1. INTRODUCTION

a. Prevalence of breast cancer

Breast cancer is the most prevalent disease and holds second rank in the mortality rate of women (after lung cancer). In 2015, approximately 231,840 new breast cancer cases and 40,730 breast cancer deaths (40,290 women, 440 men) are estimated throughout the world. According to the World Health Organization (WHO), by 2050 it is expected that 27 million new breast cancer cases and 17.5 million breast cancer deaths will occur per annum (1). Globally, the burden and incidence rates of breast cancer are enormously increasing than the other cancers. Metastatic action of the breast tumors leaves the disease condition elusive and incurable (2). Current therapies for breast cancer include radiation therapy chemotherapy and endocrine therapy has enriched the therapeutic effect but toxicity and side effects associated with these therapies are obstructing the clinical utility. Most of the cytotoxic drugs used clinically are chemotherapeutics administered into systemic circulation. Administering low molecular weight chemotherapeutics into systemic circulation exhibit rapid clearance, low pharmacokinetic profile and sub optimal tissue distribution and a small fraction reach the tumor/tumor cell. Hydrophobic natured chemotherapeutics exhibit large volume of distribution leading to higher accumulation at healthy tissue site and causes toxicity. Chemotherapeutics are highly susceptible to develop multi drug resistance (MDR) in the tumors. Exploration and development of a new technology are critical to treat breast cancer, which could effectively target tumor cells without killing healthy cells (3,4).

b. Conventional breast cancer therapy

(i). Chemotherapy

Chemotherapy is the treatment of cancers using cytotoxic drugs to kill the cancer cells or inhibiting their cell division. Even though there are no randomized controlled trials for chemotherapeutics still its been widely used in the management of advanced breast cancer to improve the quality of life (5). Advantage of chemotherapy is reducing the tumor volume preoperatively and avoiding the breast-conserving surgery. So, it has been applied to the treatment of lower-stage breast cancer (6). Anemia is the common side effect of chemotherapy, is a low red blood cell count that can cause fatigue and reduced quality of life. Chemotherapy is associated with many side-effects such as pain, nausea, vomiting, hair loss, weight changes, fatigue and anxiety; however, the most commonly reported side-effect is fatigue (7).
(II). Radiation Therapy
Radiation therapy/radiotherapy is a targeted treatment which is the most effective way to kill the cancerous cells in the breast which may remain around even post-operation. Radiation can reduce the risk of breast cancer recurrence by about 70 per cent. Side effects of radiotherapy treatment are confined to the treated area (8). Skin at the treated area turns red, dry, tender; itchy and other possible side effects cause limited oral intake, which may lead weight loss (9). Radiotherapy refers to the medical use of ionizing radiation for malignant tumors. The effects of radiation therapy are localized to the region being treated. This therapy injures or destroys cells in the area being treated (the ‘target tissue’) by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Usually, a combination of all the three treatment regimes is used against any given cancer for the maximum benefit (10).

(III). Hormonal therapy
Hormonal therapy is to treat hormone receptor-positive cancers. In this therapy, the amount of hormone can be reduced or blockade of the hormonal action. Estrogen action may be blocked or its amount is reduced to decrease the risk of recurrence (11). Hormone therapy includes aromatase inhibitors, selective estrogen receptor (ER) modulators and estrogen receptor down regulators as well as surgical treatments such as removal of ovaries and fallopian tubes. Tamoxifen use of at least five years is associated with a 12% reduction in recurrence and a 9% reduction in mortality over a 15-year follow-up period, in ER-60 positive and ER-unknown breast tumors. The benefits of tamoxifen appear to be optimized at five years, with current recommendations to discontinue adjuvant tamoxifen after five years. It can also be given for advanced-stage or metastatic disease to shrink or slow the growth of existing tumors (12). Recent trials (Arimidex-No Iviadex study in postmenopausal women (ARNO-95) and the Intergroup Exemestane Study have shown benefits of aromatase inhibitors over tamoxifen for disease-free survival and complications (13). Recently, it has been observed that some cases of breast cancer are resistant to hormonal therapy (in spite of ER positivity) either ab initio or while on treatment, and several mechanisms have been postulated to explain these, and many strategies are currently being tried to overcome this resistance.

(c). Complications associated with conventional breast cancer therapies
Majority of cancerous diseases are treated with multiple drug therapies with a combination of 2 or more anti-cancer drugs that can exhibit synergistic or additive effects by different mechanisms involved in cancer progression. As briefed above the
chemo and hormonal therapies have demonstrated elevated efficacy still significant toxicities are associated with these therapies. Underlying mechanisms for the evolution of side effects and toxicities as the anti-tumor drugs are not site-selective and thereby cause non-specific cytotoxic action over healthy cells in the bone marrow, gastrointestinal epithelia, and hair follicles (14). Serious side effects and complications are associated with the Tamoxifen, hormonal therapeutic include high risk for endometrial cancer by 2.4 times in women aged 50 years or older (15) and thromboembolic complications by 1.9 times (16). Among the chemotherapeutics, conventional doxorubicin is associated with acute toxicities include myelosuppression, mucositis, and alopecia. Irreversible congestive heart failure is one of the most serious, conventional doxorubicin- induced toxicity failure (17). Even though targeted therapies demonstrated considerable positive effect by the results of multiple clinical studies these therapies are associated with severe side effects. Severe heart complications include ventricular dysfunction and congestive heart failure in addition to common flu-like symptoms are associated with Trastuzumab alone or in combination with chemotherapy (18). Therefore, a safe, smart therapeutic approach is required for selective delivery of cytotoxic agents to the tumors which are responsible for the breast cancer progression and metastasis and also improving the therapeutic index and efficacy/toxicity balance.

