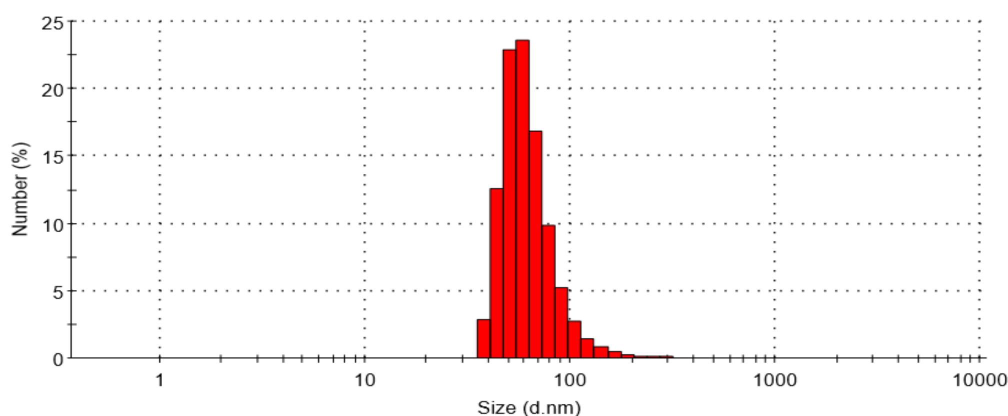

Figure 36: Size distribution curve of NL-1

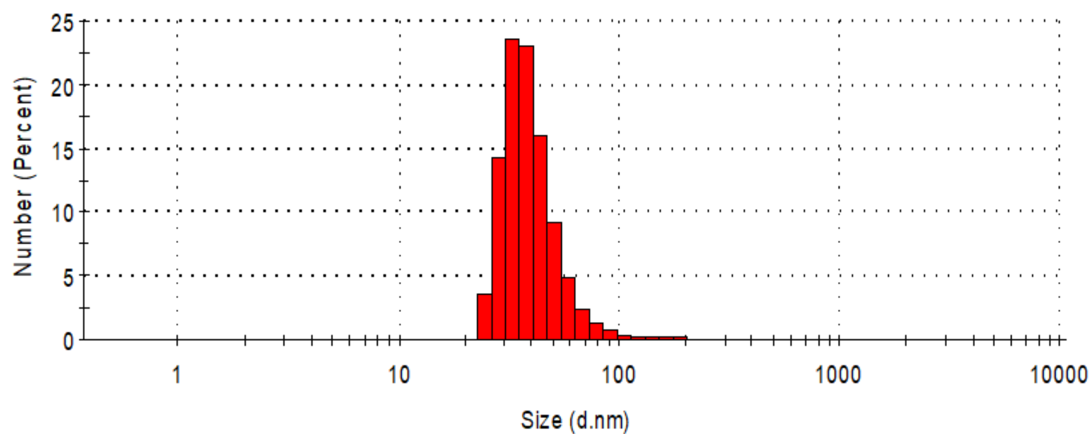

Figure 37: Size distribution curve of NL-2

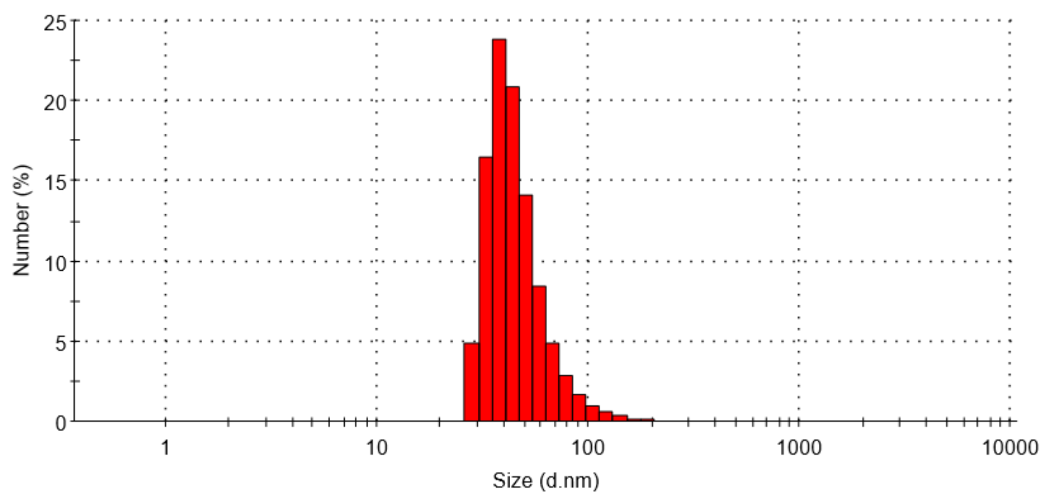

Figure 38: Size distribution curve of NL-4

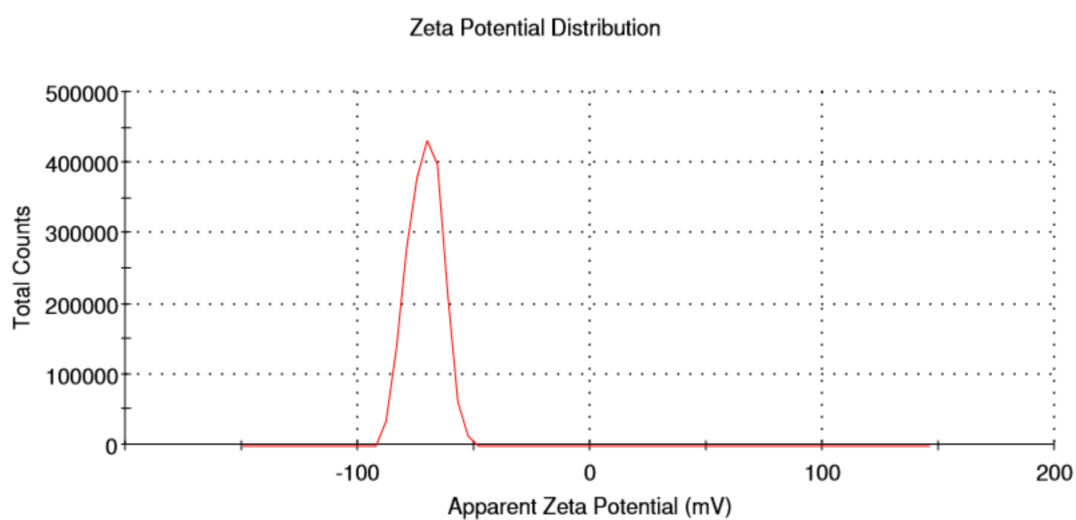

Figure 39: Zeta potential of NL-1

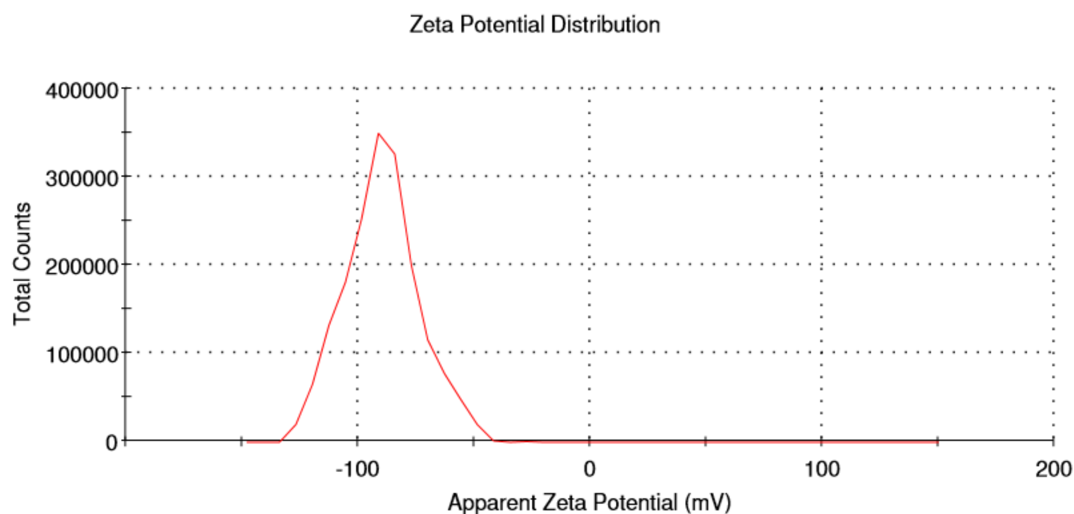

Figure 40: Zeta potential of NL-2

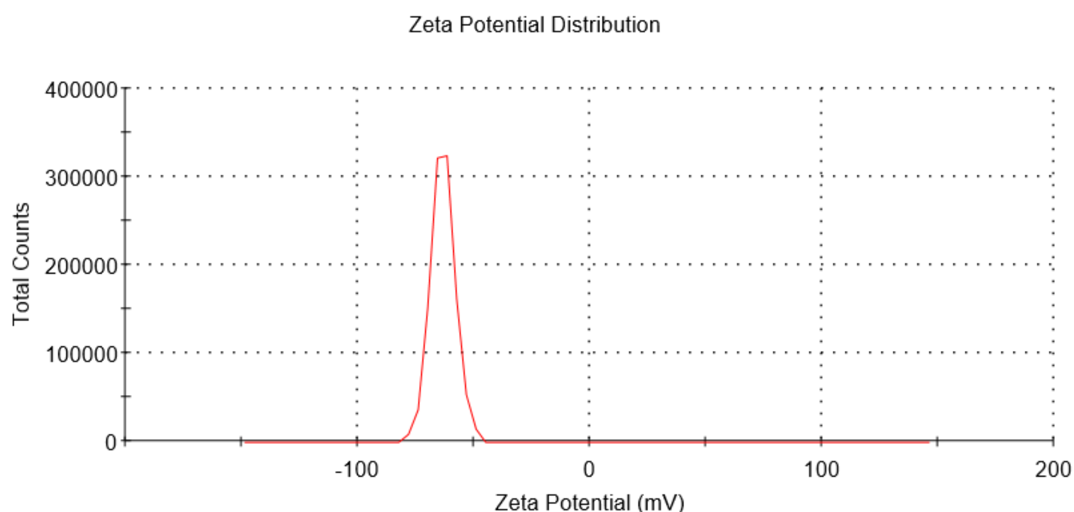

Figure 41: Zeta potential of NL-4

The data from Malvern particle size analyser revealed that the average particle size of formulations NL-1, NL-2 and NL-4 were 210.73 nm, 130.80 nm and 193.36 nm respectively. The corresponding polydispersity index (PDI) values of the experimental formulations NL-1, NL-2 and NL-4 were 0.438, 0.270 and 0.537 respectively. The PDI values indicate narrower size distribution range. The mean zeta potential values of the experimental formulations NL-1, NL-2 and NL-4 were -71.2 mV, -89.7 mV and -63.2 mV respectively. The average particle size, polydispersity index and mean zeta potential values of the experimental formulations have been shown in table 7.

Table 7: The average particle size, polydispersity index and mean zeta potential values of the experimental formulations.

Formulation Code	Average particle size (Z-average) (nm)	Polydispersity index (PDI)	Zeta potential (mV)
NL-1	210.73 ± 38.58	0.438 ± 0.05	-71.2 ± 7.24
NL-2	130.80 ± 12.01	0.270 ± 0.09	-89.7 ± 10.9
NL-4	193.36 ± 22.37	0.537 ± 0.12	-63.2 ± 5.26

