Co-delivery of Aceclofenac and Methotrexate via lipobrids renders therapeutic effect in breast cancer

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CONTENTS

Abbreviations:

2D: 2- Dimension

ACE: Aceclofenac

C-6: Coumarin-6

COX: Cyclo-oxygenase

COX-2: Cyclo-oxygenase-2

DCM: Dichloromethane

DDW: Double distilled water

DLC: Dynamic light scattering

DMF: Dimethylformamide

DMSO: Dimethyl sulfoxide

DoE: Design of experiments

ER: Estrogen receptor

F: Fucose

G: Galactose

HER-2: Human epidermal growth factor receptor-2

IL-1β: Interleukin 1β

IL-6: Interleukin 6

M: Mannose

MMP-1: Matrix metalo proteinase 1

MTT: 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide

MTX: Methotrexate

NLC's: Nanostructured lipid carrier

NPs: Nanoparticles

NSAIDs: Non-steroidal anti-inflammatory drugs

PR: Progesterone receptor

PBS: Phosphate buffered saline

PDE: Percentage drug entrapment

PDI: Polydispersity index

PDL: Percentage drug loading

PL: Phospholipids

PL S100: Phospholipon S100

Q_{24}: Cumulative drug permeation in 24 hours

Q_{48}: Cumulative drug permeation in 48 hours

RPMI-1640: Rosalind Park Memorial Institute-1640

SA: Stearyl amine

SD: Standard deviation

SEM: Scanning Electron Microscope

SLNs: Solid lipid nanoparticles

TNF-α: Tumor necrosis factor α

Figure captions

Table captions

Abstract

Inflammation and breast cancer are co-related with one another. Inflammation is one of the most rudimentary responses of the body's self defence mechanism whereas the breast cancer is one of the most complex molecular and morphological diseases. They are mainly initiated by the large number of the immune cells (mast cells), molecular mediator like chemokine, cytokine, vasoactive amines, eicosanoid (Prostaglandins, leukotrienes) and the product of the proteolytic cascade. Inflammation mainly takes place to reduce the effect of injury or to subside the effect of the foreign pathogen and the parasite at the same time playing an integral role in the removal and clearance of the dead cells. Chronic inflammation is mainly associated with the occurrence of complexities like atherosclerosis, cancer etc.

Present study surfaces the over-expression of pro-inflammatory markers at molecular level. The use of anti-inflammatory drugs namely Methotrexate and Aceclofenac when loaded onto, lipid-polymer hybrid nano-particles (LPHNPs) plays a crucial role in subsiding pathology induced inflammation. The characterizations of the lipobrids were done containing the different formulation. The size obtained was119.3±0.3; the drug loading efficiencies and the drug loaded size were 94% and 18.8 % respectively. Inflammation is mounted by rapidly dividing cancer cells. We assayed the prepared formulation on the MDA-MB-231 breast cancer cells. We mimic the inflammation in the breast cancer cells by introduction of the LPS and subsequently the amount of inflammation and drug treatment assay was carried out *in-vitro.* We had also accomplished the drug sensitivity (MTT) assay at three different time points followed by the calculation of the IC50. We observed the sustained release of drug from the lipobrid. In a nut shell the results obtained, proven that this method of drug delivery can open new vistas and be economically more feasible in addressing the inflammatory diseases.

Hypothesis

- Objective 1: Formulation and characterization of lipobrids.

Specific aim 1: Formulation and Characterization of MTX and ACL loaded lipobrids

- Objective 2 : Assessment of apoptosis in breast cancer cells induced by MTX and ACL loaded lipobrid

Specific aim 1: Assessment of molecular markers such as TNF-α, MMP-1, IL-6, IL-1β, COX–2 involved in breast cancer pathology

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 Breast cancer pathophysiology

Breast cancer is largely the disease of the breast tissues. It begins with the formation of the lump in the breast tissue which gradually increases in size majorly due to the excessive accumulation of the rapidly growing and dividing cells having specific ability of overcoming apoptosis. This phenomenon is consequently followed by the change in the breast shape, dimpling of the breast skin, fluid coming from the nipple and a red scaly patch of skin (Francie B.De, Abreu et.al, July 2013). Breast cancer is mainly a disease of great molecular, clinical and physiological variation (D.Max Parkin et.al, 2009).

Breast cancer remains one of the most lethal and fatal malignancies across the globe being the most prevalent amongst female. In the recent years nearly, 2,32,000 new incidents of breast cancer were registered with a mortality rate of 40,000 mainly due to invasive breast cancer in the United States (2016; Siegel *et al*., 2014).

1.1.1 Factors affecting breast cancer

Over the years, life-saving treatment strategies have worked and beget a ray of hope in the advancement of breast cancer understanding. Although, there has been a wide array of medications available, yet the breast cancer treatment largely depends on the physiological condition, age of the patient and the stage of the cancer (Miele *et al.*, 2009).

Breast cancer has overwhelming outcomes due to various factors which include both environmental and genetic factors. National institute of environmental health sciences (NIEHS) in association with the breast cancer and the environment research program (BCERP) made a detailed observation to study the consecutive interaction of the environmental factor and breast cancer. The study explains the transdisciplinary action of the chemical, physical, biological and social environmental factor along with the genetic characteristics. Of late, it was observed that women of younger age group (25 -40 years) implicate an increasing number of incidents of breast cancer (Connor, Thomas H., et al.2014).

1.1.2 Types of breast cancer

Breast cancer is categorized in two types: invasive carcinoma (Ductal carcinoma and lobular carcinoma) and the non-invasive carcinoma (Ductal carcinoma *in-situ* and lobular carcinoma *in-situ*) (Paul Lichtenstein et.al.July 2000). Based on the hormonal receptor expression, there are mainly ER receptor (oestrogen receptor), PR receptor (progesterone receptor) and HER 2 receptor (human epidermal growth factor receptor 2). The other minor categories mainly include inflammatory breast cancer, Paget disease of nipple, Phyllodes tumor and angiosarcoma etc.

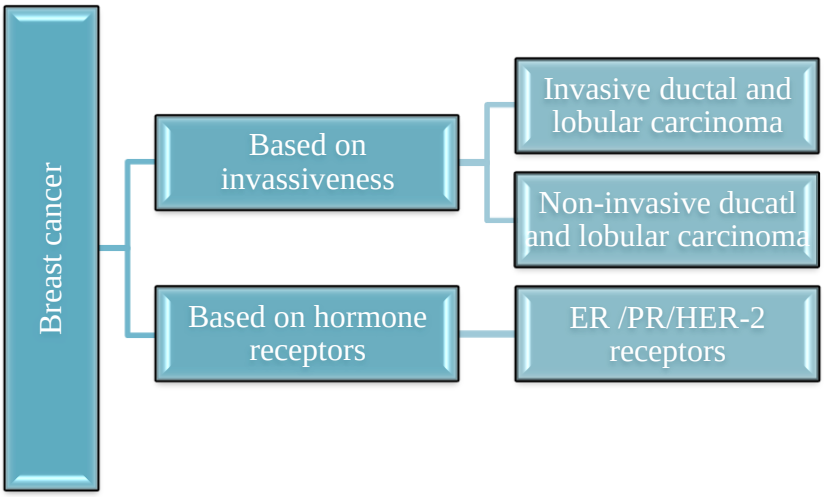


Figure1 The hierarchical presentation of the major and the different types of the breast cancer which are broadly categorized on the basis of invasiveness and hormonal receptors.

1.2 Breast cancer cells MCF-7 & MDA-MB-231 cells

Breast cancer cells MCF-7 and MDA-MB-231 are solely the triple negative breast cancer cells. The triple negative breast cancer mainly refers to the type where all the three types of hormonal receptor ER receptor (oestrogen receptor), PR receptor (progesterone receptor) and HER 2 receptor (human epidermal growth factor receptor 2) are not expressed.

The MCF-7 cells are the adherent cells. They are primarily of the mammalian origin derived from *homo sapiens*, from the metastatic site of mammary glands. Their morphology is moreover similar epithelial cells. They are aggressively metastatic and associated with the WNT7B pathway. The MCF-7 cells mainly maintain the characteristics of the differentiated mammary cells. Also, they also have got the ability to process the estradiol via the cytoplasmic oestrogen receptors and formation of domes.

The cells MDA-MB-231 is adherent prolifically growing cells. MDA-MB-231 cells are of mammalian origin and are derived from the *homo sapiens* but they are mainly associated with the TGF alpha oncogene. Both of them having a doubling time of 24-32 hrs and may be cultured *in-vitro* in the RPMI-1640 (Rosalind Parkin Memorial Institute -1640) media in the presence of glucose. The breast cancer cells overly express lectin receptor and show greater affinity towards glucose (G), mannose (M) and Fucose (F) as and when used as ligands (Garg et.al.2016).

1.3 Inflammation

Inflammation is the primary most and initial signs of disease (allergic responses, fever or injury) or any other tumorogenic response. The inflammation is mainly characterized by the cardinal signs which include redness (rubor), warmth (calor), pain (dolar), swelling (tumour) and altered function. The inflammation is mainly resulted due to an increase in the

vasodilation followed by the rise in the inflammation mediators. For example, histamine and mast cells are found to be actively circulating in blood.

Inflammation is categorized as acute and chronic inflammation. The inflammation is differentiated based on the time of manifestation, the time till they continue to mount upon in systemic circulation and the mediators responsible for evoking inflammation. The acute inflammation, a rapid and transient process lasting longer whereas the chronic inflammation is relatively slow, sustained and long-standing process. Acute inflammatory response mainly evolves with the injured tissue which mainly leads to the release of the chemokine signals followed by the activation of the inner endothelium lining and the nearby capillaries. The group of inflammatory receptors such as integrin and the select in present within the capillaries help binding to attract wandering neutrophils since they are adhesion molecules reside in activated endothelium. Once the neutrophils are attached to it, rate of their movement is dawdling down and start rolling, they further secrete the inflammatory chemicals which bind to the integrin. The neutrophils then squeeze through the spaces of the endothelium layer where they undergo transformation in the shape and size which enables reaching them to their target sites.

The inflammatory mediators playing an important role in the mounting inflammation are divided into two groups of inflammatory mediators; bradykinin and kallikerin. The mediators chiefly help in maintaining pH in microenvironment of injured tissue due to their acidic and basic nature, respectively. The mediators in association with one another excise long chain molecules trailing at the end of the chemokine signal, whereas other mediators increase the secretion of histamine and mast cells leading to dilation of the vessels. This allows the accumulation of inflammatory molecules like neutrophils, mast cells, macrophages and eventually leading to phagocytosis of the dead & damaged cells, or the pathogens.

The chronic inflammation is mainly mediated by the dense infiltration of the lymphocytes and the macrophages at the site of the injury or the tumour. However, when the macrophages enable to protect from skin damage which leads to granuloma formation. Moreover, chronic inflammation most of the time is independent of the acute inflammation.

1.3.1 Inflammation markers

The inflammation is one of the defence mechanisms in the tripe negative breast cancer which is characterized by the expression of diagnostic and prognostic markers. As and when they express themselves, it helps us measuring the efficacy of the drug. Reportedly, the most commonly expressed inflammatory markers are COX-2, MMP-1, TNF-α, IL-6, and IL-1β (Torsten O. Nielsen et.al.August 2009).

MMP-1 gene has been seen up-regulated in triple negative breast cancers. It is also inversely proportional to the survival of the patients in the advanced stages. The expression is also correlated with the expression of the P_{53} genes. MMPs are of proteolytic nature and are very efficient in degrading the extracellular membrane component (ECM*)*. The activity of this enzyme is mainly regulated by the tissue inhibitory MMP (TIMMP*)*. As they are capable of degrading ECM, they were correlated with degree of invasiveness in each cancer pathology

(Boström, Pia, et al. 2011). The up-regulation and over expression of molecular markers helps diagnose breast cancer. Also, they are the cause of short time relapse with a poor outcome in breast cancer treatment (Egeblad, Mikala, and Zena Werbet.al.2002).

Tumour associated macrophages (TAMS) are mainly associated with the cancer cell growth and proliferation.COX-2 is responsible for the appropriate functioning of TAMS which helps in the controlling growth and survival of breast cancer cells both *in-vitro* as well as *in-vivo*. This pathology is related to the increase in BCL-2 & P-gp, and decrease in BAX concentration and causes M2 phage polarization (Ramanathan, Suhashini, and Nithya Jagannathan 2014). All these events lead to the inhibition of PI3K/Akt receptor pathway when cell-cell interaction takes place. In addition, COX-2 is responsible for the secretion of prostaglandins which are one of the important contributors of inflammation (Thomadaki, Hellinida, and Andreas Scorilas et.al. 2008).

IL-6 mainly supports the spread of the ductal *in-situ* carcinoma (DCIS*)*. This gene is mainly having a paracrine mode of secretion between the DCIS and carcinoma associated fibroblast cells (CAF cells) which are group of subpopulation of the tumour cells. The interaction of the DCIS and the CAFs was mainly studied by the help of the MAME (mammary architecture and microenvironment engineering) model. The inhibition of paracrine and autocrine signalling led an increase in the level of proliferation and migration of the breast tumour which in turn forms multicellular structure. IL-6 signalling in the breast tumours helps in determining malignancy at an early stage. IL-6 is mainly a pro-inflammatory cytokine that helps in the mesenchymal transition and the modulation of tumour cells. This mainly occurs with the activation of the IL-6 receptors which then dimerizes with the universally expressed cell surface protein named glycoprotein 130 (gp-130). This is followed by the downstream activation of the JAK /STAT pathway leading to the whole of the autocrine and the paracrine loop of cell signalling (Cheng, George Z., et al 2008). This is also known as IL-6 trans-signalling pathway, and is allied with up regulation of cathepsins MMPs etc. associated with destruction of extra-cellular membrane component (Hodge, David R., Elaine M. Hurt, and William L. Farrar 2005).

