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Formulation And Ex-Vivo Evaluation Of Folate Conjugated Nanostructured Lipid Carriers (FC-Nlcs) For A549 Carcinoma Cell Targeting

Folate conjugated Nanostructured Lipid Carriers (FC-NLCs) for A549 Carcinoma Cell Targeting

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Abstract: This study presents the development and evaluation of Paclitaxel-loaded Nanostructured Lipid Carriers (NLCs), P-NLCs and folate-conjugated (FC-NLCs) formulations, prepared via solvent injection method. The formulations were characterized by SEM, TEM, particle size, polydispersity index (PDI), and zeta potential. FC-NLCs exhibited a particle size of 190.1±4.6 nm and PDI of 1.000, while PNLCs showed 231.3±2.3 nm and PDI of 0.821. Drug entrapment efficiency was higher in P-NLCs (79.4±1.6%) compared to FC-NLCs. Thermal and compatibility analyses confirmed the stability of drug-excipient mixtures. *In-vitro* drug release studies across varying pH conditions revealed sustained release profiles, with FC-NLCs showing slower release due to folate-mediated surface sealing and hydrophobic interactions. Notably, both formulations demonstrated enhanced drug release at acidic pH (4.0 and 6.4), aligning with the tumor microenvironment. FC-NLCs released 83.70% of drug over 72 hours at pH 7.4, while P-NLCs achieved 86.44% release in 30 hours. Cytotoxicity assays on A549 adenocarcinoma cells indicated dose-dependent inhibition, with FC-NLCs exhibiting superior cell growth inhibition (GI_{50} = 0.144 µg/mL) compared to P-NLCs (GI_{50} = 0.296 µg/mL) and free Paclitaxel (GI_{50} = 1.0 µg/mL). Fluorescence imaging confirmed enhanced cellular uptake of FC-NLCs, attributed to folate receptor-mediated endocytosis. These findings support the potential of folate-conjugated NLCs for targeted and sustained anticancer drug delivery.

***Index Terms*:** Nanostructured Lipid Carriers, Folate-conjugated NLCs, Paclitaxel, *In-vitro* drug release, pH-responsive release, A549 adenocarcinoma cells, Cytotoxicity assay

I. INTRODUCTION

Cancer remains a leading cause of death globally, as highlighted by the World Health Organization (WHO). In India, projections for 2020 indicated more than 17.3 lakh new cancer diagnoses and approximately 8.8 lakh deaths attributed to the disease, with breast, lung, and cervical cancers being the most prevalent. The Indian Council of Medical Research (ICMR) estimates that the number of new cancer cases in the country will rise to around 14.5 lakhs by 2025 and is expected to approach 17.3 lakhs by 2030 [1]. Lung cancer is a disease of uncontrolled cell growth in tissues of the lung. This growth may lead to metastasis, which is invasion of adjacent tissue and infiltration beyond the lungs. The vast majority of primary lung cancers are carcinomas of the lung, derived from epithelial cells. The most frequent type of cancer-related mortality among males is lung cancer and second most common in women, is responsible for1.80 million deaths worldwide annually

[2]. Global Cancer Observatory predicts 2.08 million new cancer cases by 2040. Chemotherapeutic medications like paclitaxel, docetaxel, gemcitabine, vinorelbine often combined with platinum-based cisplatin. The anticancer drugs used for the treatment have destroy normal cells of the body along with the tumor cell causes drug related side effect and severe life threading. The most common symptoms are shortness of breath, coughing, and weight loss. The most common cause of lung cancer is long-term exposure to tobacco smoke. The occurrence of lung cancer in nonsmokers, who account for as many as 15% of cases, is often attributed to a combination of genetic factors, radon gas, asbestos, and air pollution, including secondhand smoke. Possible treatments include surgery, chemotherapy, and radiotherapy. With treatment, the five-year survival rate is only 14% [3].

II. NEED OF THE STUDY

Cancer is the second most common cause of death in the developed world. Cancer occurs due to abnormal growth of cells and can invade into normal tissue or organ and spread to other parts of the body. Conventional modes of administration have limited utility
because anticancer drugs in such systems lack efficient selectivity towards melanoma cells. This not only precipitates toxicity, but also there is considerable loss of dose before reaching the desired site [4]. The site-specific delivery of drugs to the target sites has
the potential to reduce the side effects and to increase pharmaceutical response. Nanosize carrier systems are extensively used as targeted drug delivery system for efficient management of several disorders and diseases. The various systems including liposomes,
solid lipid nanoparticles, functionalized nanoparticles, dendrimer, mixed micelles have been worked extensively for their targeting potential. Nanocarrier offers an opportunity in this direction. Another strategy is the passive targeting that relies on the well-known enhanced permeation and retention (EPR) effect [5-8]. The effect first described by Maeda and Bhadra takes advantage of poorly formed (leaky) vasculature of solid tumors that allows selective accumulation of polymer-drug conjugates/complexes ranging in size between 10 to 500 nm within tumors when compared to that of free drugs. The polymeric molecules are retained following the accumulation due to their larger size whereas free drug molecules are easily eliminated from the cells. Nanocarrier has an advantage for this strategy as well because drugs can be physically encapsulated into the interior of nanometer sized nanocarrier thereby increasing the uptake of drugs into tumors [9]. In the present thesis work, it is proposed to design and develop nanostructured lipid carriers (NLCs) conjugated with folate bearing paclitaxel to target the drug directly to lung cancer, to minimize its side effects and maximize drug utilization.