Values are represented as mean ± SD (n=3)

6.4.3. Study of morphology of nanoliposome by scanning electron microscopy (SEM):

Figure represents the SEM photographs of the formulations NL-1, NL-2 and NL-4 respectively. All the three formulations were found to be nanosized and spherical in shape. The image also reveals that all the experimental formulations had mostly concavo-convex structure.

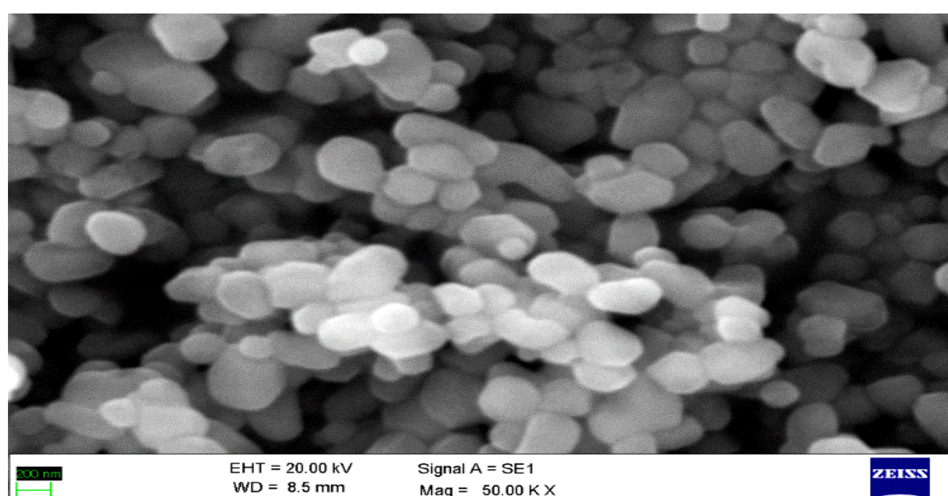

Figure 42: SEM photograph of NL-1

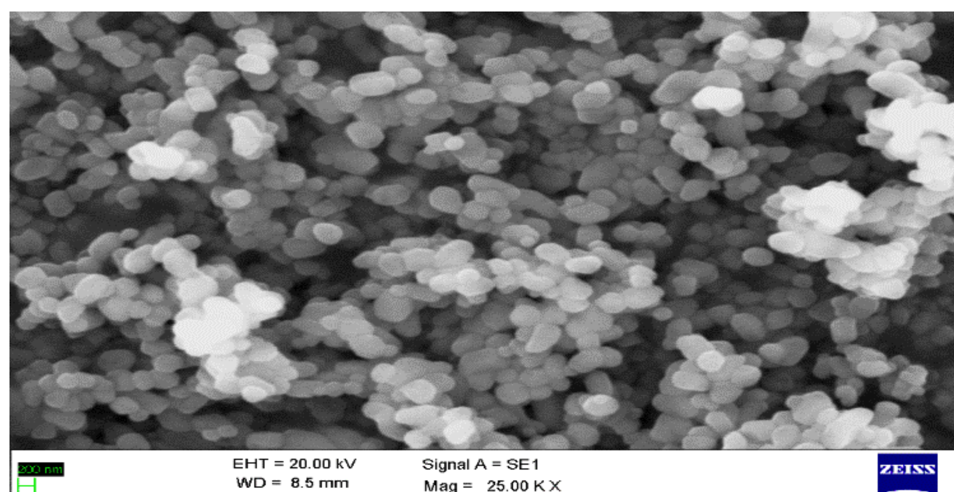

Figure 43: SEM photograph of NL-2

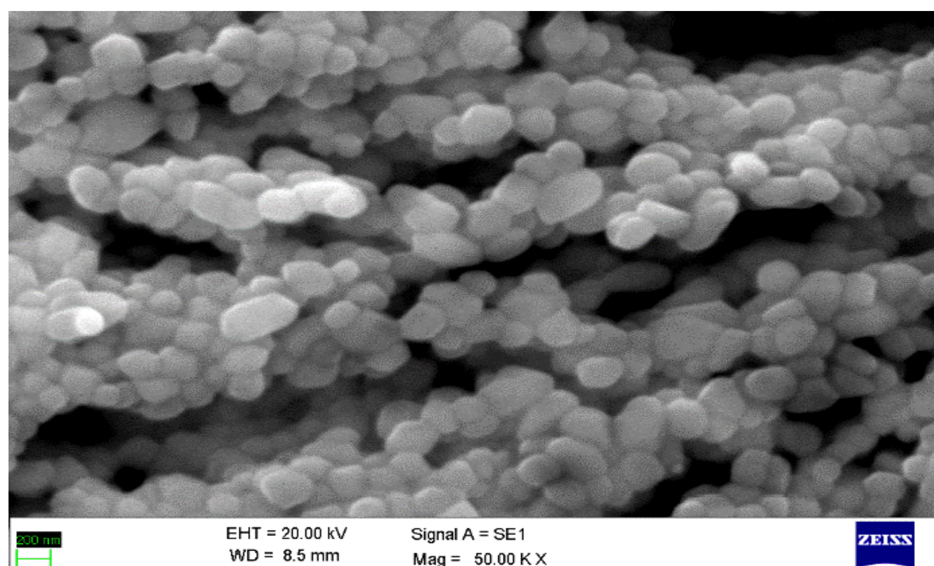

Figure 44: SEM photograph of NL-4

6.4.4. Study of morphology of nanoliposome by field emission scanning electron microscopy (FESEM):

We have given FESEM images of the selected formulation, taken from a particular portion of the samples to justify our findings. FESEM photograph showed that all the formulation had smooth surface. NL-2 showed a mixture of larger and smaller particles within nano-dimensions.

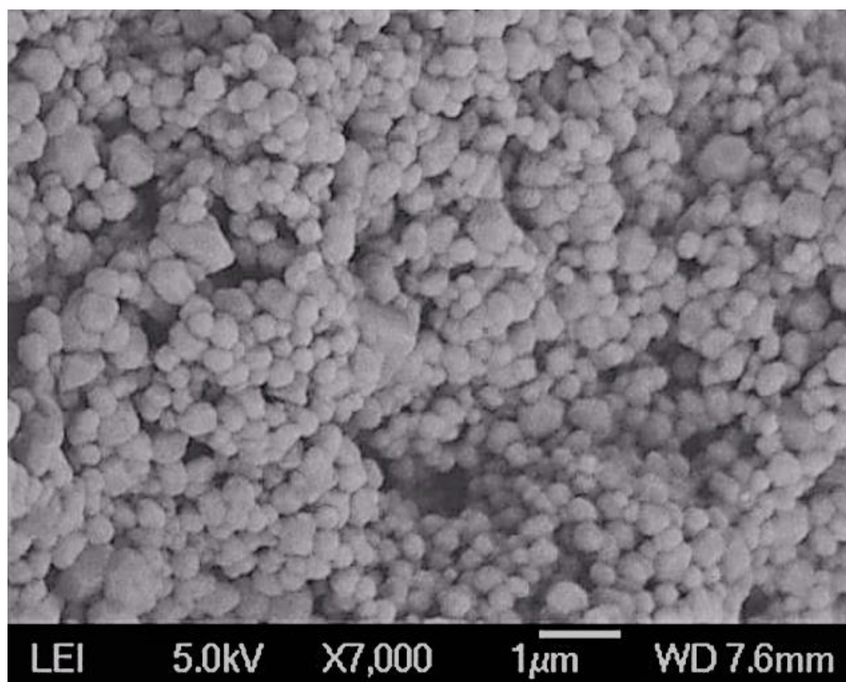

Figure 45: FESEM photograph of optimized formulation NL-2

6.4.5. Atomic Force Microscopy (AFM) analysis:

The AFM images revealed the presence of individual nanoliposome with a small marked agglomeration observed. The 2D AFM image is shown in figure 46 and its corresponding 3D view is shown figure 47. The figure show that the particles were mostly scattered and surface had dearth of smoothness in their desired condition, with a variable dimention.