TNF-α plays a crucial role in regulation of breast cancer pathway. TNF-α and IL-1β are well known inflammatory and pro-tumorogenic factors. The in vitro estimation of the expression of chemokines and cytokines was carried out through ELISA. The over-expression of these inflammatory markers reportedly affected other components of the cell microenvironment (Ben-Baruch, Adit et.al.2012). Their up regulation leads to a constant release of the CCL-2, CXCL-8 *and* CCL-5 factors. This lead to the prolong activation of the MSCs. The CAF cells which are derived from the close proximity of the breast tumour showed a heightened expression of all the above factors. CCL-2 enhanced the expression of the MSCSs which was mainly mediated by the help of the TNFR-I and TNFR-II receptors. Hence, inhibition of this factor helps in creating an anti-tumorogenic effect thereby inhibiting the occurrence of tumour. TNF-α is also associated with the relapse of the disease or the second hit precisely. (Ben-Baruch, Adit et.al.2012).

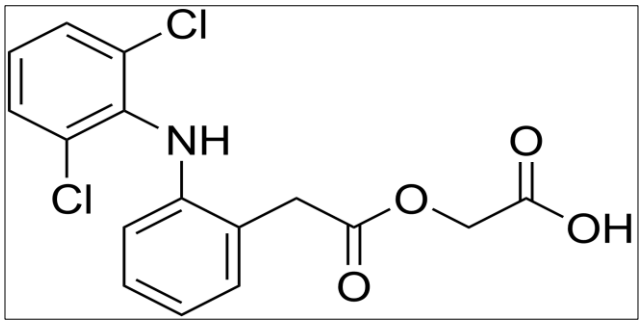
IL-1β plays a pivotal role in the cancer growth and migration. It is also known to enhance the proliferation and the progression of the breast cancer. It is the basic cytokine which is released upon injury, antigenic attacks or inflammation. This is mainly an inflammatory cytokine which acts directly on the cell inducing a fever response. This metabolism mainly takes place upon the activation of the inflammasome which are mainly recognised by the pathogen receptors or the tumour cells. Inflammasomes have been responsible for maintaining the carcinogenic and tumour microenvironment, inflammation, tumour homeostasis and progression. It is proteolytically activated by the help of inflammasomes before it can exert its tumorogenic effects. It is responsible for the excessive production of the prostaglandin which is one of the causative agents of inflammation. It also induces several genes responsible for inflammation like TGF-β and the vascular endothelial growth factor (VEGF) (Shangguan, Lei, et al 2012). All of them are responsible for the cell proliferation, angiogenesis and metastasis satisfying all the respective stages of the cancer hall marks.

1.3.2 Anti-inflammatory drugs

Anti-inflammatory drugs specifically subside the inflammation taking place in any part of the body system. The anti-inflammatory drug Methotrexate and Aceclofenac plays an instrumental role in reducing inflammation and pain. These drugs are primarily non-steroidal anti-inflammatory drugs (NSAIDs). These are the most widely accepted drugs having properties like analgesic, anti – pyretic and the anti-inflammatory characteristics. They play an important role in the chemotherapeutic treatment and reducing the pathology induced inflammation (Garg et.al. 2016)

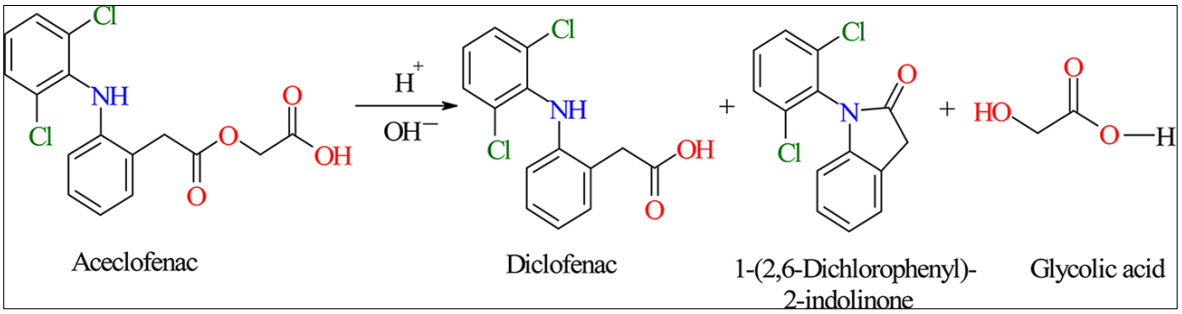
NSAIDs ACE (Aceclofenac) interfere with the production of prostaglandin, one of the major contributory inflammatory chemokines. Aceclofenac inhibits the release of prostaglandins by blocking the expression of the COX-1 and the COX-2 genes which are responsible for the release of the cyclooxygenase enzyme. This selective activity of the aceclofenac mainly takes place in the blood mononuclear and polymorph nuclear cells, respectively. The metabolism of drug mainly takes place in the hepatocytes CYPC29 pathway via the microsomes where they lead to the formation of the [2-(2',6'-dichloro-4'-hydroxyphenylamino) phenyl] acetoxyacetic acid (Bort, R., et al. (1996).

This metabolite is further conjugated and modified in the downstream processes. There is also the formation of minor metabolites [2-(2',6'-dichlorophenylamino)-5-hydroxyphenyl] acetoxyacetic acid and[2-(2',6'-dichlorophenylamino)phenyl]acetic acid, as well as the hydroxylated derivatives [2-(2',6'-dichloro-4'-hydroxyphenylamino)phenyl]acetic acid and [2-(2',6'-dichlorophenylamino)-hydroxyphenyl] acetic acid. These if not counteracted by the help of the diclofenac will continue to form the isomers of the cyclooxygenase (*isozymes).* They are bound to decrease the production of IL-6 (Gonzalez, E., et al.1994).



(Garg.et.al, *RSC Advances*, 2015)

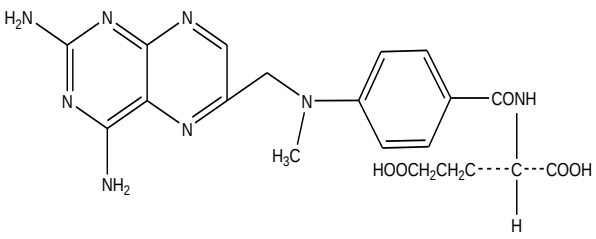
Figure 2 *C*hemical structure of aceclofenac which is popularly known for its anti-inflammatory property on the tumor cells. The chemical formulaof aceclofenac $C_{16}H_{13}Cl_2NO_4$



(Garg et.al *RSC Advances*, 2015)

Figure 3 The catabolic metabolism of aceclofenac which when into the systemic system is mainly converted into the secondary metabolites diclofenac and the hydroxyl and the acidic derivatives which largely contributes to inflammation

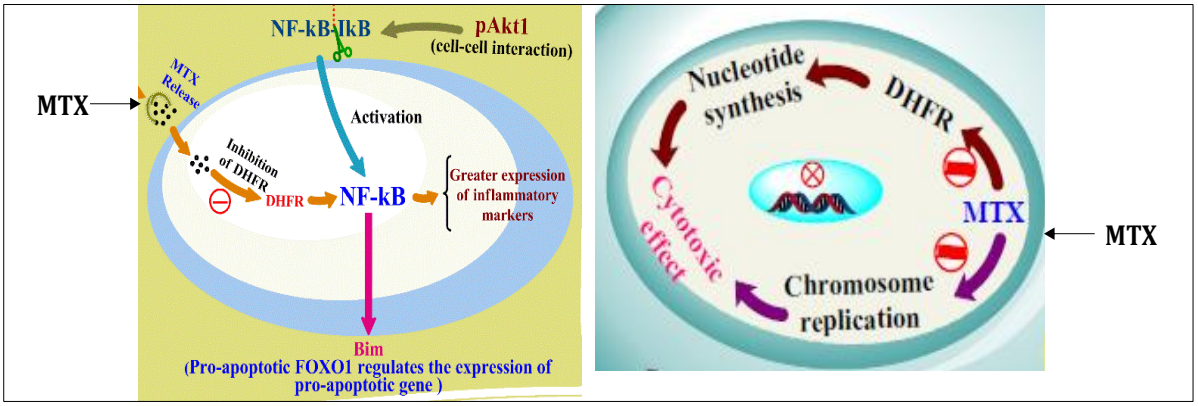
MTX (Methotrexate), folic acid and dihydrofolate reductase inhibitor (DHFR), inhibits *de-novo* DNA synthesis. MTX inhibits/block the conversion of dihydrofolate reductase into tetrahydrofolate reductase. This therefore blocks the replication of diseased DNA and reduces tumorogenic inflammation (Garg et.al.2015).



(Garg et.al.2015)

Figure 4 Chemical structure of methotrexate

The methotrexate ($C_{20}H_{22}N_8O_5$) mainly refers to the disease modifying anti rheumaticdrug (DMARD), and known as leading anti-inflammatory drug



(Garg and Tyagi *et al*, *IJP* 2015)

Figure 5 Metabolic activity of MTX

The figure represents the detailed mechanism of the methotrexate is mainly considered as the analogue of the DHFR thereby preventing its conversion into the THFR which confers the inhibition of DNA replication which tightly regulates the uncontrolled division of cells.

1.4 Tumour targeting- Novel Drug Delivery System (NDDS)

The targeted delivery via the nano carriers has drawn significant attention for their sustained and targeted drug delivery attribute. This sustained release of drug helps in obtaining the accurate results. The "bullet and the target concept of the drug delivery" are effective and targeted and controlled delivery of drugs and candidate vaccines (Tyagi et.al.*Vaccine* 2015).

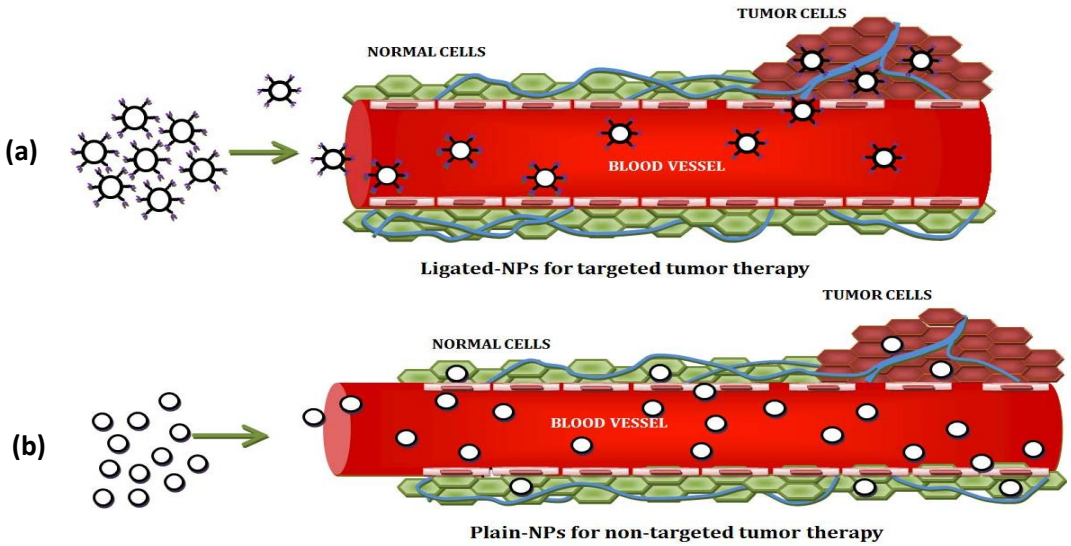
There are mainly two types of drug delivery system known as the active and the passive targeting. The delivery of the drugs by these formulations on the cells and the tissues is widely accepted and is also termed as the active targeting (Bertrand *et al.*, 2014). The active targeting mainly implies to the modified drug and the drug carrier molecule which is capable of recognising the target area such as organs and organ systems.

1.4.1 The target of tumorogenic inflammation

The constitution of human body mainly includes the cells, tissues, organs and the organ systems. Hence, the effect of the drug is also observed in the same sequence. The entire process of the drug distribution is divided into three orders which begin with the targeting the drug specifically acts on the organs followed by the distrubtion and traversal of delivered drug to the specific cell type and the eventually deep-seated tissues. Further, third order targeting refers to delivery in intracellular component of cells and tissues (Bertrand *et al.*, 2014; Brannon-Peppas and Blanchett, 2004).

1.5 Ligand conjugation/anchoring of drug loaded lipid-polymer hybrid nanoparticles (LPHNPs-lipobrids)

Lipid polymer hybrid nano particles (LPHNPs) have emerged as promising drug delivery systems. They have demonstrated to overcome the limitations of the liposomes and the solid lipid nano particles (SLNPs). LPHNPs reportedly address the issues such as low solubility, dose related toxicity, non-specific targeting, rapid diffusion throughout the body, short half-life in the blood stream and development of conventional drug resistance, and better meet the needs of translational research.