(d). Application of nanotechnology for breast cancer therapy
Nanotechnology is no longer a new concept and creating higher impact in every part of the health care system. Nanomedicine-Nanotherapeutics-Nanotheranostics are the terminologies coined up because of the exploitation of the nano sized particles in the field of health care. The keyword ‘nanoparticle’ in the Pubmed resulted about 101,198 articles (search date: Jan 2015) inferring that the progression in the field of nanotechnology is very high. Remarkable progression has been witnessed by the fact that there are more than 150 ongoing clinical trials. Tremendous efforts and time have been spent to shift this technology from pre-clinical stage to commercialization stage (19,20). Nanomedicine which may be nanoparticulated (particle itself a therapeutic agent) or nanocarriers (carrier encapsulates the therapeutic agent) are intended to design in the range nanometers (nm) to several hundred nm as per the requirement and usage. Nanoparticles can easily permeate into the leaky vasculature-tumor tissue (where healthy tissue has a uniform vasculature) and can deliver the drug in the controlled manner at the site of tumor tissue, which is well known as Enhanced Permeability and Retention (EPR) effect. Nanoparticles includes polymeric nanoparticles, liposomes, micelles, which are featured with encapsulation of poorly soluble drugs, site specific drug delivery, elevating drug bio-availability,
higher permeability across the biological membranes and controlled drug release. Most of the drugs intended for chemotherapy act on the surface receptor/within cytoplasm/within nucleus. Tumors are characterized by heterogeneity such as aberrant expression, mutation of oncogenes/tumor suppressor genes which cause modifications in cellular events such as apoptosis, cell cycle arrest, adaptive resistance and metastasis. Drugs act while trafficking in the blood circulation and the concentration at the tumor site exhibits the therapeutic efficacy of the drugs (14). To attain high concentration at the tumor site, higher dosages are administered. Due to non-specificity systemic side effects and toxicities elicit. Drugs internalize into tumors mainly through the passive diffusion or active transport whereas nanoparticles internalize into cells via endocytosis (21). Steps involved in the endocytosis are

- Cargo enters into membrane invaginations which are pinched off to turn as membrane-bound vesicles, also known as endosomes. Cells consist of heterogeneous populations of endosomes furnished with endocytic machinery, which originate at different sites of the cell membrane.
- The endosomes transport the cargo to various specialized vesicular structures, which enables to deliver the cargo towards different destinations.
- Finally, the cargo is delivered to intracellular components and sent back to the extracellular milieu or delivered across cells (22).

(e). Diallyl disulfide (DADS)

Natural antioxidants, especially dietary flavonoids and polyphenolic compounds are well-documented on their prophylactic and therapeutic action in chemical-induced carcinogenesis. They confine on various carcinogen bio-activating steps, which prevent the covalent binding of the carcinogen to cellular DNA (23). Allium species include garlic, onion, leeks and scallions possess great potential in treatment/prevention of cancers and cardiovascular diseases. Among them garlic has demonstrated anti-tumor, anti-hypertensive, anti-hypercholemic actions (24). Epidemiological studies reported that dietary intake of allium products exhibits inverse effect on risk of many cancers. The beneficiary effects of the garlic are because of the presence of organosulfur compounds namely, allylsulfides and flavanoids. Among them Diallyl disulfide (DADS) is the principal organosulfur ingredient present in garlic as it shares the major portion about 60% garlic oil (25, 26). In general, it is a hydrophobic organic compounds and exhibits anti-tumorogenic action in vitro growth of breast, colon, lung and gastric cancer cell lines, and leukemia cell lines. These evidences represent DADS as a efficient cytotoxic drug
(27-30). Despite its pronounced cytotoxic action, hydrophobic, low bio-availability and short biological half-life brings up challenge to the formulator in the designing a suitable drug delivery system. This became a major stumbling block to this wonder molecule for clinical translation.

(f). Folate targeting pathway

Folate receptor is a glycosylphosphatidylinositol-linked membrane glycoprotein which is over-expressed on the surfaces of cancer cells, including breast cancer, whereas its expression is absent in most normal cells. Folic acid permeates into the cytoplasm via folate receptor-mediated endocytosis mechanism (Figure. 1). Hartmann et al. reported the correlation between high folate over-expression and bad prognosis of breast cancer, suggesting that targeting of this receptor helpful for treating the tumors (31).

![Figure 1: Folate receptor mediated endocytosis mechanism (32)](image)

(g). Lipid based nanocarriers

Among the nanocarriers, lipid based nanocarriers have been great potential to solubilizing, encapsulate and deliver active molecules in a programmed pattern to achieve bioavailability and avoid side-effects (33,34). These drug carriers are made up of bio-compatible lipids such as phospholipids, cholesterol and triglycerides. Numerous advantages of the lipid matrix make the lipid based nanocarriers as an idealistic drug delivery system. Bio-compatibility and bio-degradability characteristics of these systems are prone to be less toxic as compared to other drug delivery systems such as polymeric nanoparticles (35,36).
(h). Solid lipid nanoparticles (SLN)
In early nineties, Mueller and coworkers and Gasco and coworkers have focused on the development of SLNs for the drug delivery. SLNs are bio-compatible; sub-micron sized colloidal drug delivery systems, which are designed by replacing the oil by solid lipid in the emulsions. They provide higher entrapment efficiency, higher loading, greater surface area, simpler scale-up & manufacture and less toxic than the polymer which potentiates the activity of the drug encapsulated in the lipid core (37-39). SLNs exhibit sustained drug release and highly stable than the liposomes and even sterilization carried out during the manufacture of liposomes can be bypassed by the SLNs. The therapeutic utility of nanoemulsions is eclipsed by unpredictable drug release and of nanocrystals by poor solubilization of drug in biological fluids can be overcome by SLNs. Lipids present in the SLNs are highly purified triglycerides or waxes, calixarenes and sterols. Lipophilic drugs face solubility and bioavailability problems, which can be overcome by delivering through SLNs. SLNs are capable of loading of hydrophilic/hydrophobic compounds, controlled and extended drug release, bypass the reticulo-endothelial system and deliver the chemotherapeutic at the site of action. Ingredients for SLNs preparation are approved by the Food and Drug Administration (FDA) and they are under Generally Recognized as Safe (GRAS) status (40,41). History and background of SLNs are very short as the findings were lacking of clinical studies in the breast cancer treatment. Pre-clinical studies such as in vitro cell line studies or in vivo animal studies for the SLNs in breast cancer treatment were promising, but this technology should become clinically significant. SLNs encapsulate poorly soluble and hydrophobic natured drugs, which are facing bioavailability and cellular uptake limitations. SLNs encapsulate poorly soluble and hydrophobic natured drugs, which are facing bioavailability and cellular uptake limitations.