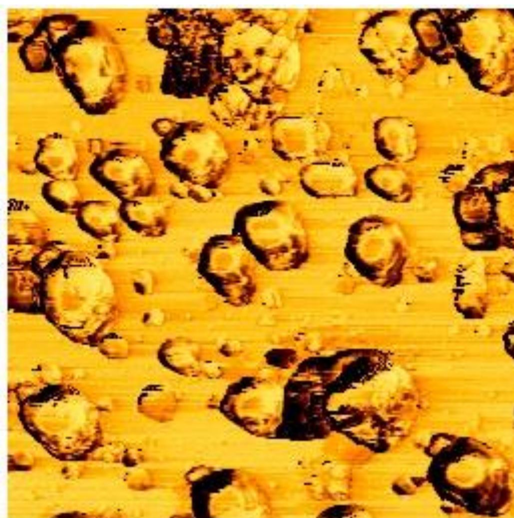

Figure 46: 2D AFM image of optimized formulation NL-2

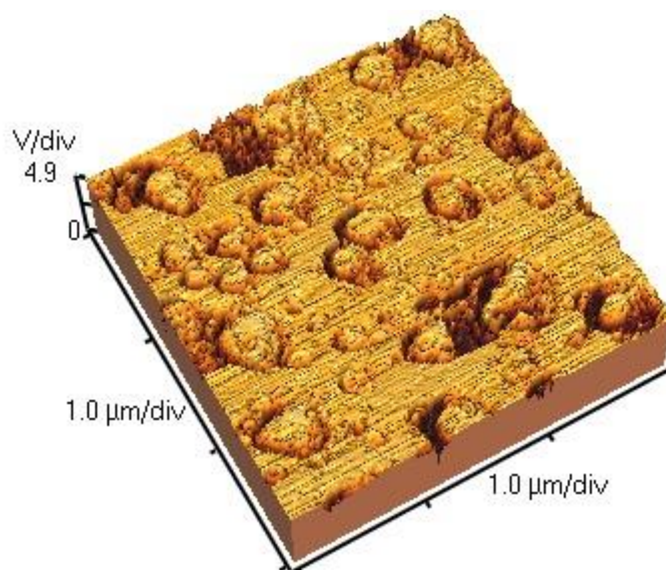

Figure 47: 3D AFM image of optimized formulation NL-2

6.4.6. Study of morphology by Cryo-TEM:

The Cryo-TEM image provide details on internal structure and lamellarity, it also showed that optimized formulation NL-2 was somewhat oval in shape and had a smooth surface. The photograph also revealed that the Abiraterone acetate nanoliposome were of submicron size and also showed the entrapment of drug scatteredly distributed (as dark spots) inside the vesicle of nanoliposome. Presence of unilamellar lipid vesicles along with bilamellar vesicles with a mixture of smaller and little larger vesicles observed.

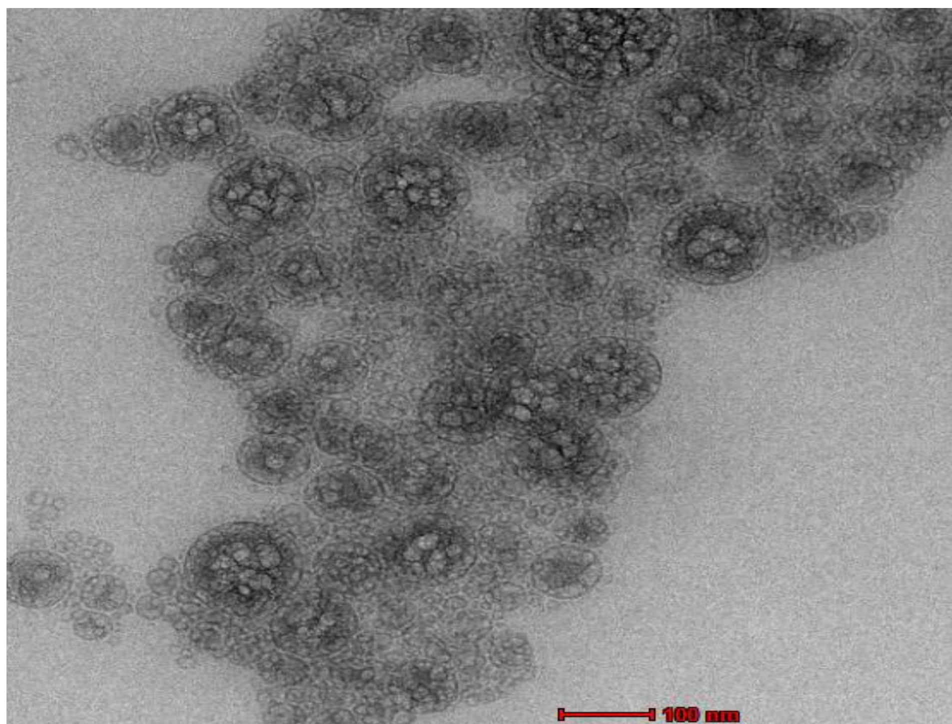

Figure 48: Cryo-TEM image of optimized formulation NL-2

6.4.7. Elemental analysis of selected nanoliposome by SEM

EDX analysis showed weight % and atomic % of different elements such as carbon (C), oxygen (O), sodium (Na) and phosphorous (P) in experimental NLs (Figure 49,50, and Table 8,9). The data showed proportional variations in values of weight % and atomic % of various element in blank nanoliposome (BNL-2) and drug loaded nanoliposome (DNL-2).

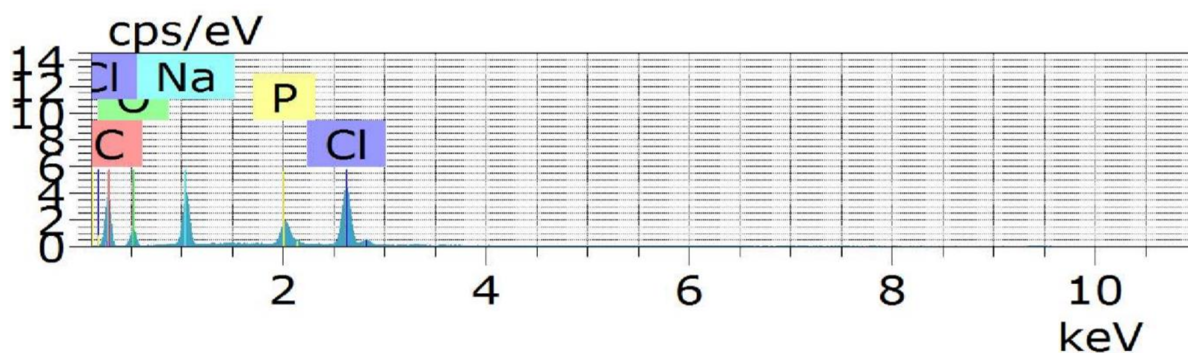

Figure 49: Energy dispersive X-ray (EDX) of DNL-2

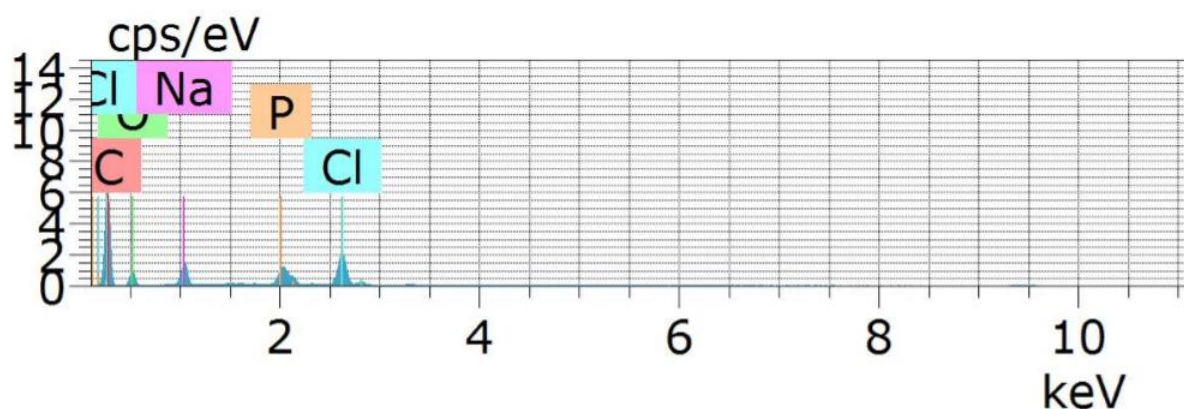

Figure 50: Energy dispersive X-ray (EDX) of BNL-2

Table 8: Weight % and atomic % of elements in experimental DNL-2

Spectrum: Objects 349

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
C	6	K-series	50.40	59.88	72.26	6.30
O	8	K-series	15.34	18.23	16.51	2.19
Na	11	K-series	7.98	9.49	5.98	0.54
Cl	17	K-series	7.84	9.31	3.81	0.29
P	15	K-series	2.60	3.09	1.44	0.13
		Total:	84.16	100.00	100.00	

Table 9: Weight % and atomic % of elements in experimental BNL-2

Spectrum: Objects 351

El	AN	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
C	6	K-series	56.72	71.99	80.51	6.64
O	8	K-series	13.60	17.26	14.49	1.94
Cl	17	K-series	3.67	4.66	1.77	0.15
Na	11	K-series	3.09	3.92	2.29	0.23
P	15	K-series	1.71	2.16	0.94	0.09
		Total:	78.78	100.00	100.00	

6.4.8. *In vitro* drug release study and analysis of drug release kinetics

On the basis of particle size, drug loading, entrapment efficiency, percentage yield and SEM study NL-2 formulation was found to be the best among the experimental formulations and it was considered for the *in vitro* drug release study. It was studied by dialysis membrane method. Primarily we had chosen two release media one was PBS (pH 7.4) and another was PBS (pH 7.4) containing 2-Hydroxypropyl-β-cyclodextrin (1% w/v).