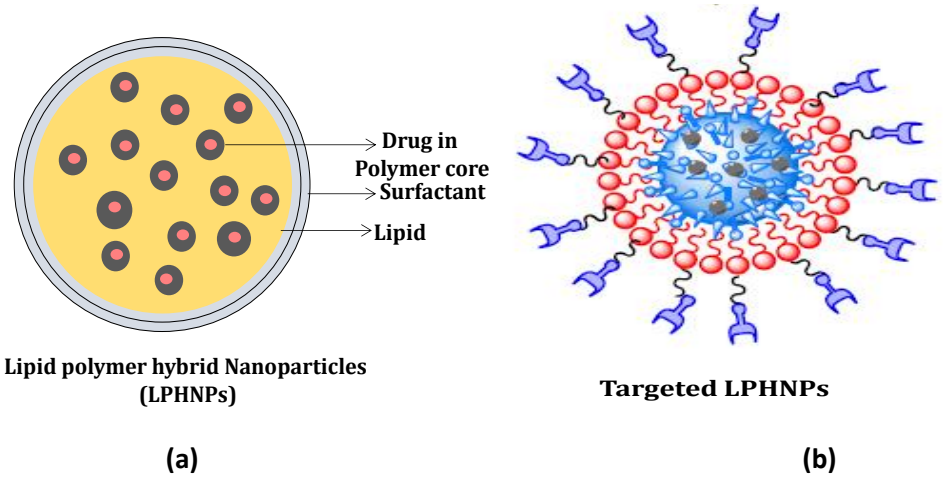
(Garg *et.al.* 2015)

Figure 6 Targeted and non-specific drug delivery (a) and (b) shows the difference in the amount of released entrapped and un-entrapped drug. When the drug is the free there is no chance of the sustained release as a result of which there is a sudden burst (oxidative burst) of the drug and non-specific release leading to the formation of free radicals and resulting into greater cell deaths.

The ligand anchored nano-carriers deliver the drug in targeted and sustained manner with the minimal possibility of formation of reactive radicles leading to the death.

1.5.1 Structure of lipobrid

Lipid polymer hybrid nano-particle is a multi-layered nano carrier which is made up of the outermost layer of the ligand attached to the second layer of the lipid. Between the first and second layer, the available surfactant helps in maintaining the integrity and the rendering stability to structure. The innermost layer containing the polymer in which the drug is entrapped significantly. The outermost lipid layer is highly hydrophobic and the inner polymeric layer is hydrophilic in nature making the mamphipathic in nature. Therefore, they conductively imitate the cell membrane "fluid mosaic model".



(Garg et.al *nanomedicine* 2015)

Figure 7 Structure of lipobrid a) illustrates the basic design of the LPHNPs which are formulated by polymer and lipid for greater drug entrapment (b) More descriptive explanation of (a) where in the lipid polymerhybrid nanoparticles is multi-layered the outer most layer being the lipid which covers the polymeric surface and further the drug being entrapped in the polymeric core. The fucose ligands present on the lipid surface are responsible for targeted delivery.

The lipid layer present in the nano structure mainly adorns the function of the biocompatible shield and also the barrier to prevent the leakage of the hydrophilic and water soluble drug from the carrier. The ligands (fucose, galactose and mannose) attached on the lipid surface matrix mainly helps in the targeted dissemination of the drugs from the carrier as they mainly interact with the pre-identified and the highly expressive lectin receptors present on the surface of the cells(Garg *et al.*, 2015; Jain *et al.*, 2010; Jain *et al.*, 2015b).

1.5.2 Advantages of the lipobrids over other nano-carriers

- ✓ There are reduced adverse effects as there are hardly any peak of drug blood levels exceeding the drug therapeutic range and falls in the acceptable range of toxicity.
- ✓ Targeted drug delivery system (TDDS) confers a reduction in drug blood level fluctuation. TDDS provides control over the sustained release.
- ✓ Enhanced patient compliance.
- ✓ Reduced dosing frequency and dosage
- ✓ Cost-effective
- ✓ The extended duration of action

2. MATERIAL AND METHODS

2.1 Preparation of lipid hybrid nanoparticles (LPHNPs)

Materials: Methotrexate, Aceclofenac, Phospholipids s-100, Casein, Zein were gifted by Dr. Neeraj Garg, UPIS, Punjab University. DMF is received as a gift from Institute of Pharmacy, Nirma University and PERD research center, Ahmedabad. DCM ordered from SRL Diagnostics.

2.1.1.Formulation of LPHNPs

LPHNPs were prepared by single step nano-precipitation method in which all the components were heated separately and mixed on magnetic stirrer. MTX (10 mg), polymer (Casein or Zein) were dissolved in 1 mL of DMF and heated at 60-70°C. Phospholipids S100 (10 mg) and Stearylamine (10 % mol ratio of PL) was dissolved in 1 mL ratio of DCM: DMF followed by heated at 60-70°C. Both the heated solutions were mixed together and stirred continuously for 10 min. These solutions were added drop by drop into 20 mL of surfactant solution of colliform at a constant flow rate of 1 mL/min. The solution was stirred on magnetic stirrer at 800 rpm for 2-3 h. The organic phase was then removed by dialysis (MWCO 10 KDa) against double distilled water for 12hr (Garg *et al*; 2015).

This illustrates the two types of formulations made from the two different polymers Casein and Zein, contains different concentrations of the lipid and the drug entrapped in it which is shown in Table 1.

Table 1: Preparation of different formulations based on various polymer

Control	Polymer	Drug	PLS-100	Stearyl amine	Fucose
1.	Casein (10mg)	Lipobrid without drug	10mg	2mg	2mg
		MTX + Lipobrid	10mg	2mg	2mg
		ACL+ Lipobrid	10mg	2mg	2mg
		MTX+ACL+Lipobrid	10mg	2mg	2mg
2.	Zein (50mg)	Lipobrid without drug	10mg	2mg	2mg
		MTX + Lipobrid	10mg	2mg	2mg
		ACL+ Lipobrid	10mg	2mg	2mg
		MTX+ACL+Lipobrid	10mg	2mg	2mg

2.1.2. Preparation of ligand anchored lipobrid

Ligand conjugated lipobrids were prepared by post insertion method as reported elsewhere (Garg *et al*; 2015) by incubating the suspension of lipobrid with equal molar ratio (equivalent to Stearyl amine present in Lipobrids) of activated fucose. This method facilitates activation

of ligand(Fucose) by ring opening at acidic pH (4.0) at 0.1 M sodium acetate buffer (pH 4.0) followed by the reaction of aldehyde group of activated ligand with free amino groups present at the outward of prepared lipobrid. The mixture was allowed to continuously agitate on a magnetic stirrer maintained at an ambient temperature for 48-72 h to ensure completion of the reaction. Ligand-anchored lipobrids were subjected to extensive dialysis against double distilled water in a dialysis tube (dialysis bag; MWCO 12 kDa for 1 h) to remove free ligand and other impurities (Garg *et al*; 2015)

2.2 Characterization of ligand-anchored lipobrids

MTX-lipobrids were characterized with respect to size, PDI, zeta potential, scanning electron microscope (SEM), percentage drug entrapment (PDE) and percentage drug loading (PDL). Size, zeta potential and PDI of NPs was determined by Zeitasizer (ILS, Ahmedabad University and CUG Gandhinagar). Also *in-vitro* release and stability studies of lipobrids were carried out.

2.2.1. Standard calibration curve of MTX and ACE

Materials: MTX, ACE, DMF, Quartz cuvette, UV spectrophotometer

The stock solutions were prepared by 1mg/mL concentration in DMF. From the stock solution a series of dilutions of different concentration ranging from 10 to 100 µg/mLwere prepared and OD were measured by UV spectrophotometer. The lambda max was found to be 275nm and 300nm for ACE and MTX respectively.

2.2.2. Drug encapsulation and drug loading efficiency

Materials: MTX loaded lipobrid, ACE loaded lipobrid, Dialysis bag, UV spectrophotometer

After formulation of lipobrid, the lipobrid were subjected to dialysis using 10 kDa for 12 hour dialysis membrane against double distilled water. The absorbance unentrapped drug was measured using spectrophotometer at 275 nm for ACE and 300 nm for MTX.

Formula for calculating drug entrapment and drug loading efficiency:

$$\% \text{ Drug Entrapment} = \frac{(\text{weight of Total Drug} - \text{weight of free drug}) \times 100}{\text{Total Drug}}$$

$$\% \text{ Drug loading} = \frac{(\text{weight of Total Drug} - \text{weight of free drug}) \times 100}{\text{Weight of lipid polymer}}$$

2.3 Cultivation of of breast cancer cells (MDA-MB-231 & MCF-7)

Cells were obtained by National Centre for Cell Science (NCCS), Pune. They were cultivated and maintained by practicing standard guidelines available through ATCC (American Type

of Cell Culture), USA. Cells were used for the estimation of inflammatory cytokines such as IL-6, IL1-β, MMP-1, and COX-2. It was also used for *in-vitro* drug release assay. Cells were cultured as a monolayer in RPMI complete medium containing 10% FBS, 2 mM L-Glutamine, 5% antibiotic solution. The cells were maintained in culture flask at 37˚C in an incubator with 5% CO_2.

2.3.1. Revival (Thawing) and sub-culturing

Materials: Micropipettes, 70% iso-propanol, 0.25% (w/v) trypsin EDTA, Complete growth medium (RPMI, 10% FBS(v/v) along with 5% antibiotic solution) after filter through 0.22 µm sterile filter, water bath, BSL II laminar air flow chamber, cooling centrifuge, CO_2 incubator.

2.3.1.1. Method of Revival

Cryopreserved vial containing cells were taken from -80˚C storage. Complete media were kept ready in T-25 flask. Once the vial is taken from -80˚C, it directly transferred in to 37˚C water bath. Vial content is transferred in to 15 mL Centrifuge tube and centrifuge it at 3,000 rpm for 5 minutes. After centrifugation, remove the supernatant and re-suspended the pellet in 1 mL of media and transferred the media into flask containing 4 ml of complete media. Incubate the flask at 37^oC CO_2 incubator, and cells were observed routinely under inverted microscope (10X, 20X) to ensure the confluency, growth and contamination of cells.

2.3.1.2. Method of sub-culturing

The cells were sub-cultured when reached 90-95% confluency. For sub-culturing, 0.25% trypsin- EDTA solution were added and incubated at 37˚C for 1-3 minutes to detach the cells from the surface of the flask. Cells were observed under microscope for complete detachment of the cells. The complete media containing serum was added to inhibit the effect of trypsin. Transfer all the materials to the 15 mL centrifuge tube and centrifuge for 5 minutes at 3,000 rpm. The supernatant was decanted off, and pellet was then re-suspended in 1 mL of growth medium. The cells were counted by haemocytometer.

2.3.2. Cell count and viability

Materials: sterile eppendrorftube, trypan blue stain, haemocytometer

The tube containing 1 mL of cell suspension, 10 µL of suspension was transferred to eppendorf (autoclaved) and mixed it with 10 µL of trypan blue. 10 µl of suspension was taken from the mixture was placed onto haemocytometer, and cells were counted using the following equation:

Formula for cell count

$$\text{Cell count} = (\text{total cell count } /4) \times \text{dilution factor} \times 10^4 \text{ Cells/mL}$$

Remaining cells suspension was transferred to new flask containing appropriate amount of fresh complete growth medium and incubate it in CO_2 incubator.

2.3.3. Cryopreservation

Materials: DMSO, cryopreserve medium (10% DMSO+90% complete growth medium), Cryogenicvials

Once cells reached to 80% confluency, it was trypsinised and cell suspension was centrifuged, and pellet was re-suspended in 1 ml cryopreserved medium and transferred to cryogenic vial. The cryogenic vial containing cells were stored at -80˚C.

2.3.4 Drug sensitive assay (MTT assay)

Materials: Flat bottom 96 well plates, cell suspension, ACE, MTX, PBS, Absolute ethanol, MTT solution, DMSO and RPMI-1640.

Day 1: For cell viability, cell suspension was incubated over-night for cell attachment in 96 well tissue culture plates (~1×10^4 cells/well).

Day 2: The growth medium was then replaced with complete medium (0.2 mL) containing the free MTX or MTX-lipobrid to the different wells so as to achieve net concentrations of 0, 0.1, 1.0, 10 and 20 µg/mL (equivalent to free MTX) for 24, 48 and 72 h.