(i). HYPOTHESIS
Intervention of breast cancer with folate receptor targeting agent and cytotoxic drug loaded solid lipid nanoparticulate drug delivery system will selectively target the folate receptor expressed in the breast tumors and dramatic drug level will be elevated and exhibits effective cytotoxic action.

(I). Implications of the hypothesis
Failure of existing therapies and development of drug resistant aggressive tumor types are the primary factors responsible for this increasing mortality rates (42). Reasons for the failure of drugs in clinical trials include (a) lack of efficacy at doses that are non-toxic; (b) failure of drugs to reach the vicinity of tumor; (c) lack of
stability in the serum; and (d) non-availability of appropriate delivery systems (43,44). In addition, recent studies also showed drug resistance development due to the activation of several bypass mechanisms in breast cancer cells. Several nanocarrier-based drugs have been approved for treating cancers. Since nanocarriers offer several advantages such as (a) protection of drug from degradation in serum; (b) prevention of interactions with various other biological constituents, which otherwise limits the drug availability; (c) improve pharmacokinetic and dynamic properties, they are the first option to choose to deliver a drug effectively into tumors. For example, nano-carrier based albumin bound paclitaxel nanoparticles (Abraxane), liposomal doxorubicin (Myocet) and pegylated liposomal doxorubicin (Doxil) have been approved for treating metastatic and recurrent breast cancers. However, several side effects, which include low WBC count, abnormal heartbeat, liver problems have been reported with nanodrugs like Abraxane and Doxil. Even though, there are several advantages associated with nano-carrier based drug delivery systems, many hurdles are also associated with these approaches, hindering the development of many nano-carrier systems (45). For example factors such as: (a) lack of biocompatibility of nanomaterials and the feasibility of functionalization; (b) poor therapeutic index and dispersibility resulting in systemic toxicity and aggregation; and (c) lack of good stability and drug release characteristics. Therefore, effective approaches and strategies are needed to develop nanoparticles that can effectively deliver pharmacological agents to the target organs without causing any major problems to the patient. As aforementioned enticing features make SLNs as potential delivery system for the drugs facing physico-chemical problems. And the hydrophobic nature of DADS assures high encapsulating efficiency when the lipid vehicle is placed in an aqueous biological environment because of their high lipid water partition coefficients. Lipid nanoparticles conjugated with folic acid are one among the targeted delivery systems have achieved reasonable success (46, 47). Folic acid can be an efficient tool to improve intracellular delivery without any collateral toxicity.

The challenge in this study is to deliver the cytotoxic drug at tumor site by active targeting. This can be accomplished with drug encapsulated surface modified SLNs recognized as a biocompatible drug delivery system which can effectively deliver the drug candidates into breast tumors.
2. AIM & OBJECTIVES

In recent years, research is extensively intensified on ‘active targeting’ to treat the tumors in cancer diseases. Novel molecules entities and established molecules are profoundly scrutinized for their course of action to target cancers. Although many novel molecules have evolved but failure in tumor site specific targeting was accompanied. Tremendous endeavors and focus is made on advancements in discovering novel targeting pathways to treat cancer. Quintessentially, to achieve tumor targeted localized chemotherapeutic action and diminish the systemic toxicity a novel drug delivery system need to be designed. So, the present work aims to develop an active targeted drug delivery system to for tumor specific treatment. In this respect the objectives of the present study were:

- To develop Diallyl disulfide (DADS) loaded solid lipid nanoparticles (SLNs) using quality by design approach for the attaining the statistical optimized and stable nano formulation.
- To conjugate folic acid for the breast tumor targeting and to achieve the site specific drug delivery.
- To determine characteristics – Particle size, Zeta potential, Morphology, Surface conjugation and drug release.
- To determine the cytotoxicity, cellular uptake and cell death mechanism of Diallyl disulfide by observing the targets they act upon inside the cell and cell signaling cascade.
3. PLAN OF WORK

a. Literature review
b. Selection of drug
d. Pre-formulation studies
   I. Fourier Transform Infrared Spectroscopy
   II. Development of calibration curve for DADS
e. Preparation of DADS-SLNs
f. Development of statistically optimized DADS-SLNs by Response
   surface methodology - Box-behnken design
g. Conjugation of Folic acid to DADS-SLNs
h. Characterization of FA-DADS-SLNs
   I. Particle Size and Zeta potential analysis
   II. Determination of morphology of FA-DADS-SLNs by scanning electron
       microscopy
   III. Determination of encapsulation efficiency and drug loading
   IV. In vitro drug release study of FA-DADS-SLNs and DADS-SLNs

h. In vitro cell line studies
   I. In vitro cytotoxicity studies
   II. Determination of Reactive oxygen species study
   III. In vitro cellular uptake study by triple fluorescence staining method
   IV. Quantitative determination of apoptosis by annexin V/propidium iodide
       dual staining method
   V. Detection of apoptotic signaling pathways- Western Blot analysis
4. REVIEW OF LITERATURE

Literature survey was carried out related to breast cancer, its treatment & limitations and nanotechnology application to treatment of breast cancer. The most relevant literature related to aforementioned topics is summarized in this section.

a. Breast cancer

Breast cancer was first described by Hippocrates, (father of Western Medicine) in 460 B.C. He postulated that body consisted of four humors - blood, phlegm, yellow bile, and black bile strange. Cancer was induced by the excess of black bile. Symptoms such as black, hard tumors are observed that may erupt if left untreated to yield a black fluid. He termed the cancer *karkinos*, a Greek term for “crab” as the tumors may have tentacles like legs of a crab. During A.D. 200, Galen, Hippocrates successor, also postulated cancer as excessive “black bile” but contrasting Hippocrates, Galen stated that some tumors were more harmful than others. Galen suggested some of the pharmaceutical agents to treat breast cancer, such as opium, castor oil, licorice, sulphur, and a variety of salves, as well as incantations to the gods(48). During 1757, Henri Le Dran, a leading French physician expostulated that surgery is the actual cure for breast cancer as long as the infected axillia lymph nodes were amputated. In similar way, Claude-Nicolas Le Cat stated that the scalpel was the only option to treat cancer. Le Cat would remove the breast, excising out the lymph nodes as well as the pectoralis major muscle. These physicians were assured that the presence of a tumor did not cause serious effect, but was a localized-site disease that can be surgically removed before it spread. This theory survived well until the twentieth century and directed to the initiation of the radical mastectomy (49).