Figures 51 and 57 show the cumulative percentage release curve of drug against time in PBS media and PBS with 2- hp-β-CD media. The release increased initially up to first 9 h of the study. Then from the 10th hour, the drug release was slightly slower in case of PBS and for PBS with 2- hp-β-CD media drug release increased initially up to 36 h then slower with time. Figure 52-56 shows the in-vitro drug release in PBS media, which was fitted in different kinetic equations to depict the drug release pattern for 96 hours. Figure 58-62 shows the in-vitro drug release kinetics in different model in PBS with 2- hp-β-CD media. Table 10 depicts the corresponding regression (R^2) values.

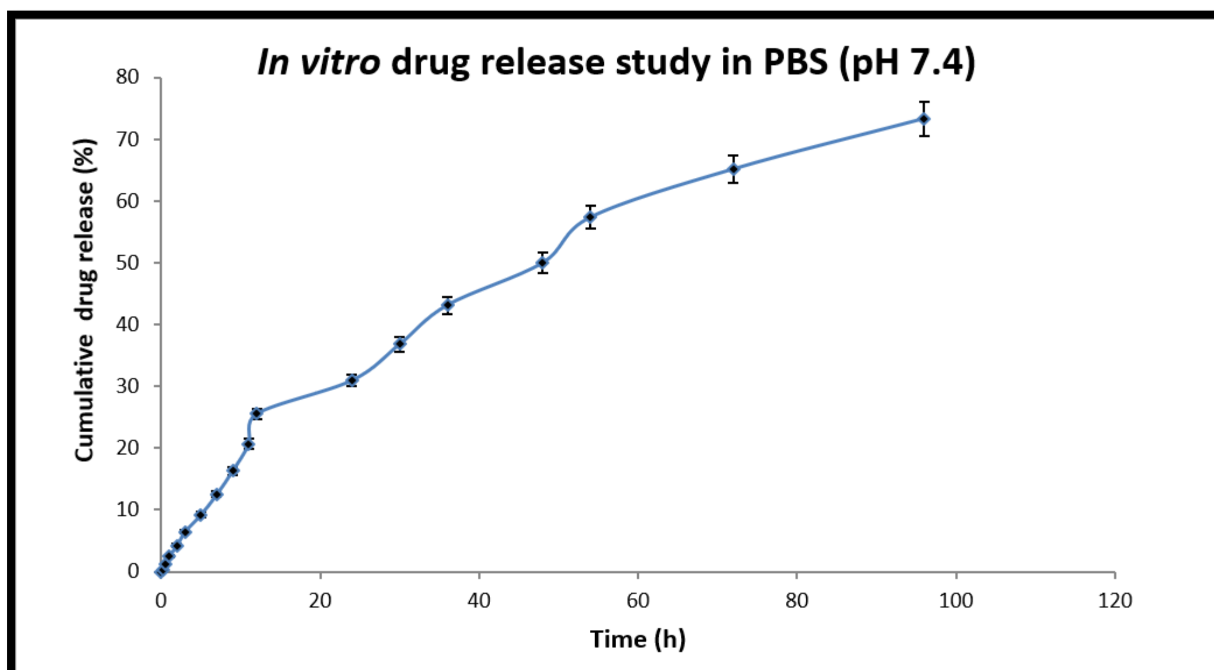

Figure 51: Cumulative drug release % of NL-2 against time (in PBS media).

When drug release data (in PBS media) were tested on various kinetic models, the following graphs along with their respective equations were obtained (Figure 52-56).

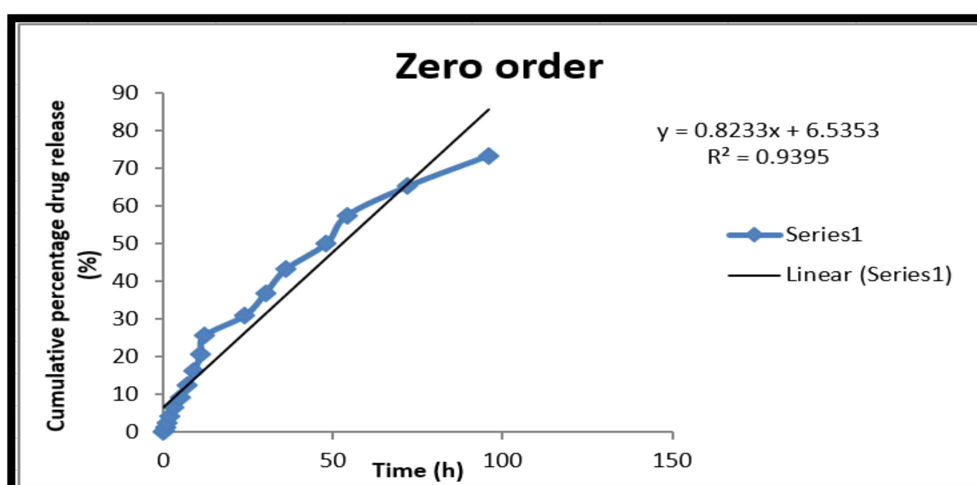

Figure 52: Zero order kinetic model (in PBS media).

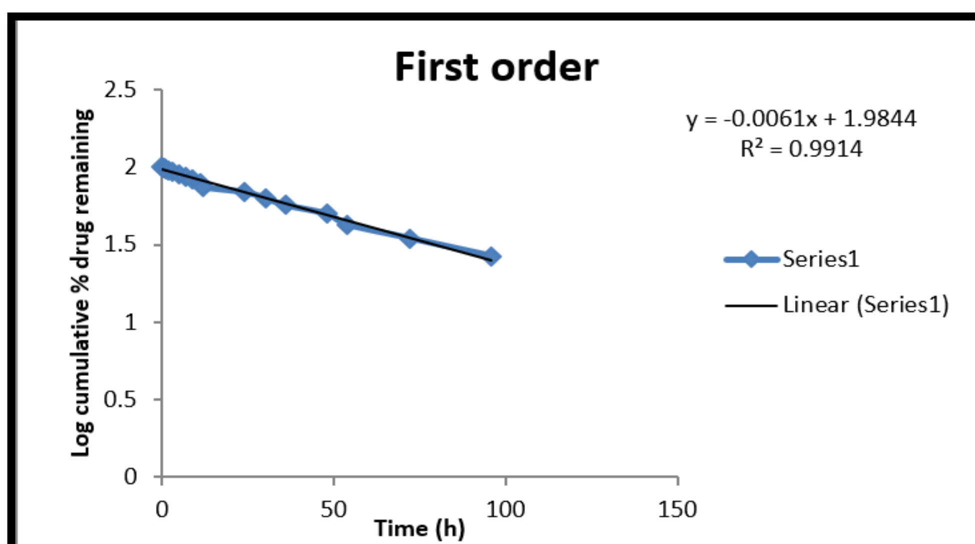

Figure 53: First order kinetic model (in PBS media).

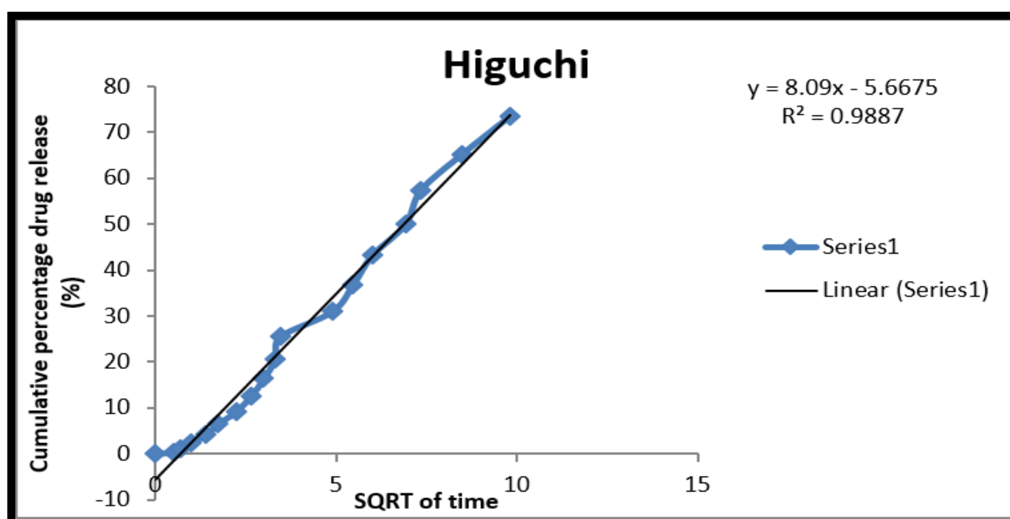

Figure 54: Higuchi kinetic model (in PBS media).