Day 3: Cells were then washed with PBS and again incubated. 150 µL of MTT solution (0.5 mg/mL in PBS) was added to each well and re-incubated for 4 h to facilitate the formation of formazan crystals. The excess solution was then aspirated carefully, and MTT formazain crystals were dissolved in 200 µL of DMSO. The optical density of 24 hour plate was read at 550 nm by the ELISA plate reader to determine the cell viability

Day 4: Repeat the same procedure as day 3 and read at 550 nm by the ELISA plate reader to determine the cell viability

Day 5: Follow the procedure of day 3 and read at 550 nm by the ELISA plate reader to determine the cell viability

2.4 *In-vitro* analysis of markers

2.4.1 RNA isolation

Once treated with drugs, remove the medium from cells and rinse the cells with PBS. Detach the cells with the help of trypsinization. Transfer the cells into centrifuged tube and centrifuge for 5 mins at 250Xg, discard the supernatant. Resuspend the cells in 600µl of lysis buffer supplemented with β-mercaptoethanol. Add 450µl of ethanol (96-100%) and mix by pipetting. After then transfer up to 700µl of lysate to the GeneJET RNA purification column

(supplied with kit) inserted in a collection tube and centrifuge the column for 1min at 12000g and discard the flow through the and place the purification column back into the collection tube. Then add 700µl of wash buffer 1 to the column and centrifuge for 1min at 12000g. After that, add 600 µl of wash buffer 2 to the column and centrifuge for 1min at 12000g followed by addition of same wash buffer 2(250µl) added in column and centrifuge for 2min at 12000g. More than 90% of RNA is eluted during 1st step. For maximum RNA yield, repeat the elution step. For maximum RNA yield, repeat the elution step with an additional 100µl of nuclease free water. This RNA will be used for analysis of gene expression. After RNA isolation, it is quantified with help of 1.5% gel electrophoresis.

2.4.2 cDNA synthesis

Once RNA is isolated, synthesis of cDNA will be done (work in progress) with the help of Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. As per the protocol given in the the kit, in a nuclease free tube add 0.1µg-5µg of total RNA and 1µl of primer. Make total volume 12 µl with the help of nuclease free water. Followed by addition of 4 µl of 5X reaction buffer, 4 µl RiboLock RNase Inhibitor, 1 µL 10 mM dNTP Mix and 1µl reverse transcriptase. Mix gently and centrifuge the tubes. For oligo (DT) 18 or gene-specific primed cDNA synthesis, incubate for 60 min at 42°C. For random hexamer primed synthesis, incubate for 5 min at 25°C followed by 60 min at 42°C. Terminate the reaction by heating at 70°C for 5 min. The reverse transcription reaction product can be directly used in PCR applications or stored at -20°C for less than one week. For prolonged period of time storage at -70°C is more preferable.

2.4.3 Polymerase Chain Reaction (PCR) amplification of cDNA

After cDNA is synthesized, it is used for polymerase chain reaction. The reagents such as 12.5 µl of PCR master mixed, 5.5 µl of nuclease free water, 2 µl of forward primer and reverse primer (individually) and 3 µl of template. Each mixture should be of 25 µl. After, the mixture is kept for PCR at respected temperature of the markers, the result can be interpreted for the study the gene expression pattern. Once gene is amplified with the help of PCR, the product is quantified by 2% agarose gel.

3. RESULTS AND DISCUSSIONS

3.1 Preparation of lipobrid

The present study reports a single step self-assembled nano precipitation method of MTX and ACE encapsulated lipid polymer hybrid nanoparticles (LPHNPs) as reported elsehwere (Garg et.al 2015).Lipid polymer hybrid nanoparticles (LPHNPs) were prepared by hydrophobic polymeric core. All materials employed in the formulation of nanoparticles were clinically safe and biodegradable. The drug and polymers were encapsulated and another lipid and surfactant were added to establishing shell around the polymeric core.

LPHNPs formulations were viewed under compound microscope as spherical vesicles were observed. In the first attempt to formulate the Casein and the Zein polymer, by standardizing the entire procedure, we found quite a good density of the lipobrids which were observedunder 100X (Oil immersion) magnification (Fig 8). The size of the lipobrids did not appear regular, however. The compound microscope gives us the basic information about the distribution and the density of the formulation.

A) Casein formulated **B) Zein formulated LPHNPs**

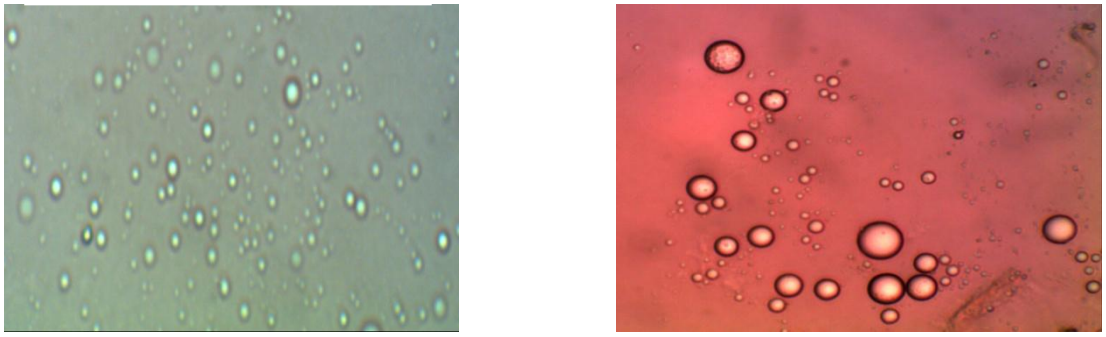
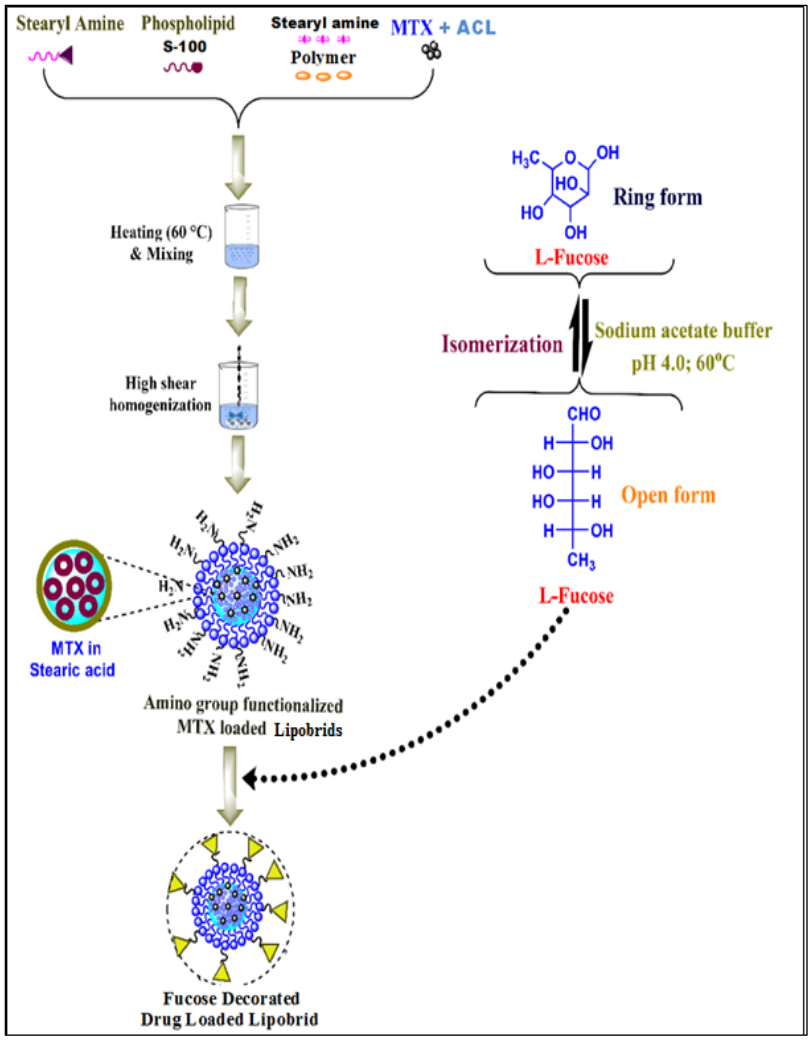


Figure 8: Microscopic image of lipobrid. The images showthe microscopic view of lipobrids in 100-X magnification ofa) casein and b) Zein. The formulated lipobrids in both the images show significant nmber.



(Garg et.al,*Colloids and surfaces B: Biointerfaces* 2016)

Figure 9 Schema of the preparation of ligand anchored lipobrid

The image illustrates the steps for formulation of drug loaded lipobrid. Here loaded polymeric nanoparticles are mixed with lipid and Stearyl amine. Stearyl amine provides free $–NH_2$ group. Once LPHNPs is prepared with help of constant steering, this formulation is subjected to ligand anchoring. Ring opening of sugar is carried out at acidic pH. After the ring opening of sugar free aldehyde group of sugar reacts with free $–NH_2$. This process leads to the formation of Schiff's base (-N=CH-) which gets reduced to secondary amine (-NH-CH_2-) and accomplishes the state of equilibrium with Schiff's base(Garg et.al, 2016)

3.2 Characterization of lipobrid

3.2.1 Characterization of casein-LPHNPs

The Particle size of whole formulation was measured using Zeta- sizer (Malvern, Nano ZS).

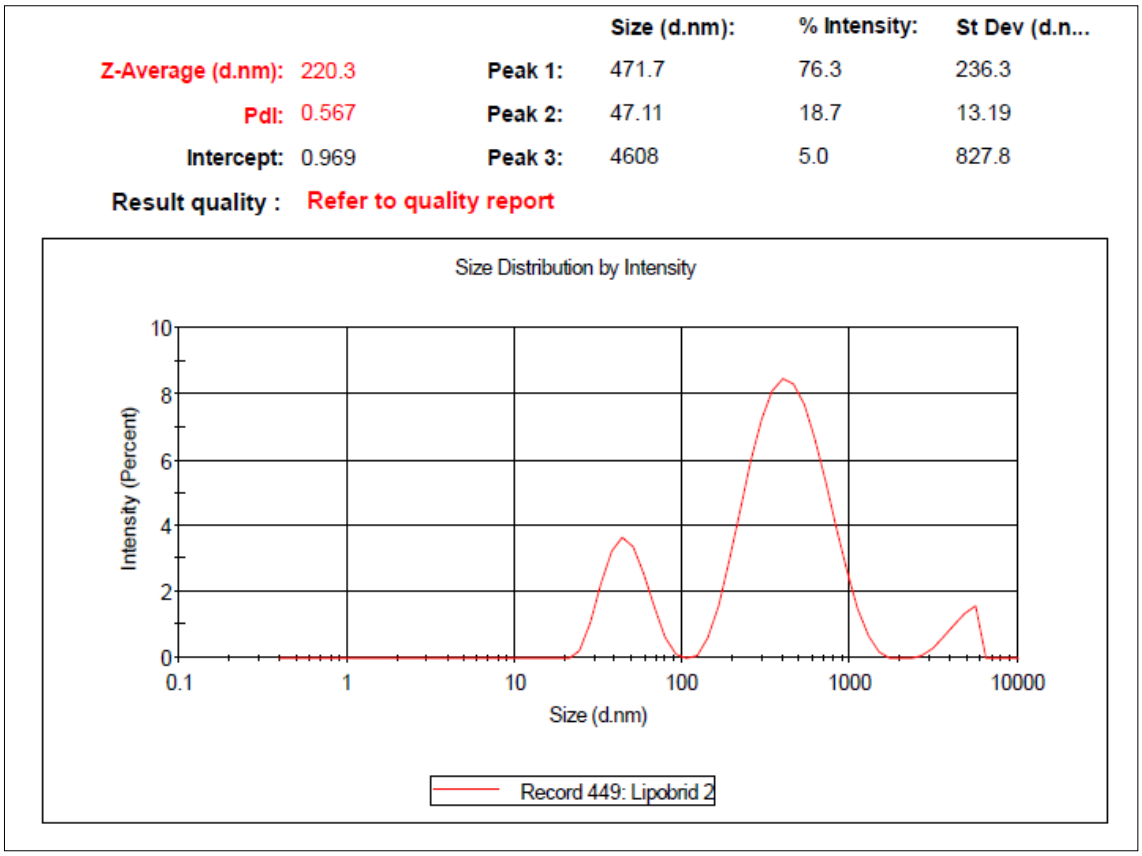


Figure 10 Particle size distribution of lipobrid. Three obvious peaks with all of them in two extremes of the range >200 nm.

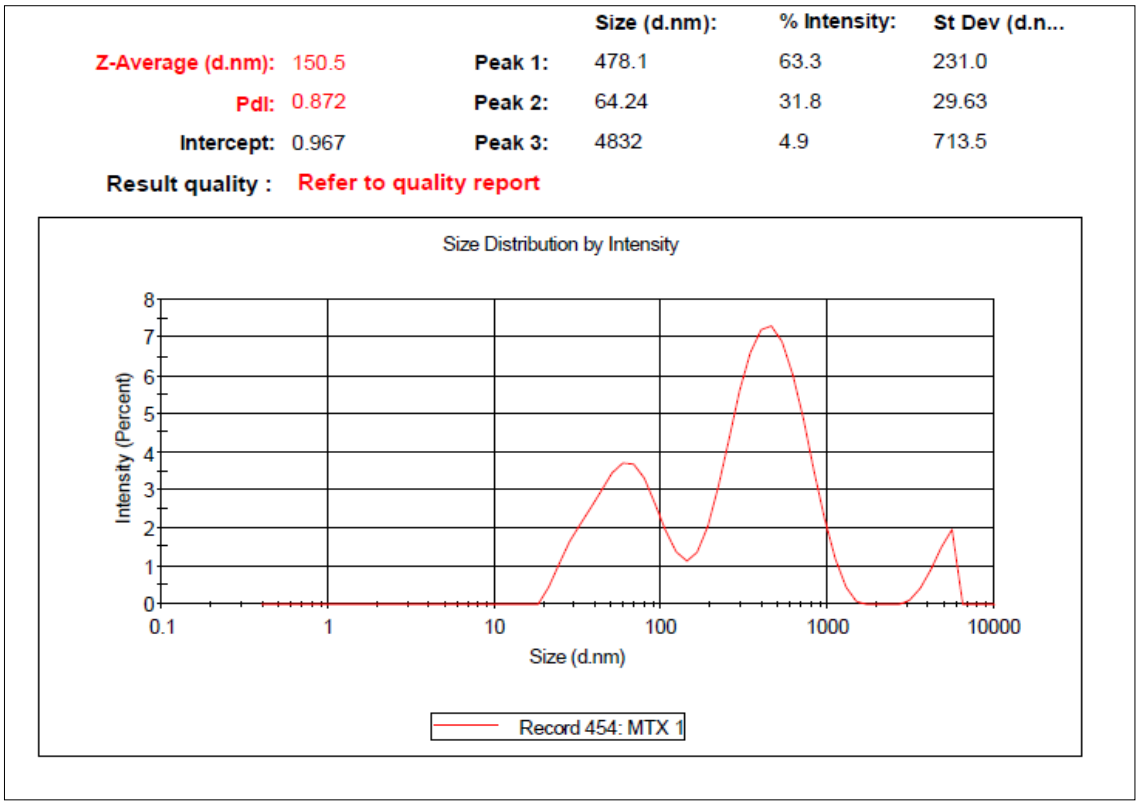


Figure 11 Particle size distribution of MTX. The three clear peaks with all other in two extremes of the range >200 nm.