**Pilar Eroles et al., 2012** described that Breast cancer is a complex disease including clinical, morphological and molecular very distinct entities. This heterogeneity cannot be explained only by clinical parameters such as tumor size, lymph node involvement, histological grade, age; or by biomarkers like estrogen receptor (ER), progesterone receptor (PGR) and epidermal growth factor receptor 2 (HER2) routinely used in the diagnosis and treatment of patients. During the last decade research has focused in depth on the molecular biology of this disease. Technological breakthroughs and in particular high throughput approaches, have allowed researchers to inquire into the nature of breast cancer revealing that this disease requires the interconnection of several signaling pathways and that both the cellular micro-environment, and the innate characteristics of the patient influence disease pathophysiology, outcome and treatment response. These findings have led
us to understand, that this is not just one disease, but many, and that each patient entails a particular case where personalized medicine could play a crucial role (50).

**Johannes Bange et al., 2001** explained the molecular targets for breast cancer therapy and prevention. With the identification and characterization of signaling mechanisms that govern cell growth, differentiation, motility and apoptosis, and the elucidation of their relevance for the development of the malignant phenotype, a new era of cancer therapy has begun. Based on advances in the molecular understanding of normal and pathologically disturbed cellular signaling networks, new target-selective drugs for therapy and prevention have been developed, and many studies are underway to investigate their clinical efficacy worldwide (51).

**Sunil R Lakhani et al., 2005** explained the molecular analysis of invasive breast cancer and its precursors related to breast cancer progression. New multi-step pathways of breast cancer progression have been delineated through genotypic–phenotypic correlations. They concluded that only through the combination of comprehensive morphological analysis and cutting-edge molecular tools can this knowledge be translated into clinical practice and patient management (52).

b. Nanotechnology to treat breast cancer

**Tanaka et al., 2009** briefed on nanotechnology for breast cancer therapy. A pegylated form of liposomally encapsulated doxorubicin is routinely used for treatment against metastatic cancer, and albumin nanoparticulate chaperones of paclitaxel were approved for locally recurrent and metastatic disease in 2005. These drugs have yielded substantial clinical benefit, and are steadily gathering greater beneficial impact. Clinical trials currently employing these drugs in combination with chemo and biological therapeutics exceed 150 worldwide. Despite these advancements, breast cancer morbidity and mortality is unacceptably high. Nanotechnology offers potential solutions to the historical challenge that has rendered breast cancer so difficult to contain and eradicate: the extreme biological diversity of the disease presentation in the patient population and in the evolutionary changes of any individual disease, the multiple pathways that drive disease progression, the onset of ‘resistance’ to established therapeutic cocktails, and the gravity of the side effects to treatment, which result from generally very poor distribution of the injected therapeutic agents in the body. A fundamental requirement for success in the development of new therapeutic strategies is that breast cancer specialists in the clinic, the pharmaceutical and the basic biological laboratory and nanotechnologists engineers, physicists, chemists and mathematicians optimize their ability to work in close collaboration. Different generations of nanotechnology tools
for drug delivery are reviewed, and our current strategy for addressing the sequential bio-barriers is also presented, and is accompanied by an encouragement to the community to develop even more effective ones (14).

**Grobmyer et al., 2012** reviewed on emerging nanotechnologies promise new approaches to early detection and treatment of metastatic breast cancer. Curative options are limited for patients with breast cancer metastatic beyond regional nodes. Fulfilling the promise of nanotechnologies for patients with metastatic breast cancer will require delivery of nanomaterials to sites of metastatic disease. Future translational approaches will rely on an ever increasing understanding of the biology of breast cancer subtypes and their metastases. These concepts were highlighted and elucidated (53).

**Sareen et al., 2013** have discussed the role of nanoparticles with respect to oncology, by particularly focusing on the breast cancer and various nanodelivery systems used for targeting action. Breast cancer nanotherapeutics is consistently progressing and being used to remove the various limitations of conventional methods available for the diagnosis and treatment of breast cancer. Nanoparticles provide an interdisciplinary area for research in imaging, diagnosis, and targeting of breast cancer. With advanced physicochemical properties and better bioavailability, they show prolonged blood circulation with efficient tumor targeting. Passive targeting mechanisms by using leaky vasculature, tumor microenvironment, or direct local application and active targeting approaches using receptor antibody, amplification in the ability of nanoparticles to target specific tumor can be achieved. Nanoparticles are able to reduce cytotoxic effect of the active anticancer drugs by increasing cancer cell targeting in comparison to conventional formulations. Various nanoparticles-based formulations are in the preclinical and clinical stages of development; among them, polymeric drug micelles, liposomes, dendrimer, carbon nanotubes, and nanorods are the most common (54).

**Ferrari et al., 2014** reviewed the current clinical state of nanoparticle based therapeutics in breast cancer, as well as highlighted several platforms that exemplify the future generation of innovative approaches to chemotherapy in breast cancer. Nanoparticle-based drug delivery platforms are emerging as powerful chemotherapeutic modalities in breast cancer. Doxorubicin and paclitaxel nanoparticle formulations are currently used clinically, yielding distinct pharmacokinetic parameters that prolong blood circulation times, enhance drug accumulation in tumors, and limit adverse side effects to patients. And while these nanoconstructs have shown substantial improvements in patient tolerability and survival, several emerging trends stand to make a significant impact on future
generations of nanoparticle platforms for breast cancer therapy. Firstly, there is a heightened understanding of several processes involved in tumor growth, potentiation, and invasion, resulting in the identification of several attractive molecular targets. This in turn has given rise to antibody-based therapeutics, drug repositioning, and the burgeoning field of RNA interference (RNAi). Secondly, an enhanced understanding of transport phenomena involved in delivery of chemotherapeutics has led to a rethinking and retooling of nanoscale drug carrier designs. Nanoparticle platforms are now incorporating features meant to overcome biological barriers and enhance drug accumulation within tumors, all the while incorporating unique chemistries that enable for controlled release of therapeutic payloads (55).