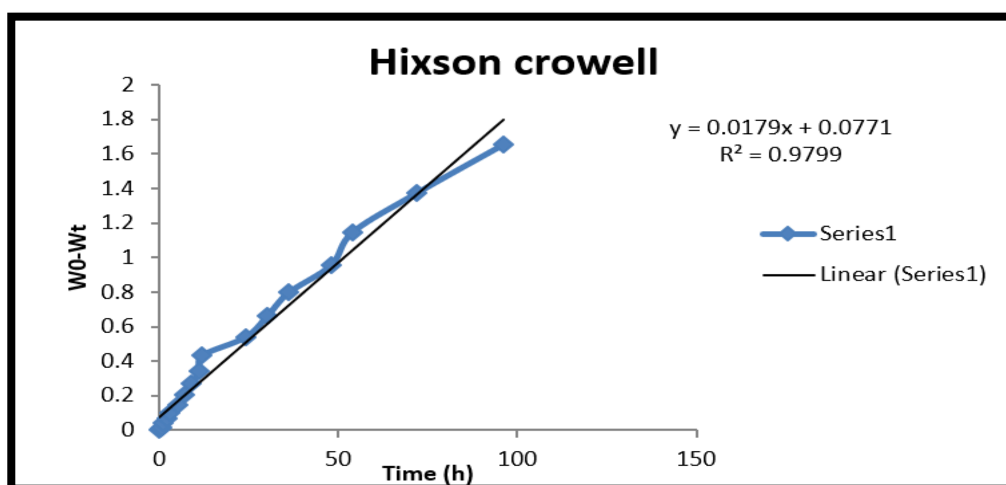

Figure 55: Hixson-Crowell kinetic model (in PBS media).

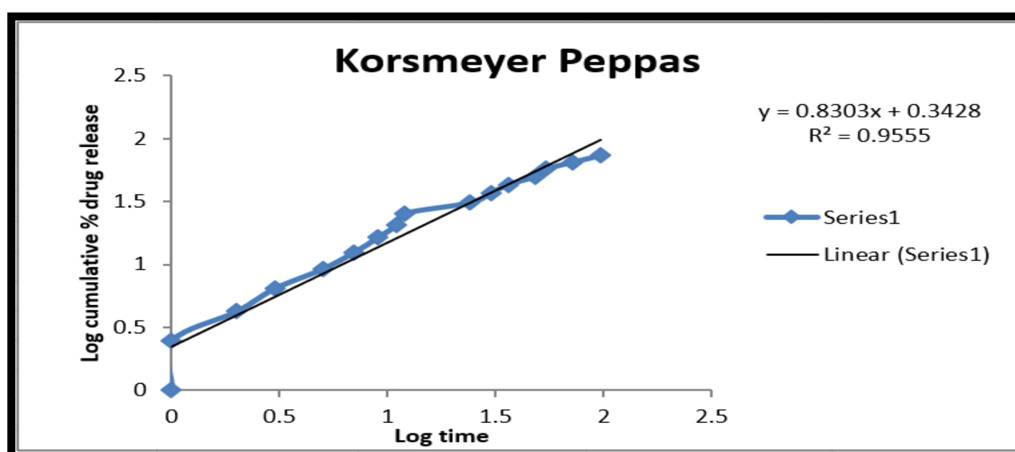

Figure 56: Korsmeyer-Peppas kinetic model (in PBS media).

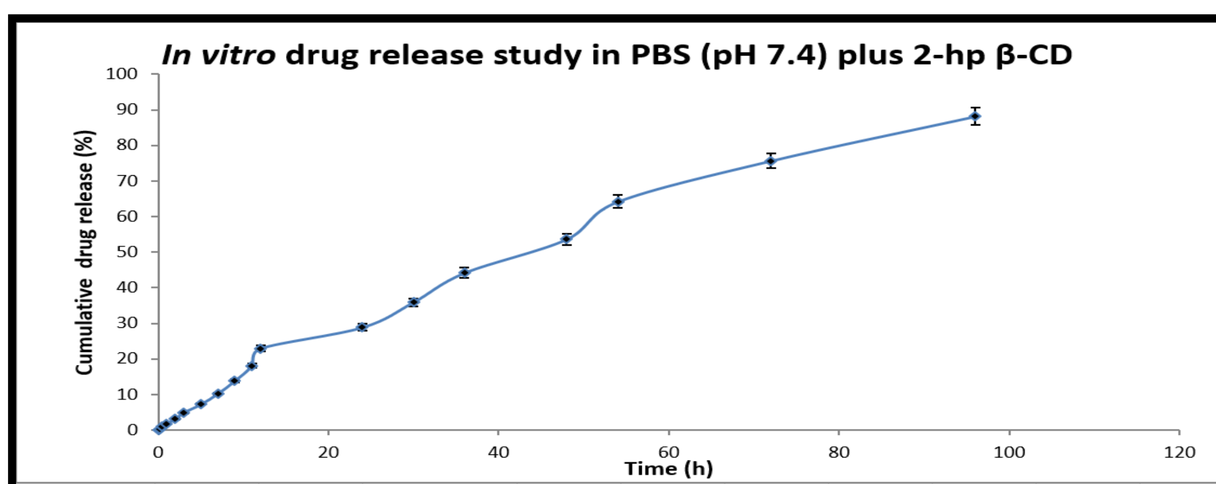

Figure 57: Cumulative drug release % of NL-2 against time (in PBS with 2- hp-β-CD media).

When drug release data (in PBS with 2- hp-β-CD media) were tested on various kinetic models, the following graphs along with their respective equations were obtained (Figure 58-62).

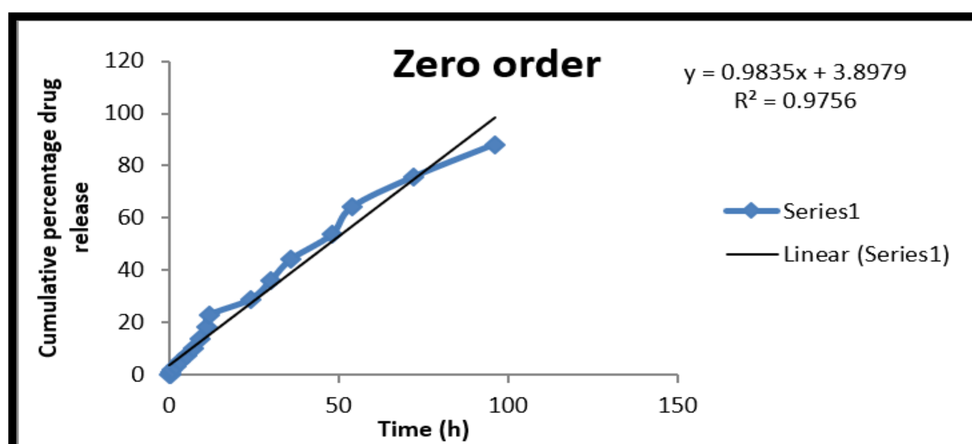

Figure 58: Zero order kinetic model (in PBS with 2- hp-β-CD media).

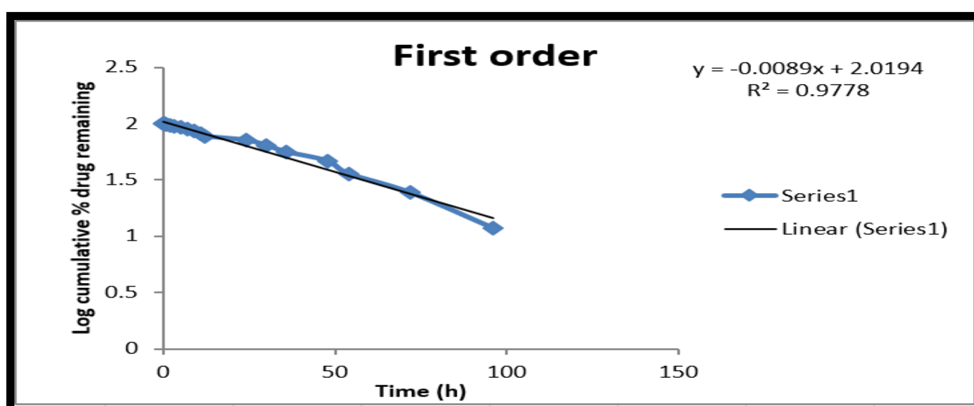

Figure 59: First order kinetic model (in PBS with 2- hp-β-CD media).

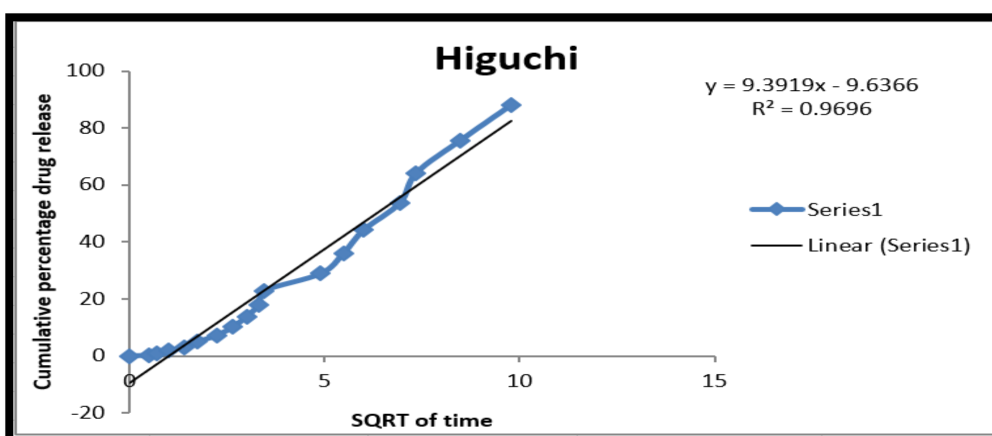

Figure 60: Higuchi kinetic model (in PBS with 2- hp-β-CD media).