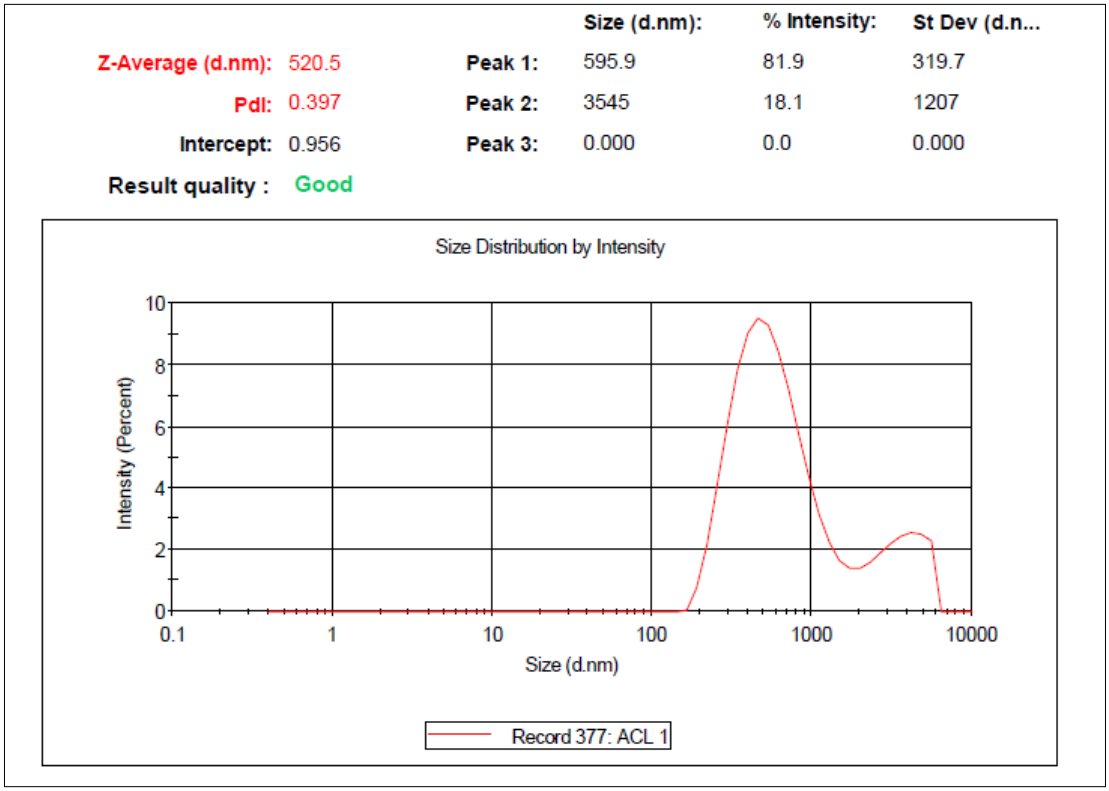


Figure 12 Particle size distribution of the ACE found at more than 200 nm.

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 668.7	Peak 1:	835.0	83.5	580.9
PdI: 0.485	Peak 2:	4219	16.5	985.6
Intercept: 0.955	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

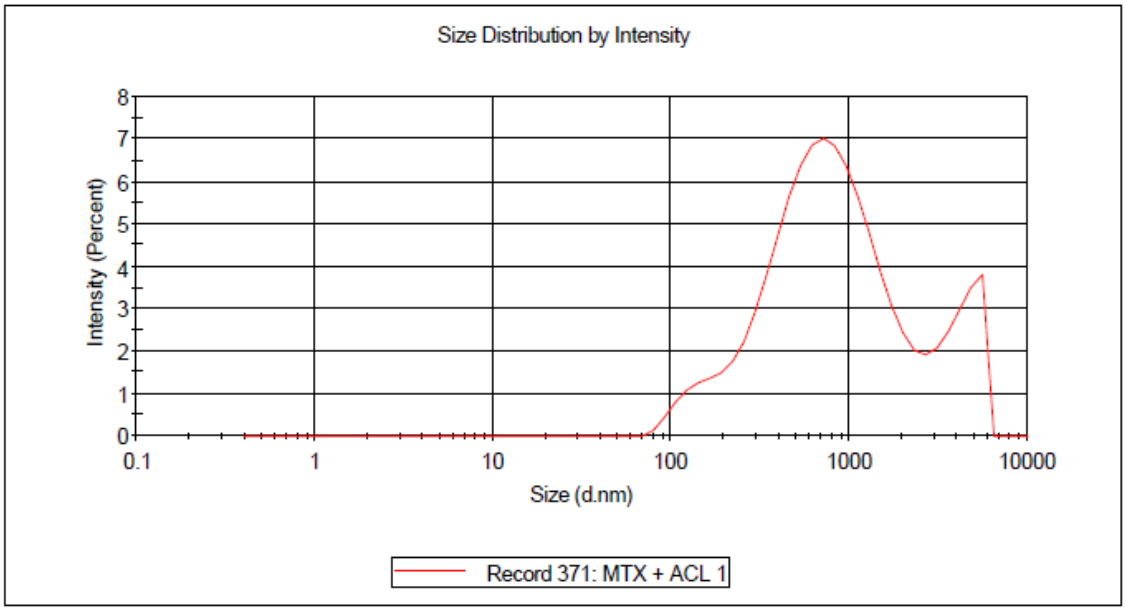


Figure 13 Particle size distribution of MTX & ACE. Two clear peaks with all of them in two extremes of the range >200 nm

Characterization of the preapred lipobrid formulation is one of the important steps providing an insight on the use and efficacy of lipobrid. The LPHNPs were characterized to determine the stability of lipobrid and interaction of the nano particles in the blood stream, which, in turn, confer sustained drug release attribute.

The formulated casein nanoparticles were characterized with respect to size through zeta sizer, and results were obtained between 5,00-2,000 nm.The greater size of nanoparticles might lead to the higher cell toxicity. These formulations were seen to show different size peak, with different density which could not fulfil the basic criteria of characterization of nanoparticles.

3.2.2 Characterization of zein LPHNPs

The Zeta-potential, particle size and Polydispersity index (PDI) of whole formulation was measured using Zeta-Sizer (Malvern, Nano ZS).

The parameters such as particle size and polydispersity index of all Zein formulations are presented in Table 2

Table 2 Particlesize and polydispersity index (PDI) of different formulation

No.	Name of formulation	Zeta size (nm)	Polydispersity index
1.	Lipobrid	119.3	-
2.	MTX loaded lipobrid	940	0.372
3.	ACE loaded lipobrid	158.1	0.272
4.	MTX and ACE loaded lipobrid	615.6	0.372 and 0.272

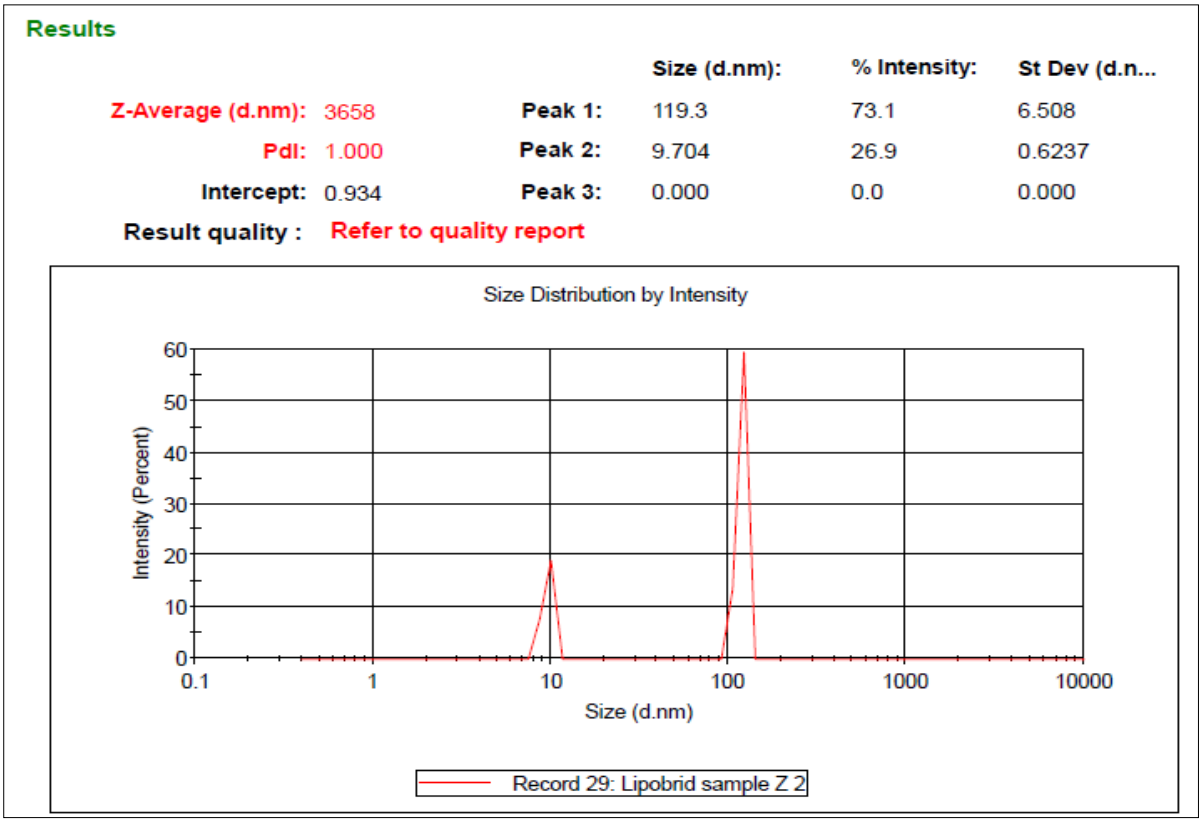


Figure 14 Particle size distribution of the lipobrid showing two peaks within the range of 200 nm.

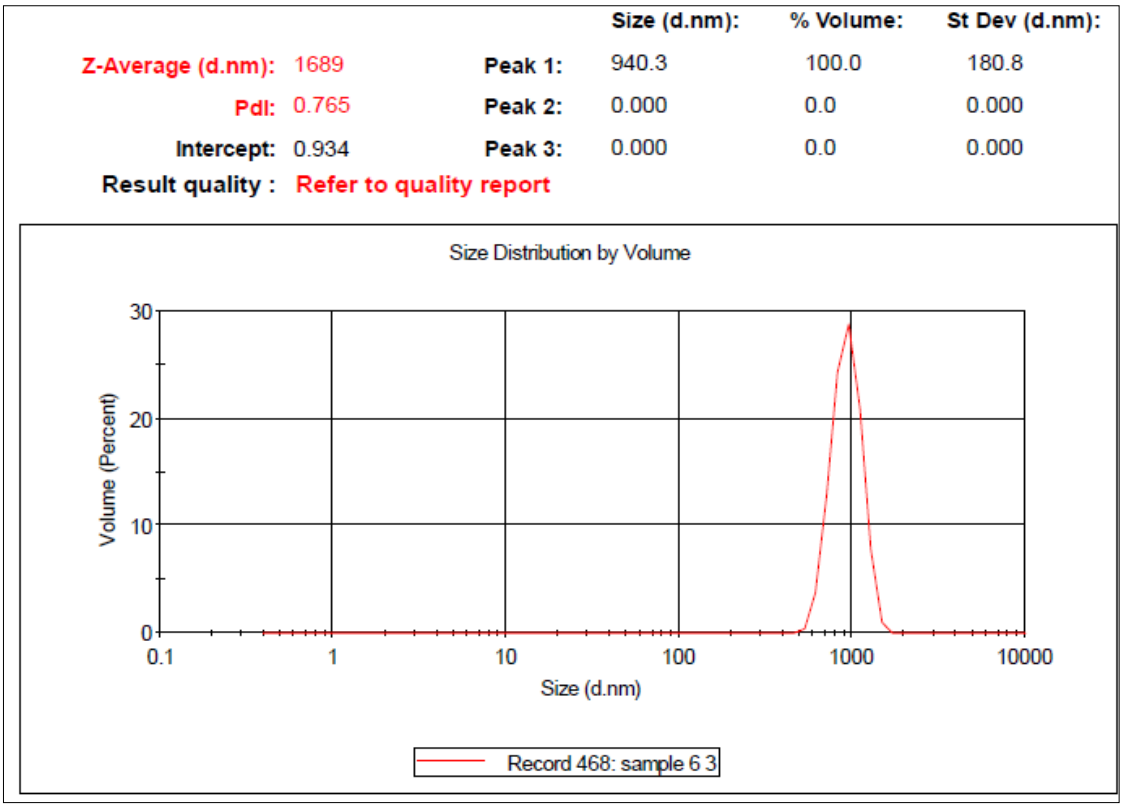


Figure 15 The graph illustrates distribution of the particle size of MTX showing the single Peak exceeding the range of 200 nm.