c. Lipid nanocarriers for breast cancer treatment
Miller et al., 2013 addressed the usage of lipid nanoparticles for cancer treatment and diagnosis. Of particular interest here are lipid-based nanoparticles that are genuine particles (approximately 100 nm in dimension) assembled from varieties of lipid and other chemical components that act collectively to overcome biological barriers (biobarriers), in order for lipid-based nanoparticles to preferentially accumulate in or around disease-target cells for the functional delivery of therapeutic agents for treatment or of imaging agents for diagnosis. The capabilities of these lipid-based nanoparticles will clearly vary depending on functional requirements, but the nanoscale allows for an impressive level of diversity in capabilities to enable corresponding lipid-based nanoparticles to address an equally diverse range of functional requirements (56).
Rodney et al., 2014 reviewed the emerging research and clinical developments of lipid nanoparticle drug delivery systems. Lipid nanoparticles are loaded with therapeutics and may not contain an enclosed bilayer. The majority of those clinically approved have diameters of 50–300 nm. The growing interest in nanomedicine has fueled lipid–drug and lipid–protein studies, which provide a foundation for developing lipid particles that improve drug potency and reduce off-target effects. Integrating advances in lipid membrane research has enabled therapeutic development. At present, about 600 clinical trials involve lipid particle drug delivery systems. Greater understanding of pharmacokinetics, biodistribution, and disposition of lipid–drug particles facilitated particle surface hydration technology (with polyethylene glycol) to reduce rapid clearance and provide sufficient blood circulation time for drug to reach target tissues and cells. Surface hydration enabled the liposome-encapsulated cancer drug doxorubicin (Doxil) to gain clinical approval in 1995. Fifteen lipidic therapeutics are now clinically approved. Although much research involves attaching
lipid particles to ligands selective for occult cells and tissues, preparation procedures are often complex and pose scale-up challenges. With emerging knowledge in drug target and lipid–drug distribution in the body, a systems approach that integrates knowledge to design and scale lipid–drug particles may further advance translation of these systems to improve therapeutic safety and efficacy (57).

Darwis et al., 2013 discussed the novel lipid-based nanoformulations and their lymphatic delivery via different routes, as well as the in vivo and in vitro models used to study drug transport in the lymphatic system. Physicochemical properties that influence lymphatic delivery as well as the advantages of lipid-based nanoformulations for lymphatic delivery are also discussed. The delivery of drugs and bioactive compounds via the lymphatic system is complex and dependent on the physiological uniqueness of the system. The lymphatic route plays an important role in transporting extracellular fluid to maintain homeostasis and in transferring immune cells to injury sites, and is able to avoid first-pass metabolism, thus acting as a bypass route for compounds with lower bioavailability, ie, those undergoing more hepatic metabolism. The lymphatic route also provides an option for the delivery of therapeutic molecules, such as drugs to treat cancer and human immunodeficiency virus, which can travel through the lymphatic system. Lymphatic imaging is useful in evaluating disease states and treatment plans for progressive diseases of the lymph system. Novel lipid-based nanoformulations, such as solid lipid nanoparticles and nanostructured lipid carriers, have unique characteristics that make them promising candidates for lymphatic delivery. These formulations are superior to colloidal carrier systems because they have controlled release properties and provide better chemical stability for drug molecules. However, multiple factors regulate the lymphatic delivery of drugs. Prior to lymphatic uptake, lipid-based nanoformulations are required to undergo interstitial hindrance that modulates drug delivery. Therefore, uptake and distribution of lipid- based nanoformulations by the lymphatic system depends on factors such as particle size, surface charge, molecular weight, and hydrophobicity. Types of lipid and concentration of the emulsifier are also important factors affecting drug delivery via the lymphatic system. All of these factors can cause changes in intermolecular interactions between the lipid nanoparticle matrix and the incorporated drug, which in turn affects uptake of drug into the lymphatic system. Two lipid-based nanoformulations, ie, solid lipid nanoparticles and nanostructured lipid carriers, have been administered via multiple routes (subcutaneous, pulmonary, and intestinal) for targeting of the lymphatic system (58).

Trotta et al., 2003 established and confirmed the preparation methodology of solid lipid nanoparticles by a solvent emulsification–diffusion technique. A preparation
method for nanoparticles based on the emulsification of a butyl lactate or benzyl alcohol solution of a solid lipid in an aqueous solution of different emulsifiers, followed by dilution of the emulsion with water, was used to prepare glyceryl monostearate nanodispersions with narrow size distribution. To increase the lipid load the process was conducted at 47±2°C and in order to reach submicron size a high-shear homogenizer was used. Particle size of the solid lipid nanoparticles (SLN) was affected by using different emulsifiers and different lipid loads. By using lecithin and taurodeoxycholic acid sodium salt, on increasing the GMS percentage from 2.5 to 10% an increase of the mean diameter from 205 to 695nm and from 320 to 368nm was observed for the SLN prepared using benzyl alcohol and butyl lactate, respectively. Transmission electron micrographs of SLNs reveal nanospheres with a smooth surface (59).

Wu et al., 2007 discussed the prospects of improvement of chemotherapeutics using SLNs as drug delivery system. Several obstacles frequently encountered with anticancer compounds, such as normal tissue toxicity, poor specificity and stability and a high incidence of drug- resistant tumor cells, are at least partially overcome by delivering them using SLN. The emergence of the newer forms of SLN such as polymer– lipid hybrid nanoparticles, nanostructured lipid carriers and long-circulating SLN may further expand the role of this versatile drug carrier in cancer treatment. This review focuses on the current use of SLN for the encapsulation and delivery of cytotoxic anticancer compounds. It also discusses more recent trends in the use of SLN as vehicles for delivery of chemosensitizers and cytotoxic therapeutic molecules. It is anticipated that, in the near future, SLN will be further improved to deliver anticancer compounds in a more efficient, specific and safer manner (41).