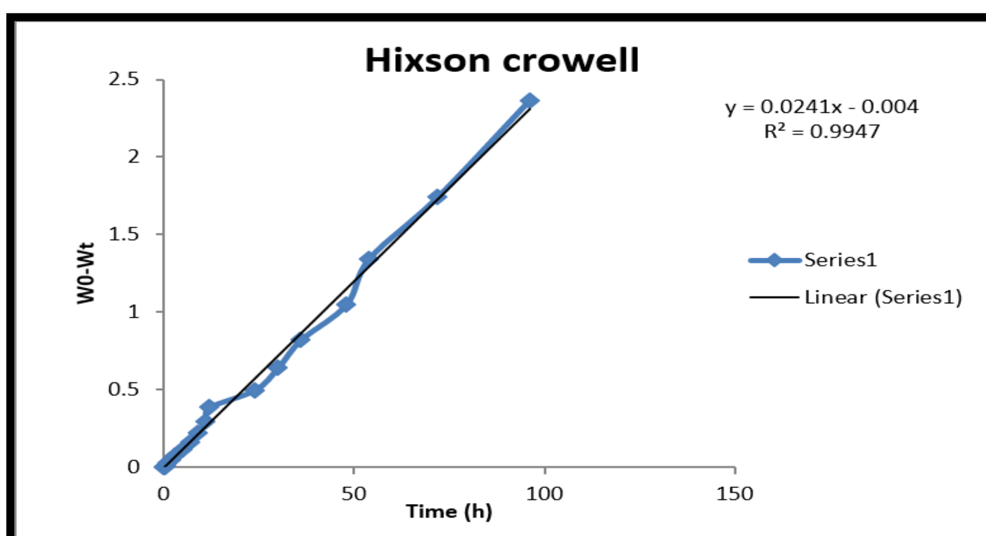

Figure 61: Hixson-Crowell kinetic model (in PBS with 2- hp-β-CD media).

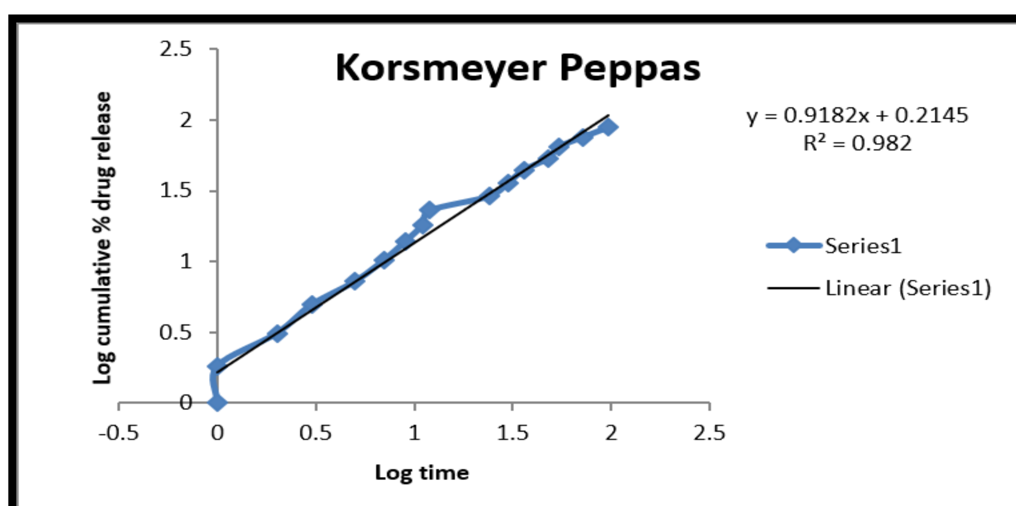

Figure 62: Korsmeyer-Peppas kinetic model (in PBS with 2- hp-β-CD media).

Table 10: The regression values (R^2) of NL-2 by using various release kinetic models.

Kinetic Model	Regression values (R^2)	
	In PBS	In PBS with 2- hp-β-CD
Zero order	0.9395	0.9756
First order	0.9914	0.9778
Higuchi model	0.9887	0.9696
Hixson Crowell	0.9799	0.9947
Korsmeyer-Peppas	0.9555	0.982

6.5. Stability study

After a successive stability study NL-2 formulation which was kept in stability chamber with 75% RH (relative humidity) in different temperature, i.e. at 4°C, 25°C and 40°C respectively for three months. All formulations were further analysed by FTIR and DLS.

6.5.1. FTIR spectra

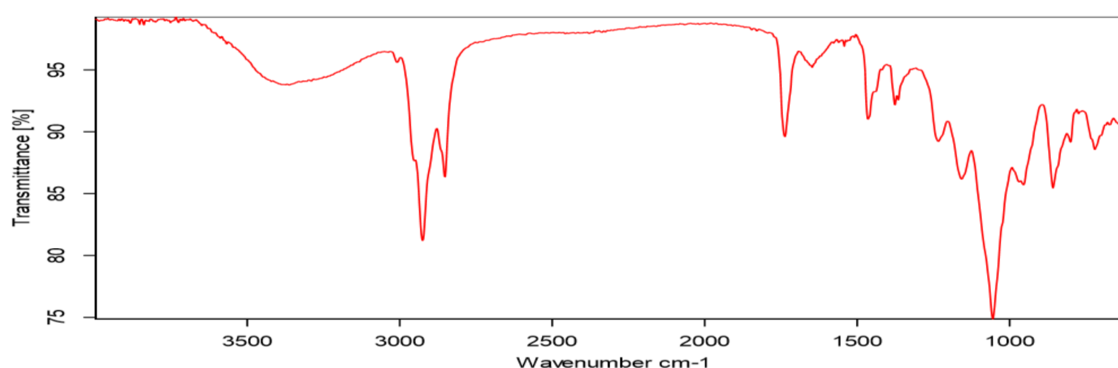

Figure 63: FTIR spectrum of NL-2 after kept at 4°C for three months.

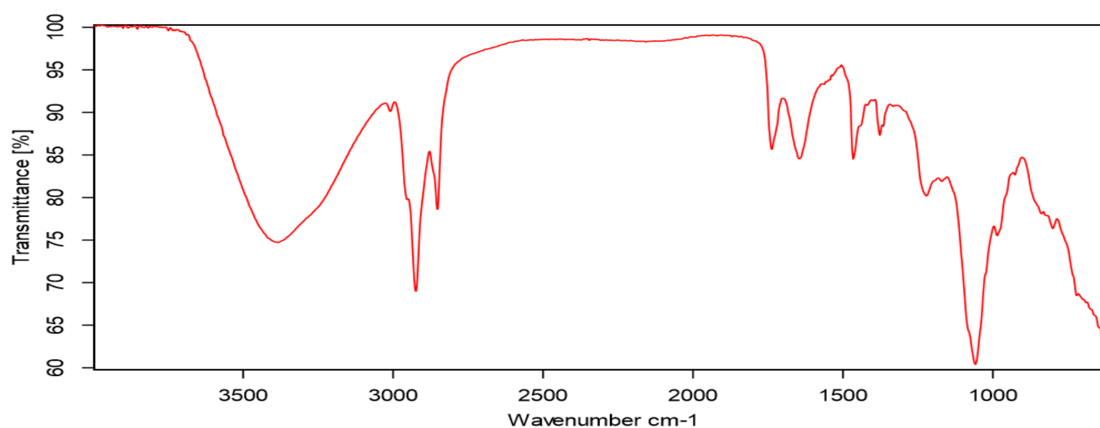

Figure 64: FTIR spectrum of NL-2 after kept at 25°C for three months.

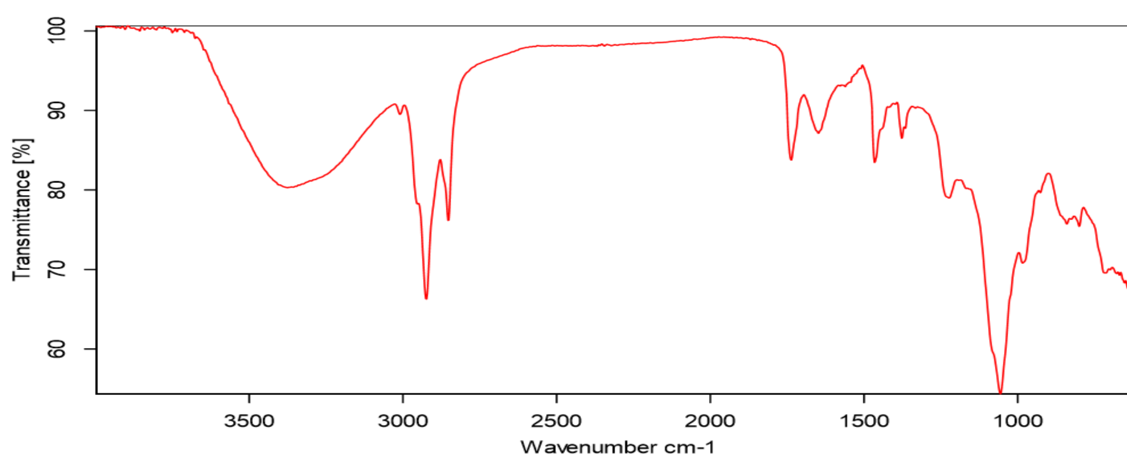

Figure 65: FTIR spectrum of NL-2 after kept at 40°C for three months.