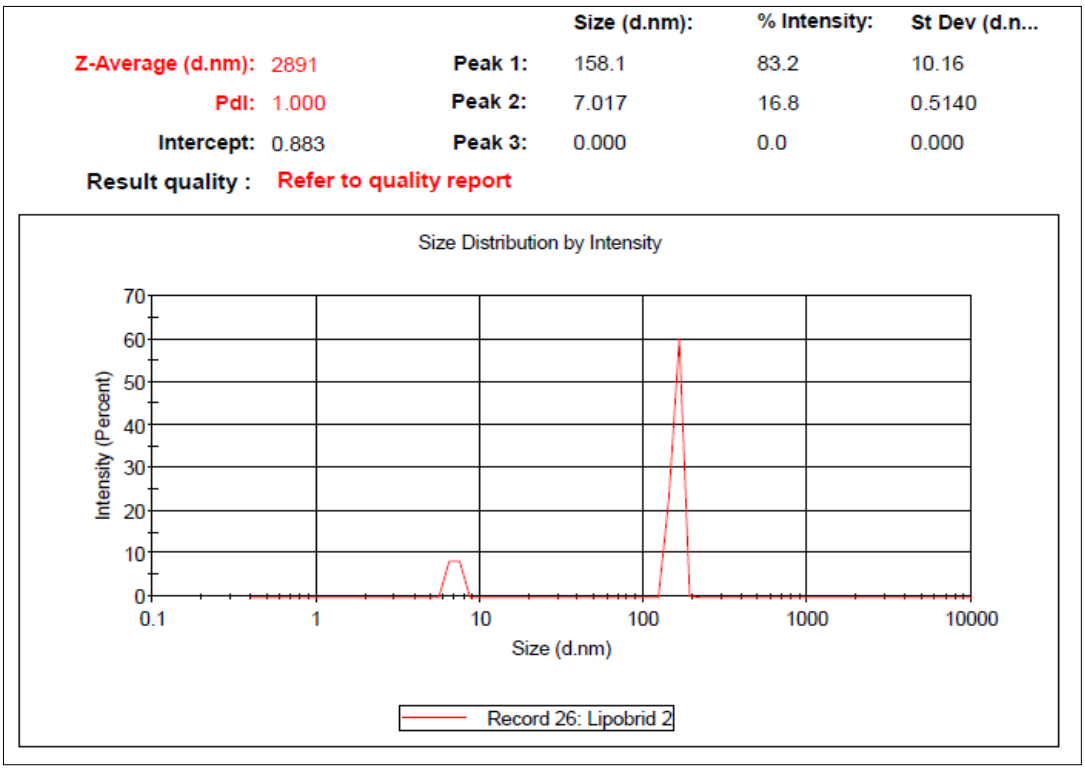


Figure 16 The graph depicts the particle distribution size of ACL in which two peaks were obtained. The first peak was seen in the range of 200 nm

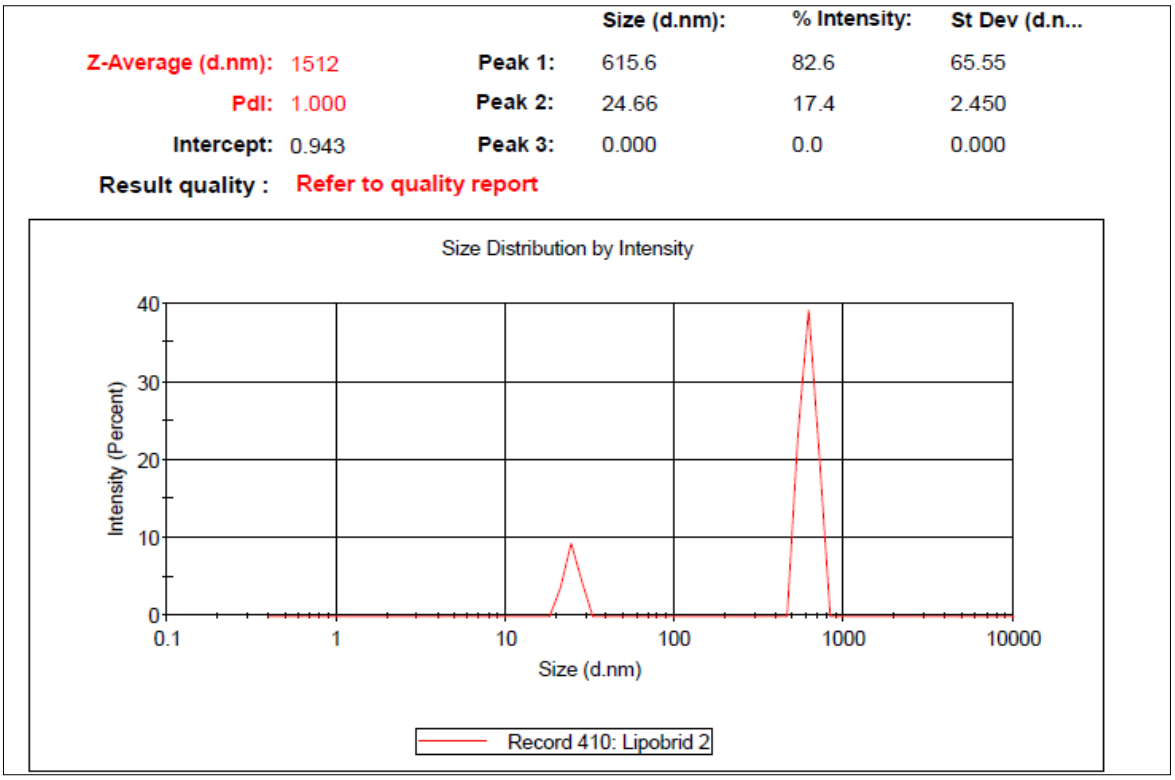


Figure 17 Particle size distribution of the MTX &ACE was seen at more than 200 nm

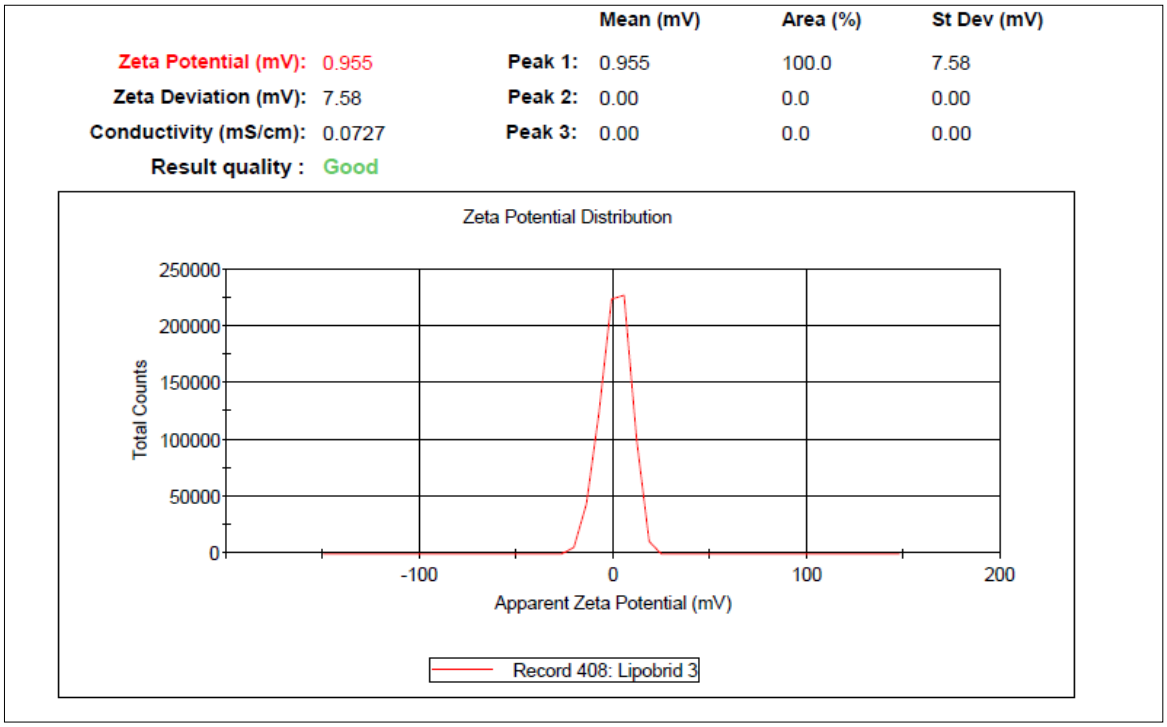


Figure 18 The graph illustrates the zeta potential of lipobrid loaded with MTX &ACE. The zeta potential gives a single peak and the potential is within the acceptable region

The smaller particle size helps the nanoparticles to escape the immune system and especially the wandering macrophages, and allowing to enhance the their ability and stability in systemic circulation.

The ideal size of the nano particles are suggested to fall with a range of <200 nm (Garg et.al. 2012). As we are the first group who had formulated (as per our best knowledge) the Zein nanoparticles but encountered difficulty in maintaining uniformity of particle size. We therefore interpret that size of only Zein lipobrid and ACE loaded Zein lipobrid is within the range of <200 nm, whereby we assumedthe said formulations to have considerable efficacy.

Whereas the MTX loaded lipobrid and the MTX+ACE loaded lipobrid is having size more than the acceptable range of 200 nm seen in entrapped MTX drug.

3.2.3 Scanning Electron Microscopy (SEM) of the formulation

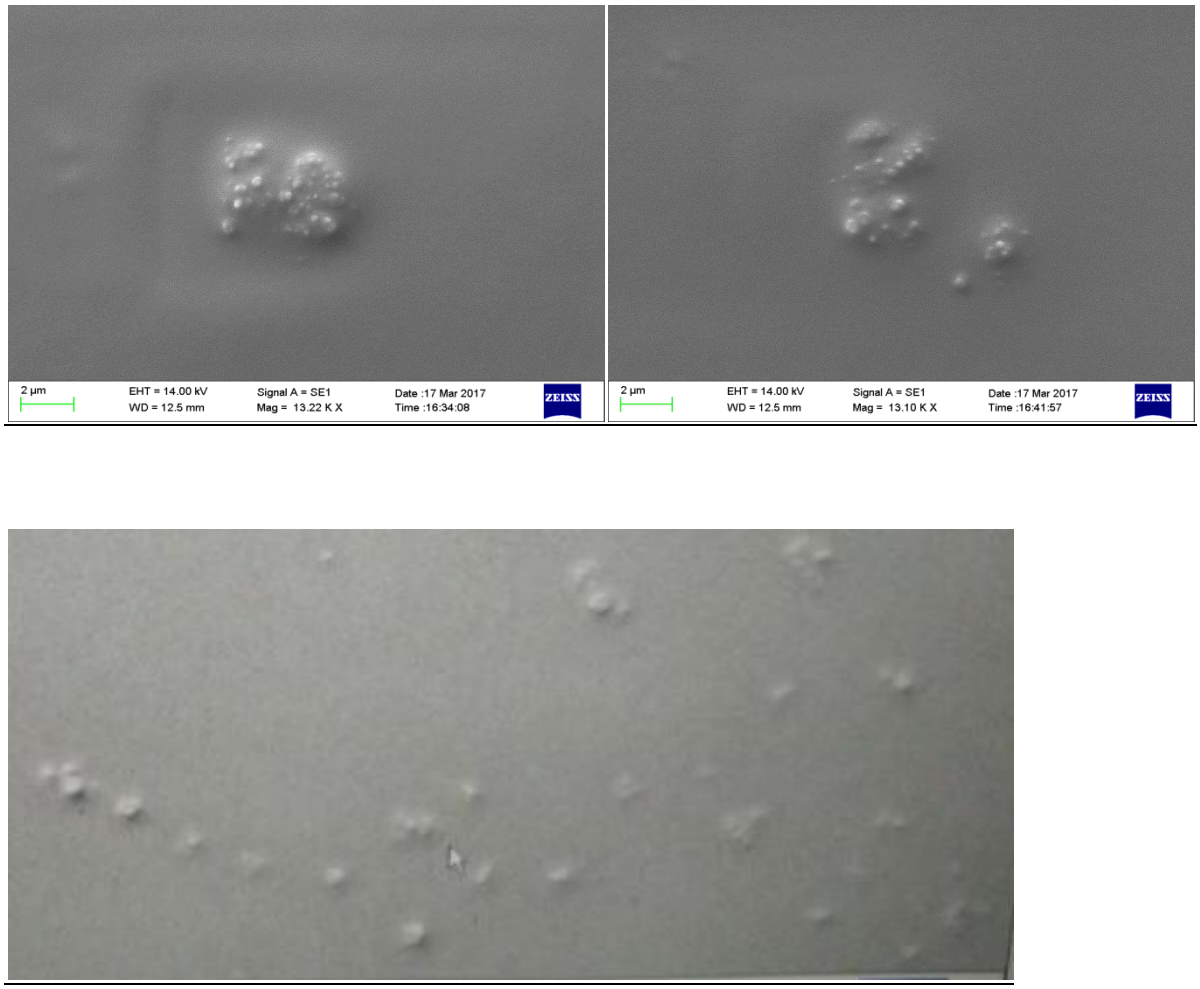


Figure 19 Scanning Electron Microscopy determined the surface morphology of lipobrid formulation.