Muller et al., 2004 described the application of SLNs for the parenteral administration. Firstly, different types of nanoparticles based on solid lipids such as “solid lipid nanoparticles”, “nanostructured lipid carriers” and “lipid drug conjugate” nanoparticles are introduced and structural differences are pointed out. Different production methods including the suitability for large scale production are described. Stability issues and drug incorporation mechanisms into the particles are discussed. In the second part, the biological activity of parenterally applied SLN and biopharmaceutical aspects such as pharmacokinetic profiles as well as toxicity aspects are reviewed (37).

Huang et al., 2014 discussed necessity of the SLNs for the insoluble chemotherapeutics in cancer therapy. Nanoparticle drug formulations have been extensively reviewed and developed in the field of drug delivery as a means to efficiently deliver insoluble drugs to tumor cells. By mechanisms of the enhanced
permeability and retention effect, nanoparticle drug formulations are capable of greatly enhancing the safety, pharmacokinetic profiles and bioavailability of the administered treatment. Here, the progress of various nanoparticle formulations in both research and clinical applications is detailed with a focus on the development of drug/gene delivery systems (60).

**Muller et al., 2009** discussed the applications of lipid nanoparticles mainly solid lipid nanoparticles, nanostructured lipid carriers and lipid drug conjugates in parenteral delivery of pharmaceutical actives. The attempts to incorporate anticancer agents, imaging agents, anti-parasitics, anti-arthritis, genes for transfection, agents for liver, cardiovascular and central nervous system targeting have been summarized. The utility of lipid nanoparticles as adjuvant and toxicity caused by these kinds of lipid nanoparticles with a glance on the fate of lipid nanoparticles after their parenteral delivery *in vivo* viz., the protein adsorption patterns (61).

**Silva et al., 2014** explained in detail about the safety of the lipid carriers. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) were developed as alternative to other colloidal carriers. They were designed to overcome lipid nanoemulsions and liposomes in stability and ability to control the release of an encapsulated substance, and at the same time to be better tolerated than polymeric nanoparticles. Since the patenting of SLN discovery, large amount of data became available on the behavior of these systems in vitro. SLN/NLC has many prerequisites to be a well tolerated carrier – the currently available data seem to confirm it, but there are also some contradictory results. In this review, we collected the available data from cytotoxicity, oxidative stress and hemo-compatibility studies in vitro and analyzed their outcomes (62).

**Huwyler et al., 2008** discussed the liposomal antineoplastic drugs for tumor targeting. During the last years, liposomes (microparticulate phospholipid vesicles) have been used with growing success as pharmaceutical carriers for antineoplastic drugs. Fields of application include lipid-based formulations to enhance the solubility of poorly soluble antitumour drugs, the use of pegylated liposomes for passive targeting of solid tumors as well as vector- conjugated liposomal carriers for active targeting of tumor tissue. Such formulation and drug targeting strategies enhance the effectiveness of anticancer chemotherapy and reduce at the same time the risk of toxic side-effects (63).

**Jia et al., 2014** analyzed the pros and cons of nanomedicines versus traditional chemotherapy, and evaluated the importance that nanomaterials can bring in to significantly improve cancer metastasis treatment. Traditional chemotherapy used today at clinics is mainly inherited from the thinking and designs made four decades
ago when the Cancer war was declared. The potency of those chemotherapy drugs on in-vitro cancer cells is clearly demonstrated at even nanomolar levels. However, due to their non-specific effects in the body on normal tissues, these drugs cause toxicity, deteriorate patient's life quality, weaken the host immune-surveillance system, and result in an irreversible damage to humans own recovery power. Owing to their unique physical and biological properties, nanotechnology-based chemotherapies seem to have an ability to specifically and safely reach tumor foci with enhanced efficacy and low toxicity (64).

**Wong et al., 2007** discussed the prospects of improved cancer chemotherapy using solid lipid nanoparticles (SLN) as a drug delivery system. Several obstacles frequently encountered with anticancer compounds, such as normal tissue toxicity, poor specificity and stability and a high incidence of drug- resistant tumor cells, are at least partially overcome by delivering them using SLN. The emergence of the newer forms of SLN such as polymer–lipid hybrid nanoparticles, nanostructured lipid carriers and long-circulating SLN may further expand the role of this versatile drug carrier in cancer treatment. This review focuses on the current use of SLN for the encapsulation and delivery of cytotoxic anticancer compounds. It also discusses more recent trends in the use of SLN as vehicles for delivery of chemo-sensitizers and cytotoxic therapeutic molecules (41).

d. Diallyl disulfide for cancer treatment

**Lei et al., 2008** investigated the effect of diallyl disulfide (DADS), a component of garlic, on apoptosis in human mammary cancer cell line (MCF-7) and its mechanisms. Cytotoxicity was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Morphology of apoptotic cells was detected by acridine orange and ethidium bromide staining. Apoptotic cells stained with propidium iodide were examined using flow cytometry. Protein levels were detected by Western blot analysis DADS inhibited the proliferation of MCF-7 cells and induced the apoptotic ratio to increase rapidly. Cleavage of the caspase-3 and caspase-3 substrate poly(ADP-ribose) polymerase was observed in MCF-7 cells after 24 h of treatment with DADS. When the MCF-7 cells were treated with 200 micromol x L DADS, the stress-activated protein kinase extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase, was inhibited after 6 h; c-Jun N-terminal kinase (JNK), that is stress-activated protein kinase (SAPK), and p38 mitogen-activated protein kinase were activated after 6 h. These results suggest that DADS both inhibits the proliferation of MCF-7 cells and induces apoptosis of MCF-7 cells.
The mechanisms may include the inhibition of ERK and the activation of the SAPK/JNK and p38 pathways (65).