6.5.2. Particle (vesicle) size, polydispersity index and zeta potential

Table 11: The average particle size, polydispersity index and mean zeta potential values of NL-2 formulations, after stability study.

Stability chamber temperature	Average particle size (Z-average) (nm)	Polydispersity index (PDI)	Zeta potential (mV)
4°C	148.8 ± 21.35	0.386 ± 0.08	-79.0 ± 8.68
25°C	238.23 ± 36.17	0.270 ± 0.10	-68.5 ± 10.4
40°C	331.6 ± 32.65	0.409 ± 0.13	-60.2 ± 6.35

Values are represented as mean ± SD (n=3)

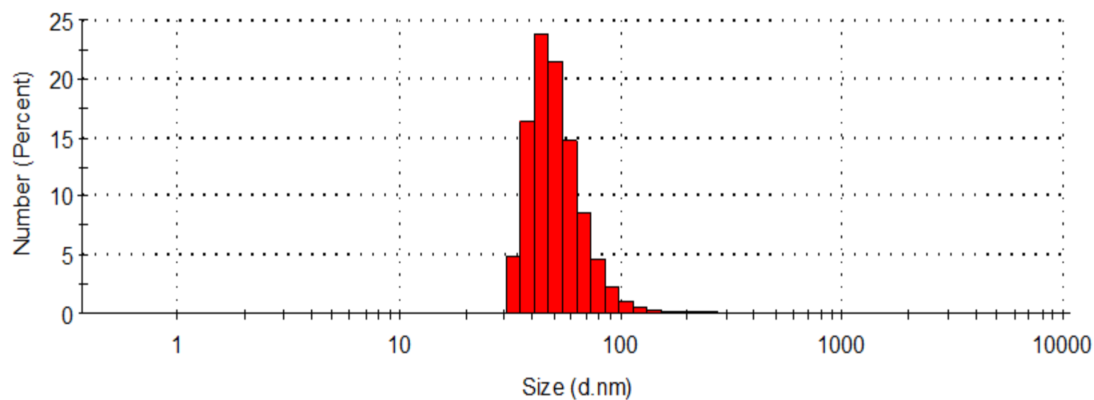

Figure 66: Size distribution curve of NL-2 after kept at 4°C for three months.

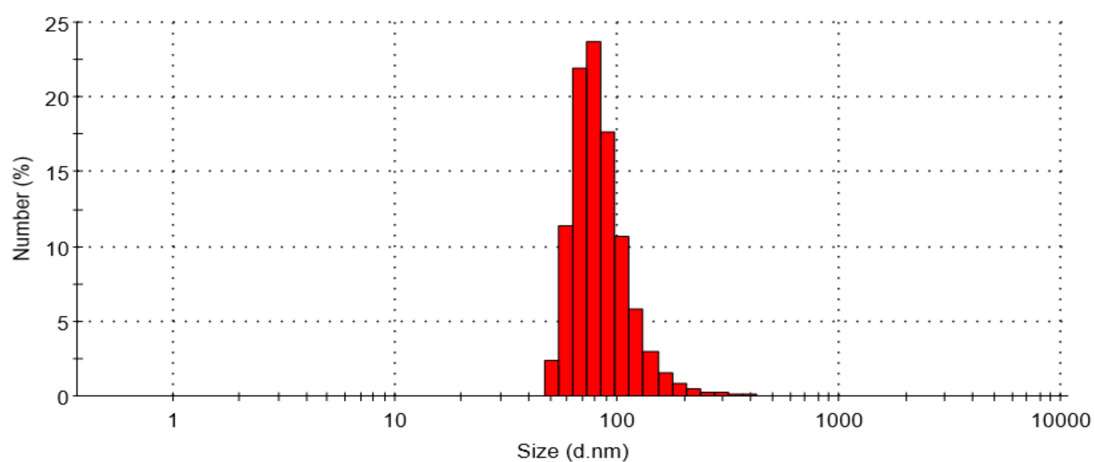

Figure 67: Size distribution curve of NL-2 after kept at 25°C for three months.

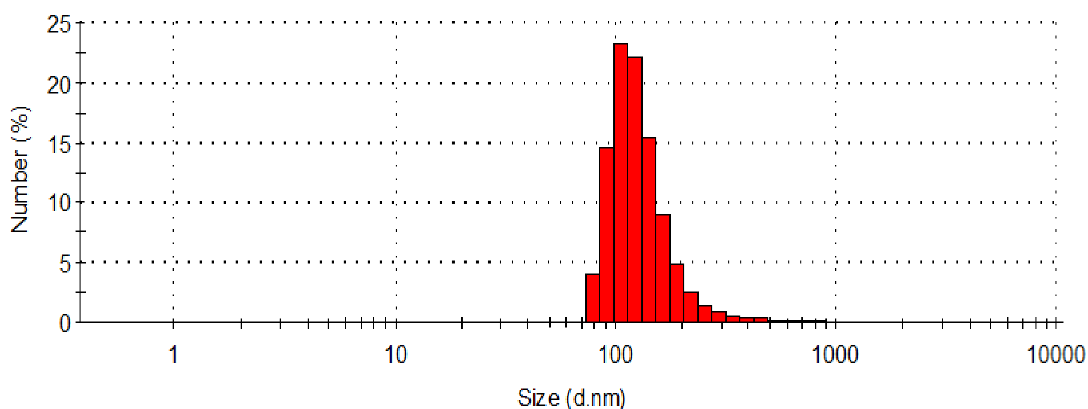

Figure 68: Size distribution curve of NL-2 after kept at 40°C for three months.

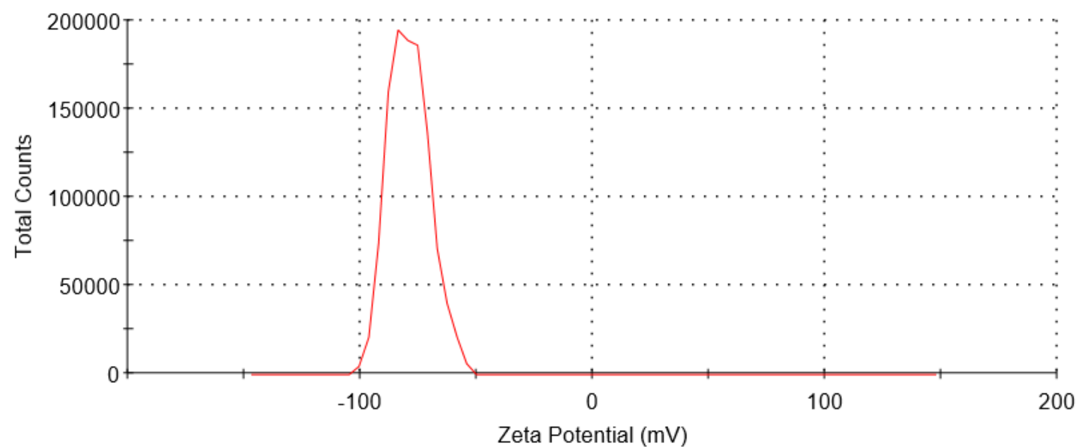

Figure 69: Zeta potential of NL-2 after kept at 4°C for three months.

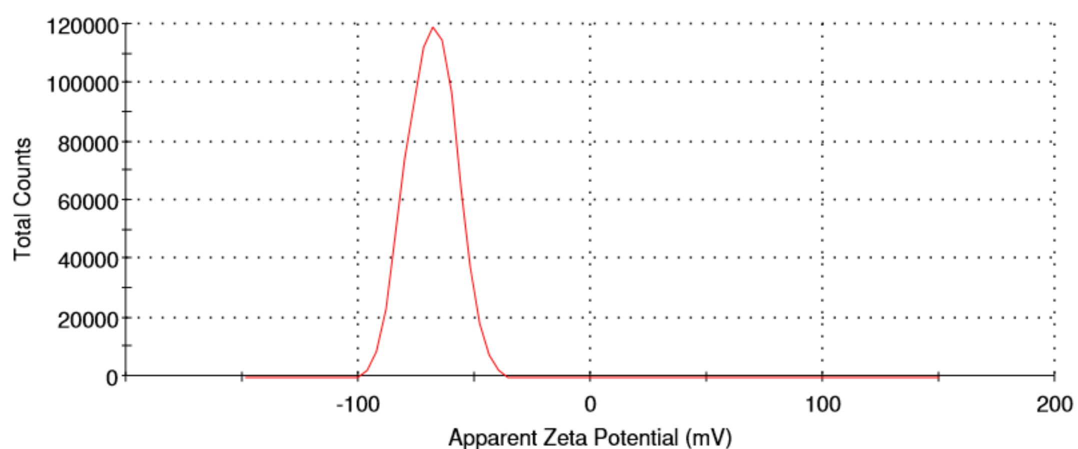

Figure 70: Zeta potential of NL-2 after kept at 25°C for three months.