3.2.4 Standard calibration curve of drugs

Different solubility tests were carried out for both drugs. The MTX and ACE both were dissolved in DMF (Dimethyl Formamide). The standard curve for both drugs was constructed for the estimation of drug entrapment efficiency, and chart shows the regression value 0.990 for MTX and 0.993 for ACE.

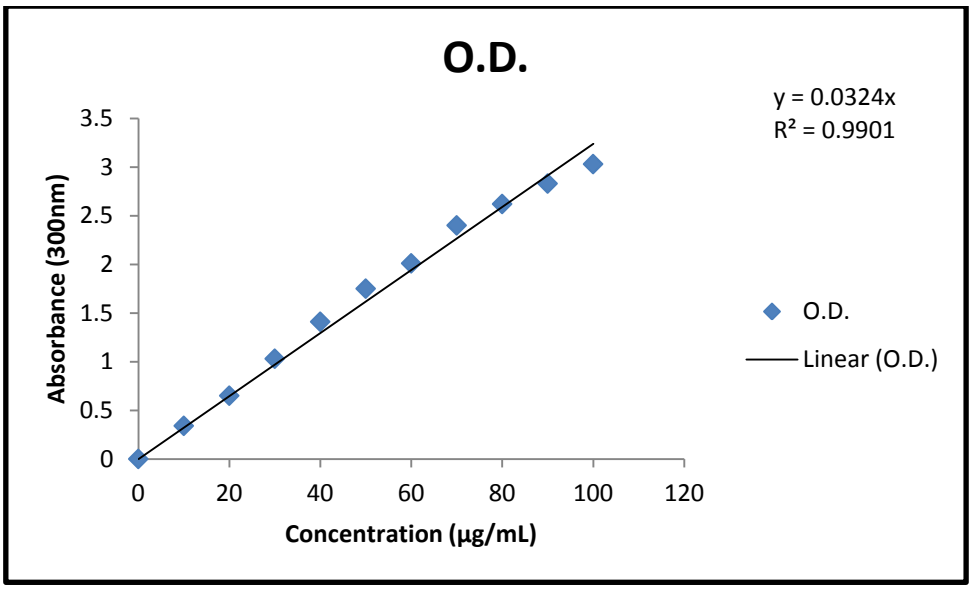


Figure 20 Standard calibration curve of MTX at 300 nm

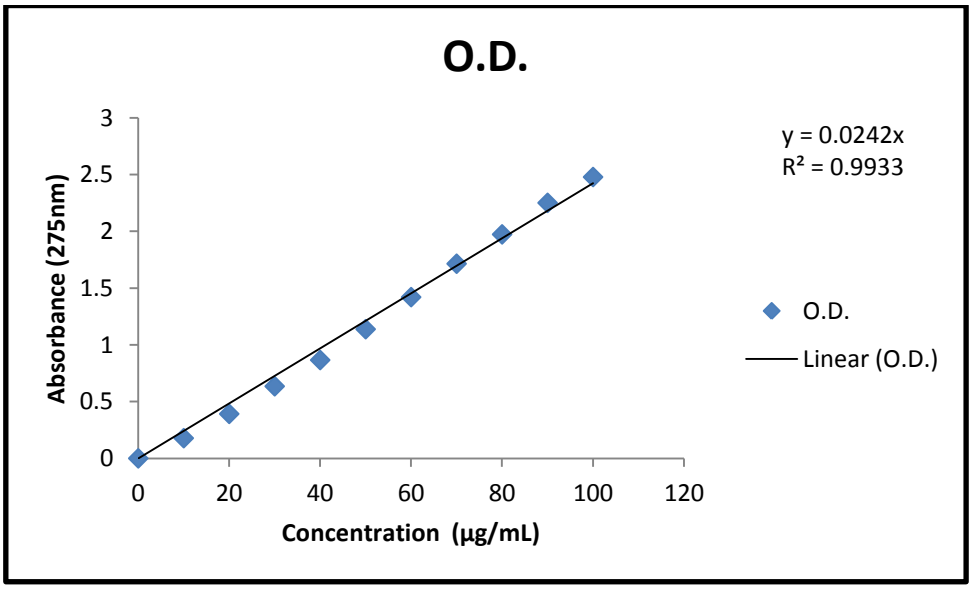


Figure 21 Standard calibration curve of ACL at 275 nm

3.2.5 Drug entrapment & Drug loading efficiency

The **drug (MTX and ACE) entrapment & drug loading efficiency** for Zein formulated lipobrid is presented in Table 3.

Table 3: Entrapment efficiency and drug loaded efficiency of zein-LPHNPs

Sr. No.	Formulation	Drug Entrapment efficiency	Drug loading efficiency
1	Lipobrid only	-	-
2	MTX loaded lipobrid	85%	17%
3	ACE loaded lipobrid	89.6%	17.96%
4	MTX &ACE loaded lipobrid	94%	18.8%

The formulation of lipobrid second most important factor that is to be considered is the amount of the drug loading and drug entrapment efficiency. A nanoparticles with a substantially higher entrapment efficiency than drug loading efficiency is considered as an ideal nano particle formulation. The entrapment and drug loading efficiency of MTX+ACE loaded lipobrid was shown to have better entrapment efficiency than loading efficiency. Whereas MTX and ACE loaded lipobrid had entrapment efficiency within the range of 85-89% than the drug loading efficiency.

3.3. Cell line studies (MDA-MB-231)

Cells (MDA-MB-231) were adherent mammalian epithelial breast cancer cells. All the steps involving cell line maintenance were carried out in accordance with ATCC guidelines. The cells at the low density 20-30% cell population and confluency levels of 80-90% showed cytoplasmic projection.

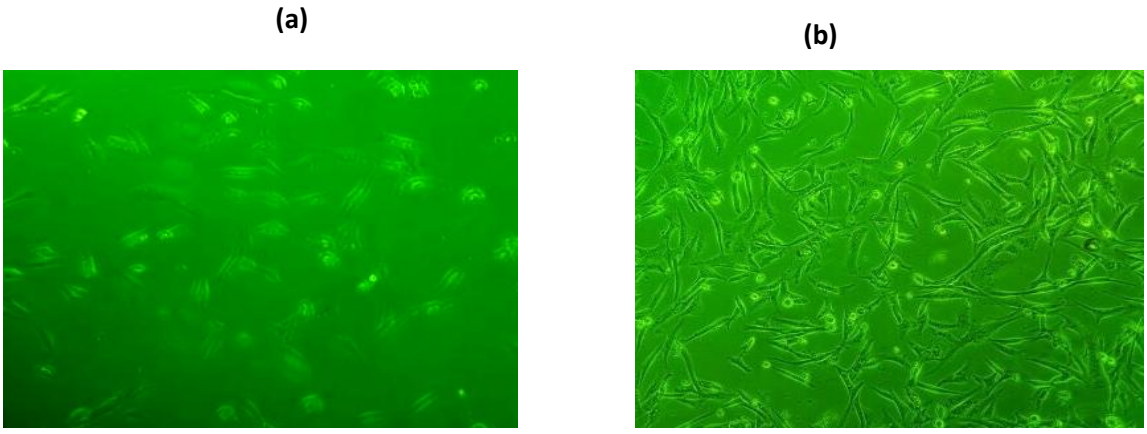


Figure 22 Microscopic image of MDA-MB-231(20X, Green Filter) (a) & (b) showing confluency of MDA-MB-231 cells during the Cultivation.

The cell density was seen 20-30% in the primary phase a. The adaptation allow them to grow prolifically by 80-90% (b) MDA-MB-231 being the slow growing cells took time to proliferate in RPMI-1640 complete media. The doubling time of cell is seen to 32 hr

3.3.1 Drug sensitivity assay (MTT assay)

The cell viability was assessed by the MTT assay of different various lipobrids formulations. MTT assay was performed in flat bottom 96 well plates, cells were seeded in a 96 well plate at ~1x10^4 cell density per well at four different concentrations 0.1,1.0,10 and 20 µg/mL as reported elsewhere (Garg et.al.2015). The graph was plotted against the percentage of the cell viability versus the concentration of the drug.

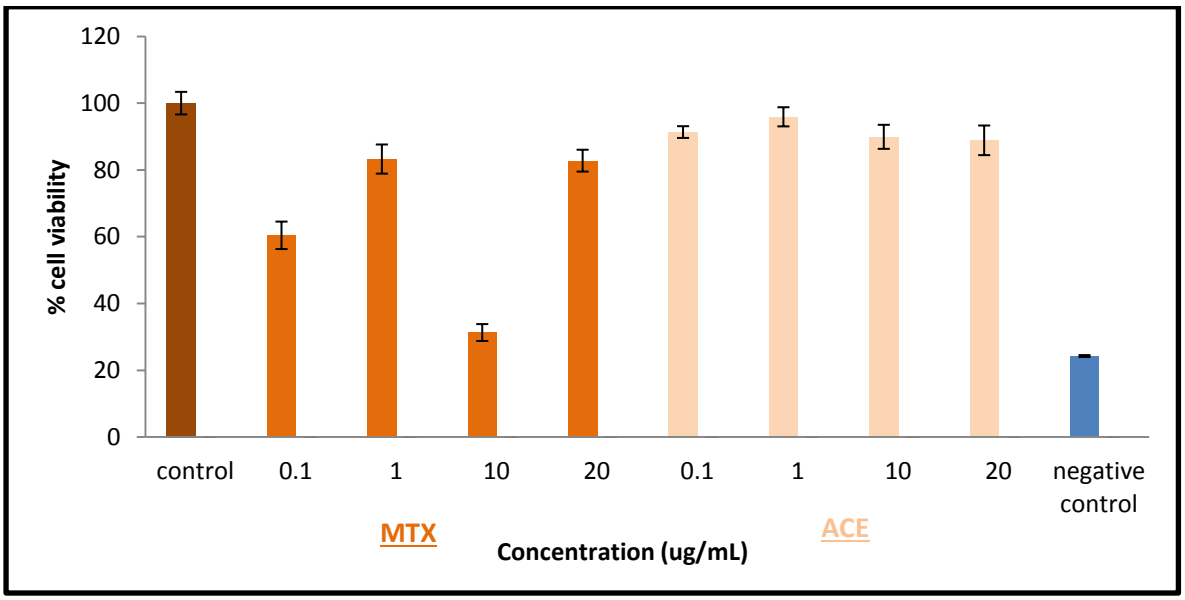


Figure 23 The graph depicts cell viability of free drug of MTX and ACE at 24 hrs, performed with MDA-MB-231 cells.