**Nakagawa et al., 2001** investigated the cytotoxicity effects in four breast cancer cell lines. Diallyl disulfide (DADS) is an oil-soluble organosulfur compound found in garlic. The effect of synthetic DADS on the growth of estrogen receptor (ER)-positive (KPL-1 and MCF-7) and -negative (MDA-MB-231 and MKL-F) human breast cancer cell lines was examined. In an *in vitro* MTT assay, regardless of ER status, DADS at an IC$_{50}$ of 1.8–18.1 μM after 72 h incubation caused inhibition of growth in all four cell lines examined. Growth inhibition was due to apoptosis as seen by the appearance of a sub $G_1$ fraction. In MDA-MB-231 cells, the apoptosis cascade comprised up-regulation of Bax protein (142%), down-regulation of Bcl-X$_L$ protein (38%) and activation of caspase-3 (438%) compared with controls. In an *in vivo* assay by orthotopic (right thoracic mammary fat pad) transplantation of KPL-1 cells in female nude mice, intraperitoneal injection of 1 or 2 mg DADS three times a week from the day of tumor cell inoculation until the end of the experiment (after 35 days) caused growth retardation and 43% reductions in primary tumor weight, respectively, compared with DADS-untreated mice without apparent side effects. Cell proliferation as evaluated by proliferating cell nuclear antigen (PCNA)-labeling in transplanted tumor of DADS-untreated mice was 59.6%, and 1 and 2 mg DADS-treated mice was 44.6 and 44.5%, respectively. In MDA-MB-231 cells, DADS antagonized the effect of linoleic acid (LA), a potent breast cancer cell stimulator (at DADS $= 1.8 \mu$M and LA $\geq 6.5\times 10^2 \mu$M concentration), and synergized the effect of eicosapentaenoic acid (EPA), a potent breast cancer cell suppressor (at DADS $>$3 $\times 10^{-3}\mu$M and EPA $>$ 6.3 $\times 10^{-1}\mu$M concentration). Thus, DADS could be a promising anticancer agent for both hormone-dependent and -independent breast cancers, and may harmonize with polyunsaturated fatty acids known as modulators of breast cancer cell growth (66).

**Su et al., 2013** discussed the molecular mechanisms for the anti-cancer action of diallyl disulfide. Considerable evidence in recent years suggests that garlic has anti-proliferative effects against various types of cancer. Garlic contains water-soluble and oil-soluble sulfur compounds. Oil-soluble compounds such as diallyl sulfide, diallyl disulfide, diallyl trisulfide and ajoene are more effective than water-soluble compounds in protection against cancer. DADS, a major organosulfur compound derived from garlic, can decrease carcinogen-induced cancers in experimental animals and inhibit the proliferation of various types of cancer cells. Its mechanisms of action include: the activation of metabolizing enzymes that detoxify carcinogens; suppression of the formation of DNA adducts; antioxidant effects; regulation of cell-
cycle arrest; induction of apoptosis and differentiation; histone modification; and inhibition of angiogenesis and invasion (67).

e. Folic acid for targeting breast cancer

Sahoo et al., 2012 developed Folate decorated dual drug loaded nanoparticle: Role of curcumin in enhancing therapeutic potential of nutlin-3a by reversing multidrug resistance. research has step towards reversing Multi drug resistance (MDR) by using curcumin, however its clinical relevance is restricted by plasma instability and poor bioavailability. In the present investigation we tried to encapsulate nutlin-3a and curcumin in PLGA nanoparticle (NPs) surface functionalized with folate to enhance therapeutic potential of nutlin-3a by modulating MDR. We document that curcumin can inhibit the expression of MRP-1 and LRP gene/protein in a concentration dependent manner in Y79 cells. In vitro cellular cytotoxicity, cell cycle analysis and apoptosis studies were done to compare the effectiveness of native drugs (single or combined) and single or dual drug loaded nanoparticles (unconjugated/folate conjugated). The result demonstrated an augmented therapeutic efficacy of targeted dual drug loaded NPs (Fol-Nut-Cur-NPs) over other formulation. Enhanced expression or down regulation of proapoptotic/antiapoptotic proteins respectively and down-regulation of bcl2 and NFκB gene/protein by Fol-Nut-Cur-NPs substantiate the above findings (68).

Hussain et al., 2012 developed folate conjugated 17-allylamino-17-demethoxygeldanamycin (17-AAG) loaded polymeric nanoparticles for breast cancer. Low water solubility and hepatotoxicity limited the clinical use of 17-allylamino-17-demethoxy geldanamycin (17-AAG), an inhibitor of heat shock protein 90 (HSP90). Folate targeted polylactide- co-glycolide–polyethylene glycol–folic acid (PLGA–PEG–FA) nanoparticles containing 17-AAG were prepared and characterized. Cellular uptake and in vitro cytotoxicity of the prepared nanoparticles were determined in MCF-7 human breast cancer cells. The particle size of 17-AAG loaded folate targeted nanoparticles was 238.67 ± 3.52 nm, drug loading was 8.25 ± 2.49% and about 80% of drug was released from the nanoparticles over 10 days. Cellular uptake studies showed much higher intracellular uptake of folate targeted nanoparticle as compared to nontargeted nanoparticles. Cytotoxicity study showed 2 fold increase (P < 0.05, n = 3) in the cytotoxicity of folate targeted nanoparticle in comparison to free drug or nontargeted nanoparticles. Due to their targeting ability, nanometer size, high drug loading and con- trolled release behavior, 17-AAG loaded PLGA–PEG–FA nanoparticles might be developed as a targeted delivery system for breast and other cancer treatment (31).
f. Application of Box-behnken design

**Hu et al., 2008** overcame multi-drug resistance (MDR) of cancer cells, paclitaxel (PTX) and doxorubicin (DOX)-loaded nanostructured lipid carriers (NLC) were prepared by solvent diffusion method using monostearin as solid lipid and oleic acid as liquid lipid matrix. The cytotoxicities and reversal activity of drug-loaded NLC were tested against human breast cancer (MCF-7) cells, human ovarian cancer (SKOV3) cells and their multi-drug resistant (MCF-7/ADR and SKOV3-TR30) cells. The chemical conjugant of folic acid and stearic acid (FA–SA) was further synthesized to prepare folated NLC. Comparing with taxol and doxorubicin solution, the NLC loading PTX exhibited high cytotoxicities in MCF-7 and MCF-7/ADR cells, while the NLC loading DOX only indicated high cytotoxicity in MCF-7/ADR cells. The reversal powers of the NLC loading PTX and DOX were 34.3 and 6.4 folds, respectively. The NLC loading PTX and DOX showed the same trends of enhanced cytotoxicity against SKOV3 and SKOV3-TR30 cells. The reversal powers were 31.3 and 2.2 folds for the NLC loading PTX and DOX, respectively. The modification of NLC with FA–SA could further enhance the cytotoxicities of drug in drug sensitive and drug resistant cells (46).