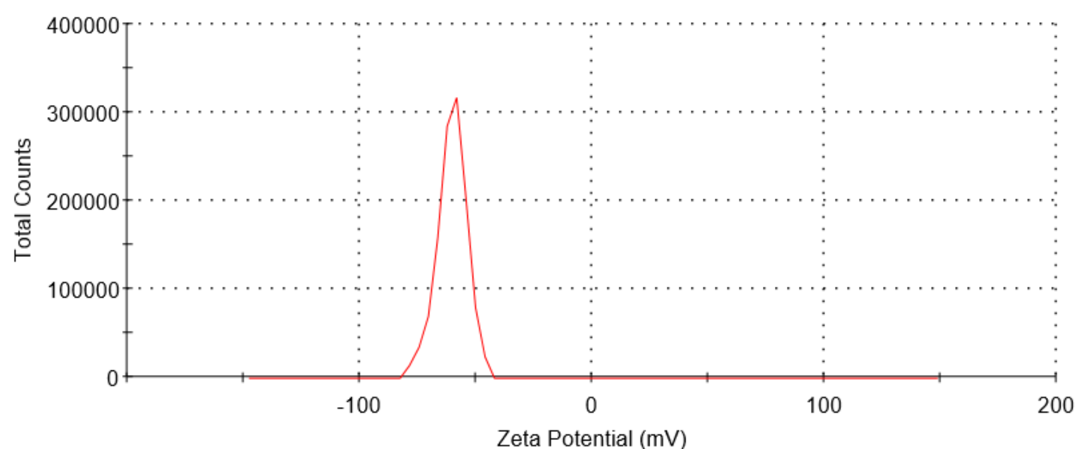

Figure 71: Zeta potential of NL-2 after kept at 40°C for three months.

Chapter-7

DISCUSSIONS

7. DISCUSSIONS

7.1. The UV absorption spectrum of abiraterone acetate (AA) in acetonitrile-dichloromethane (3:1) and ethanol-PBS (pH 7.4) mixture (1:3)

UV scanning of AA in acetonitrile-dichloromethane (3:1) and ethanol-PBS (pH 7.4) mixture (1:3) yielded lambda max values at 253.0 nm in both cases, which was very close to the reported peak of abiraterone acetate at 254.0 nm (www.fda.gov). This confirms the authenticity and purity of the product being used and yielded the reference wave length for spectrophotometric calculation in further studies.

7.2. The calibration curves of abiraterone acetate (AA) in acetonitrile-dichloromethane (3:1) and ethanol-PBS (pH 7.4) mixture (1:3)

Two different calibration curves were prepared- one in ethanol: PBS (pH 7.4) mixture (1:3) for studying the *in vitro* drug release and the other in acetonitrile: dichloromethane (3:1) for determining the drug loading of nanoliposome. Each reading was made in triplicate. In the developed calibration curve the values of R^2 were 0.9996 and 0.9999 in ethanol: PBS (pH 7.4) mixture (1:3) and acetonitrile: dichloromethane (3:1) respectively. The values favour the accuracy of the calibration curves used for further analysis.

7.3. The drug excipient interaction study by FTIR Spectroscopy

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.

When the FTIR spectrum of the physical mixture of AA and the selected excipients were compared, no major shifting of the predominant peaks of drug and the excipients was found 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 (Maji et al., 2014) among the various functional groups of SLE, CHL 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 and as a result no free drug was available on the liposome surface.

7.4. Physicochemical characterisation and evaluation of prepared formulation

Drug loading, surface morphology, average size (Z-average), PDI of the prepared formulation varied with the changes in the quantity of drug and excipients, and in different process parameters, such as speed and duration of hydration, hydration temperature, duration of sonication, speed and duration of centrifugation, lyophilization duration etc.

7.4.1. Drug loading and entrapment efficiency

With the changes made in different formulation the drug loading and entrapment efficiency varied. Among the five formulation NL-2 has the highest drug loading (4.166%) and entrapment efficiency (88.337%); NL-5 has the lowest drug loading (2.28%) and a medium entrapment efficiency (59.748%), while NL-1 has drug loading of 3.566% and entrapment efficiency of 57.777%. Initially, we found that with an increase in phospholipid concentration (at a fixed drug amount), percentage of drug loading increased. When we increased CHL and SLE ratio loading as well as entrapment efficiency increased, it was found in NL-4 where entrapment efficiency had become 70.641%.

7.4.2. Particle (vesicle) size, PDI and zeta potential of nanoliposome

Particle size plays a vital role in the in the biodistribution of nanoliposome and very small size (less than 10 nm) are very prone to renal elimination whereas size more than 400 nm are very much prone to phagocytosis (Mukherjee et al., 2014). As the vesicle size of the developed nanoliposomes were much higher than the cut-off size so it can be concluded that the developed nanoliposomes have escaped renal elimination. Also in case of the optimized formulation NL-2, it can be concluded that it was much less prone to phagocytosis as the size was 130.80 nm.

Formulation NL-1 had average particle size (Z-average) 210.73 nm and polydispersity index 0.438, NL-2 had average particle size (Z-average) 130.80 nm and polydispersity index 0.270 and NL-4 had average particle size (Z-average) 193.36 nm and polydispersity index of 0.537, suggesting for narrow range of size distribution.

The zeta potential value of NL-1, NL-2 and NL-4 were -71.2, -89.7 and -63.2 mV respectively. For a particle system to be stable in the liquid dispersion it needs to have a zeta potential of more negative than -30 mV or more positive than +30 mV. So, our results predict 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. Hence the prepared nanoliposome should be stored in the lyophilized state and reconstituted only before injection.

7.4.3. Study of morphology by SEM, FESEM, Cryo-TEM and AFM

The surface morphology of the particles of NL-1, NL-2 and NL-4 as assessed by SEM has shown that the nanoliposomal vesicle were spherical in shape and their size was in the nanoscale range and they had a smooth surface. 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 image of the optimized formulation NL-2 suggests that drug was distributed in particulate form throughout the vesicles and also proved the lamellarities of the fornulation. Further while in suspension for longer time as SEM provides three-dimensional picture the particles were actually oval in shape. The AFM image revealed the presence of individual nanoliposome with a small marked agglomeration observed.

7.4.4. *In vitro* drug release study

In vitro drug release data were collected over 96 hours and the cumulative releases percentage of drug from the optimized formulation NL-2 were plotted against time to determine the drug release pattern in various media. From the figures, it is seen that almost 70% of the initial drug content released in 96 hours in PBS media and almost 87% in PBS with 2- hp-β-CD media. The graphs clearly showed the sustained release profile of the drug from the formulation. It was also proved that 2- hp-β-CD act as a release enhancer.

The drug release pattern was also correlated with the established models of release kinetics namely Zero Order, First Order, Higuchi, Hixson-Crowell and Korsmeyer-Peppas. Considering the R^2 values (0.9395, 0.9914, 0.9887, 0.9799, 0.9555) in all the above-mentioned kinetics model in PBS media, it has been found that the drug release pattern from NL-2 formulation followed First Order Kinetics model and Higuchi model more than the other kinetics models for PBS media, indicating that erosion mechanism of the matrix polymer and may play some role to the formation of pore like systems. The R^2 values (0.9756, 0.9778, 0.9696, 0.9947, 0.982) in all the above-mentioned kinetics models in PBS with 2- hp-β-CD media, showed that the formulations followed Hixson-Crowell and Korsmeyer-Peppas Kinetics model more than the other kinetics models. This suggests that coupling of diffusion and erosion mechanism so called anomalous diffusion or non-Fickian diffusion (based on n values) was instrumental in controlling the drug release pattern from the nanoliposomal formulation. of the matrix polymer and may play some role to the formation of pore like systems

7.5. Stability study

For the stability study, NL-2 formulations which were kept in stability chamber with 75% RH (relative humidity) in different temperature at 4°C, 25°C and 40°C respectively for three months, were further analysed by FTIR and DLS. FTIR spectra of the stored NL-2 formulation were compared with those of the freshly prepared formulations. No distinguish changes in spectrum were observed for the formulations stored at 4°C. However, the formulation stored at 25°C and 40°C showed a little deformation of structure. In DLS study no prominent changes in size distribution were observed for 4°C stored formulation, but vesicles size was increased in case of 25°C and 40°C stored formulation. So, from the above statement, it’s mean that formulation should be stored in refrigerator for its longer stability.

Chapter-8

CONCLUSION

8. CONCLUSION

On successful completion of the project, in the present study a novel delivery vehicle for cancer therapy, a nanosized lipid carrier containing abiraterone acetate with size in the nanometric range and having a smooth morphology was developed. The optimized nanoliposomal formulation showed substantial amount of drug loading and a sustained release profile as it was shown in the release curve which will fulfil reduction in systemic side effects of abiraterone acetate. However further development and studies like conjugation of a ligand to the surface of the nanoliposome then quantification of the drug in mice prostate needs to be done in order to substantiate that abiraterone acetate attains the desired concentration for being therapeutically active against prostate cancer. Moreover, the potential of the ligand conjugated abiraterone acetate nanoliposome formulation in metastatic castration-resistant prostate cancer (mCRPC) and metastatic high-risk castration-sensitive prostate cancer (mCSPC) model is required to be established in order to proceed from laboratory to clinic and stability testing of the formulation is required to be done according to ICH guidelines. After fulfilment of *in vivo* investigation if we get a desirable result then, this will open a new era of treatment over the existing oral medications.

Chapter-9

REFERENCES

9. REFERENCES

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