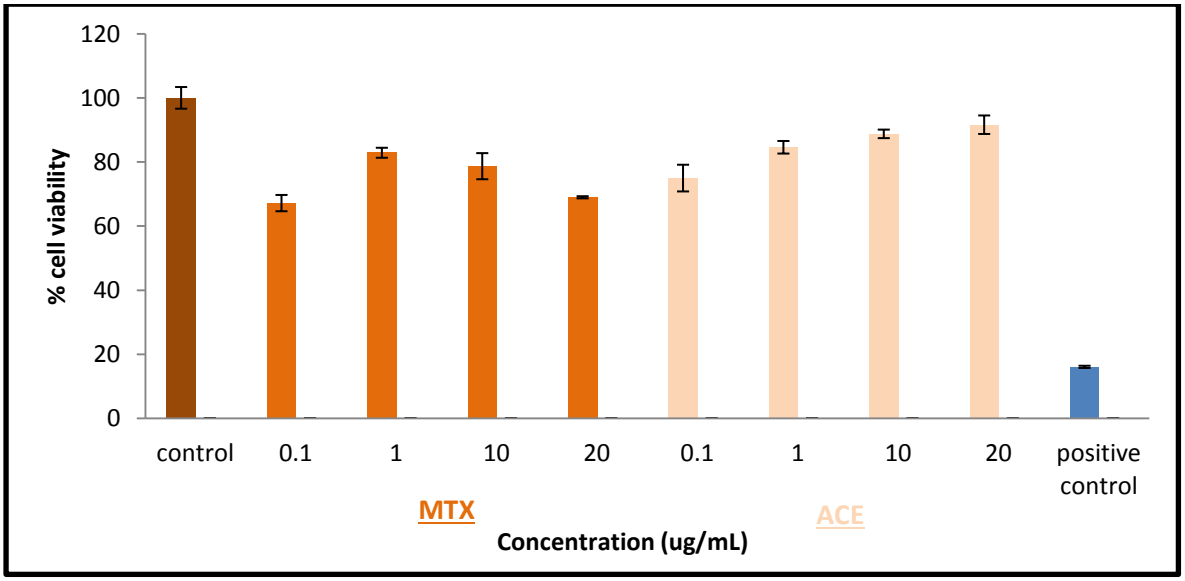


Figure 24: This graph shows the 48 hr for the free MTX and ACE carried out with cells

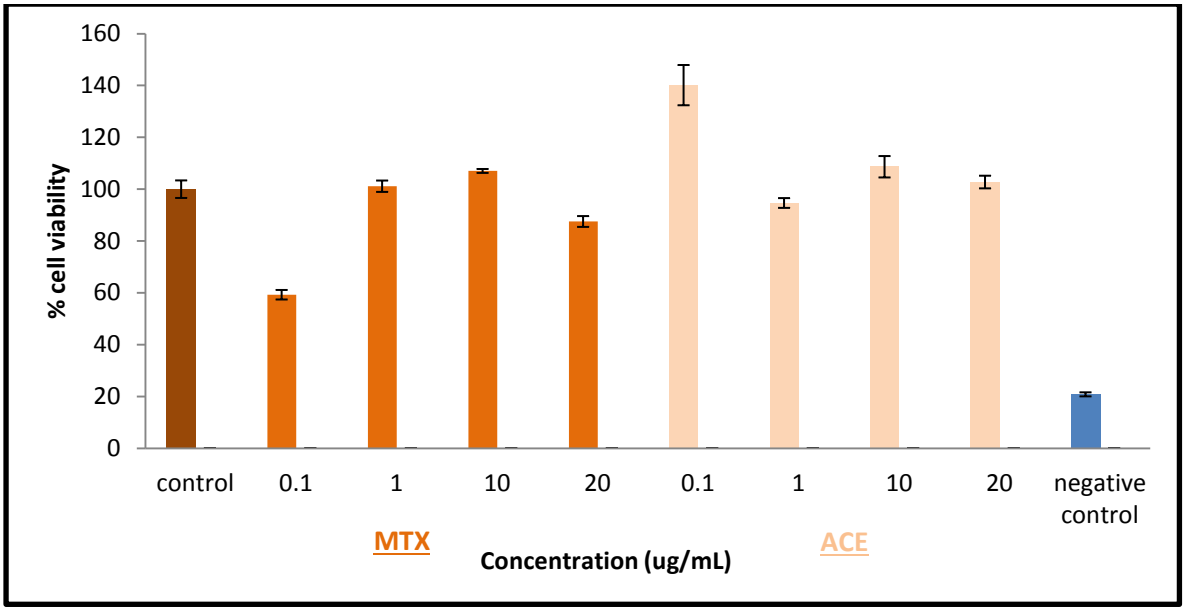


Figure 25 MTT assay performed for free drug MTX and ACE for 72 hr assayed on MDA-MB-231 cells

The MTT assay performed on free drugs with different concentrations (0.1, 1, 10, 20 µg/mL) and incubated at different time period. At 24 hrs & 48 hrs its shows the significant decreases in cell viability at 10 & 20 µg/mL, whereas at 72 hrs due to higher cell toxicity the result was not up to the mark as rapid metabolism of all drug components. Our results show that MTX is more effective as compared to ACE. It shows significantly higher ($P<0.05$) release of free drug. Therefore, we chose 48 hours incubation period for further experimentation to ensure sustain release of drug when delivered through lipobrids in comparison to free drugs.

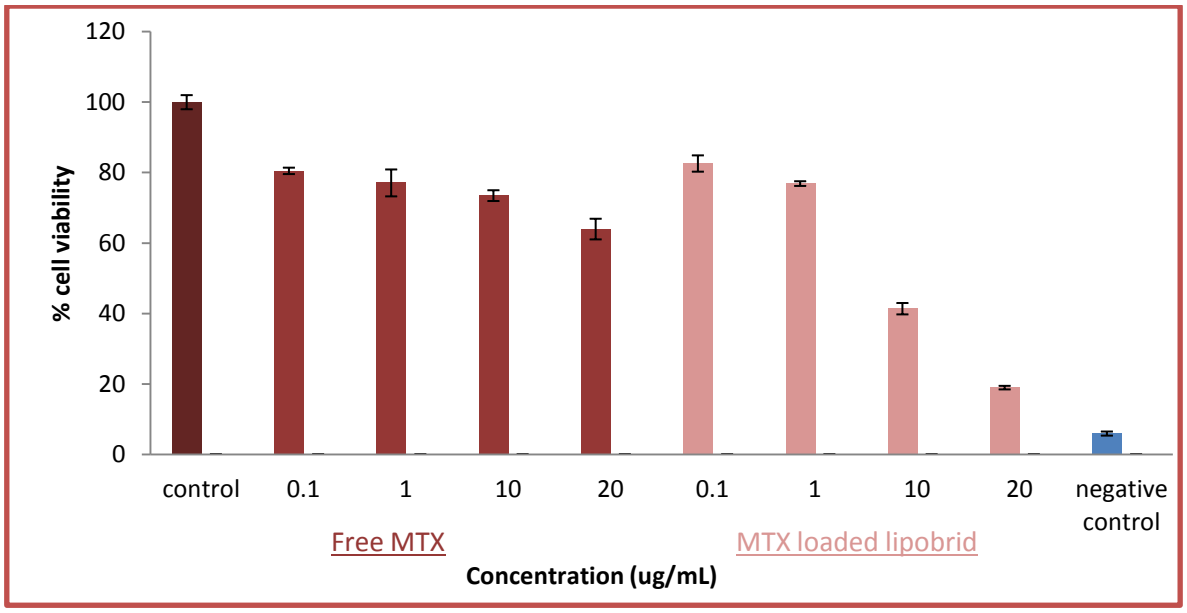


Figure 2 The MTT assay results of free drug and drug loaded lipobrid for the incubation period of 48 hrs

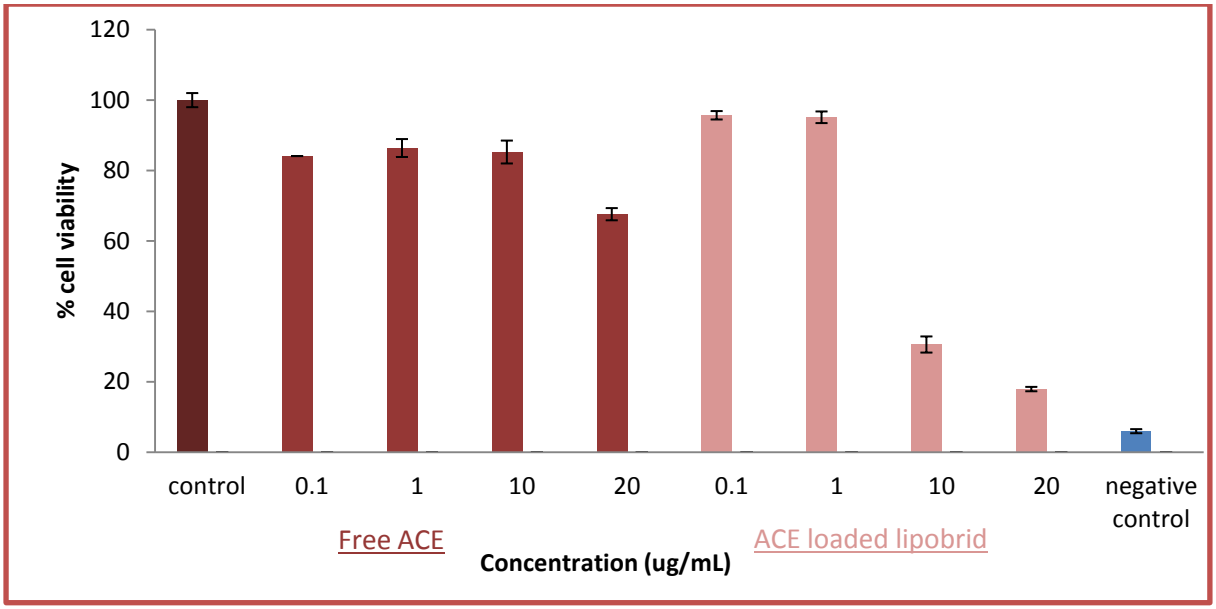


Figure 27 The MTT assay of free ACL &ACL loaded lipobrid for 48 hrs incubation

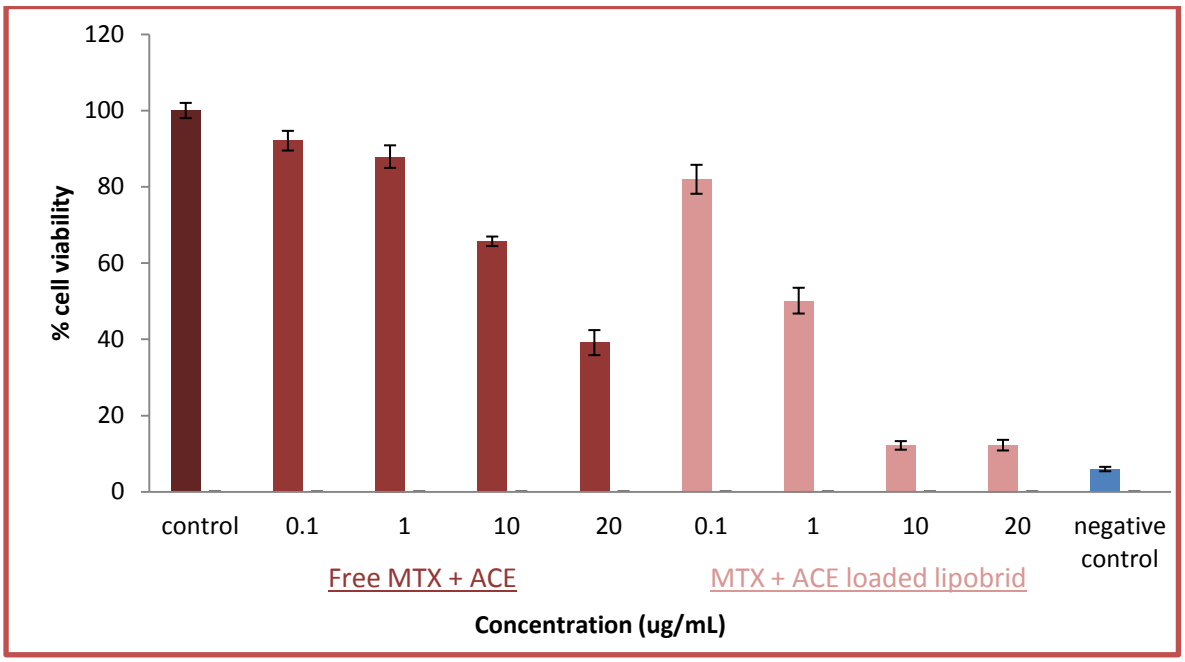


Figure 28 MTT assay of free MTX + ACL and MTX + ACL loaded lipobrid for 48 hrs incubation

This study was designed to see cell viability plotted against drug concentration with free drug and drug loaded onto lipobrid. We saw the burst release with the different concentrations of free drug tested and advocated the burst release of drug at 48 hrs incubation. Whereas MTX loaded lipobrid saw the sustained release of drug at 0.1 & 1 µg/mL concentration both free drug and drug loaded lipobrid hardly saw any significant difference in cell viability. Whereas 10 & 20 µg/mL concentrations are more effective in showing the significant decrease in cell viability.

We saw concentration dependent reduction in cell viability at given time point of 48 hrs with MTX and MTX-LPHNPs, ACE and ACE-LPHNPS, MTX+ACE and MTX+ACE LPHNPS. Here, MTX and ACE loaded lipobrid has highest effect on reduction in cell viability. It shows significantly higher ($P<0.05$) release of free drug and drug loaded lipobrids.Our results are suggestive of the fact that drugs maintained its integrity and retained its anti-tumor efficacy even upon loaded onto polymeric NPs. The encapsulation procedure did not leave any negative impact on its anti-tumor potential.

MTX loaded Ligand anchor lipobrid formulation showed a significant decrease in cell viability at different concentration and time dependent manner compare with free drug.

CONCLUSION AND FUTURE PERSPETIVES

The novel LPHNPs shows that they release their content in sustained and controlled manner as compared to that seen with free drug(s).Also, they show the sustained release of the drug plays a crucial role in the reduction of pathology driven inflammation of cells when tested in different concentrations. The *in-vitro* delivery of drugs through lipobrid formulation has the capability to form nano-dimmers for approximately 119.3±0.3 of size, and renders greater entrapment efficiency (85-93%) for lipobrids and ACE loaded lipobrids, respectively.The genetic expression of inflammatory will show the reduced expression. We observed a significant change in the number of viable cell before and after the treatment with both free and the entrapped drug which again suggests the sustained and controlled drug release to address breast cancer condition.The surface engineered lipid nano-particles could be used to deliver drugs to develop therapeutic interventions against inflammatory diseases such as rheumatoid arthritis, brain cancer etc.. In conclusion, our results show that the developed novel drug delivery vehicle (LPHNP) will better meet the needs of translational research and help finding plausible solution to various pathologies.

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