**Khan et al., 2010** optimized Risperidone loaded SLNs using box-behnken design. Subsequently, they characterized the SLN by non-destructive methods of analysis. Box–Behnken DOE was constructed using drug (X1), lipid (X2) and surfactant (X3) level as independent factors. Compritol 888 ATO and sodium lauryl sulphate were used as lipid and surfactant, respectively. The SLN was prepared by solvent evaporation method and characterized by transmission electron microscopy (TEM), differential scanning calorimetry (DSC), X-ray powder diffraction (XRD), fourier infrared spectroscopy (FTIR), near infrared spectroscopy (NIR) and NIR-chemical imaging (NIR-CI). Responses measured were entrapment efficiency (Y1), D90 (Y2), zeta potential (Y3), burst effect (Y4) and cumulative release in 8 h (Y5). Statistically significant (p < 0.05) effect of X1 on the Y1, Y2, Y3 and Y4 were seen. FTIR revealed no interaction between risperidone and compritol 888 ATO. TEM showed spherical and smooth surface SLN. Compritol retained its crystalline nature in the SLN formulation revealed by DSC and XRD studies. Homogenous distribution of risperidone and compritol 888 ATO was revealed by NIR-CI. Principal component analysis (PCA) and partial least square (PLS) were carried out on NIR data of SLN formulation. PLS showed correlation coefficient > 0.996 for prediction and calibration model of both risperidone and compritol 888 ATO. The accuracy of models in predicting risperidone and compritol 888 ATO were 1.60% and 11.27%, respectively (69).
**Review of Literature**

Peng et al., 2011 optimized a solid lipid nanoparticle (SLN) of chloramphenicol by investigating the relationship between design factors and experimental data using response surface methodology. A Box-Behnken design was constructed using solid lipid (X1), surfactant (X2), and drug/lipid ratio (X3) level as independent factors. SLN was successfully prepared by a modified method of melt-emulsion ultrasonication and low temperature-solidification technique using glyceryl monostearate as the solid lipid, and poloxamer 188 as the surfactant. The dependent variables were entrapment efficiency (EE), drug loading (DL), and turbidity. Properties of SLN such as the morphology, particle size, zeta potential, EE, DL, and drug release behavior were investigated, respectively. As a result, the nanoparticle designed showed nearly spherical particles with a mean particle size of 248 nm. The polydispersity index of particle size was 0.277 ± 0.058 and zeta potential was -8.74 mV. The EE (%) and DL (%) could reach up to 83.29% ± 1.23% and 10.11% ± 2.02%, respectively. In vitro release studies showed a burst release at the initial stage followed by a prolonged release of chloramphenicol from SLN up to 48 hours. The release kinetics of the optimized formulation best fitted the Peppas–Korsmeyer model. These results indicated that the chloramphenicol-loaded SLN could potentially be exploited as a delivery system with improved drug entrapment efficiency and controlled drug release (70).

Pokharkar et al., 2013 explained the process of optimization of iloperidone nanostructured lipid carriers for oral bioavailability enhancement using quality by design approach- Box behnken design. The current work was carried out by exploring the principles of quality by design approach to develop an optimized nanostructured lipid carrier (NLC) formulation of poorly water soluble active iloperidone (ILO) through systematic statistical study. The potential of NLC for improving the oral bioavailability of ILO was also evaluated. A 3-factor, 3-level Box–Behnken factorial design was explored to predict the responses such as particle size (Y1) and % entrapment efficiency (EE) (Y2) when concentration of lipid (X1), concentration of drug (X2) and concentration of surfactant (X3) were selected as independent variables. Particle size analysis revealed that all the batches were within the nanometer range. The % EE was found to be between 63% and 96%. In-vitro release study demonstrated sustained release profile of ILO NLC. The pharmacokinetic study in Wistar rats over the period of 24 h demonstrated 8.30-fold increase in oral bioavailability of ILO NLC as compared with ILO pure drug suspension. The NLC formulation remarkably improved the oral bioavailability of ILO and demonstrated a promising perspective for oral delivery of poorly water-soluble drugs (71).
5. METHODOLOGY

a. Materials

Table 1: List of chemicals

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the Chemical</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Diallyl disulfide</td>
<td>Alfa Aesar, India</td>
</tr>
<tr>
<td>2.</td>
<td>Sulpha-Rhodamine B</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>3.</td>
<td>Poloxamer 188 (F68)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>4.</td>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>Sigma Aldrich, India</td>
</tr>
<tr>
<td>5.</td>
<td>Stearic acid</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>6.</td>
<td>Palmitic acid</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>7.</td>
<td>Ethanol</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>8.</td>
<td>Potassium Bromide (KBr)</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>9.</td>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>10.</td>
<td>Formaldehyde</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>11.</td>
<td>Sodium Chloride</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>12.</td>
<td>Sodium Bicarbonate</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>13.</td>
<td>Sodium Hydroxide</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
<tr>
<td>15.</td>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)</td>
<td>Sisco Research Laboratories Pvt. Ltd, Chennai, India</td>
</tr>
<tr>
<td>16.</td>
<td>N-hydroxysuccinimide (NHS) 98%</td>
<td>Sisco Research Laboratories Pvt. Ltd, Chennai, India</td>
</tr>
<tr>
<td>17.</td>
<td>Dialysis bag (MWCO-12,000-14,000 g/mL)</td>
<td>Himedia labs, Mumbai, India</td>
</tr>
<tr>
<td>18.</td>
<td>Potassium dihydrogen orthophosphate</td>
<td>S.D. Fine Chemicals Ltd., Mumbai, India</td>
</tr>
</tbody>
</table>