III. MATERIALS AND METHODS

Preparation of Folate Conjugated Paclitaxel Loaded Nanostructured Lipid Carriers (FC-NLCs)

The FC-NLCs were prepared by Ethanol Injection Method with little modifications. Briefly, Tristearin, oleic acid and FA-PEG-DSPE were dissolved in ethanol at a concentration of 10 mg/ml, after this paclitaxel (10 mg) was added and shake for 10 min. for complete homogenization and injected into 20 ml of aqueous phase contain 0.5% w/v of Polaxamer-188 as surfactant solution. and stirrer at 3000 rpm for 45 min. Both phases were pre warmed to and kept at 50˚C during the mixing. The liquid suspension was prepared, then further sonicate for 5 min using a probe-sonicator to form NLCs. The NLCs were then concentrated by centrifugation at 5000 rpm for 10 min and re-suspended in phosphate buffer saline (pH 6.4). Same procedure adopted for preparation of paclitaxel Loaded Nanostructured Lipid Carriers (FC-NLCs) by utilizing DSPE in place of FA-PEG-DSPE [10].

Particle size determination

Average particle size and poly-dispersity index of the NLCs was determined by the photon correlation spectroscopy using a Zeta-sizer (DTS Ver. 4.10, Malvern Instruments, England) was used. The formulations were diluted with 1:9 v/v deionized water. The particle size distribution is represented by the average size (diameter) and the variance (poly-dispersity) of the Gaussian distribution function in logarithmic axis mode [11].

Surface charge measurement

The zeta potential of the nanoparticles was determined by laser Doppler anemometry using a Malvern Zetasizer also called Doppler Electrophoretic Light Scatter Analyzer [12]. The instrument is a laser-based multiple angle particle electrophoresis analyzer. Using Doppler frequency shifts in the dynamic light scattering from particles, the instrument measures the electrophoretic mobility (or zeta potential) distribution

together with the hydrodynamic size of particles (size range 10 to 30 µm) in liquid suspensions by photon correlation spectroscopy measurements [13].

Transmission electron microscope (TEM)
Transmission electron microscope (TEM) was used as a visualizing aid for studying the particle morphology. The sample (10µL) was placed on the grid and allowed to stand at room temperature for 90 sec. Excess fluid was removed by touching the edge with filter paper [14]. The samples were examined under a transmission electron microscope (Philips Morgagni 268, Eindhoven, Netherlands) at an acceleration voltage of 100 kV, and photomicrographs were taken at suitable magnification [15].

Scanning electron microscope (SEM)
The analytical techniques such as FESEM, (FEI, Nova NanoSEM 450 model) was used to confirm the exact particle size and shape of the prepared NLCs. The sample was drop-coated on a clean silica wafer, after drying for 48h, silica wafers were subjected to gold sputter coating at 20 mA for 2 nm thickness and there after FESEM imaging was performed and photomicrographs were taken at suitable magnification [16]

Estimation of Drug Entrapment and Percent Drug Loading
Drug entrapment of the anticancer drug paclitaxel in NLCs was determined by using Sephadex mini column. To prepare Sephadex mini column, firstly 1 gm of Sephadex G-50 was allowed to swell in 0.9% NaCl aqueous solution for 8 hrs and then the hydrated gel was filled in the barrel of 2 ml disposable syringe plugged with filter pad. The barrel was centrifuged at 2000 rpm for 2 minutes to remove excess of saline solution to form the Sephadex separating column. To separate free drug from NLCs formulation 0.2 ml of NLCs dispersion was applied drop wise on the top of the Sephadex column and then centrifuged at 2000 rpm for 2 min. to expel and remove void volume containing NLCs in to the centrifuged tubes. This eluted NLCs dispersion was collected and lysed by disrupting with 5% Triton-X100 and then the amount of entrapped drug was analyzed using spectrophotometric method. The amount of drug entrapped in folate conjugated NLCs was determined employing similar procedure as reported for paclitaxel loaded Nanostructured Lipid Carriers [17].

$$\textbf{Percent Entrapment Efficiency} = \frac{\boldsymbol{W}\ (\textbf{Amount of drug in SLNs})}{\boldsymbol{W}\ (\textbf{Total amount of drug added})} \times \mathbf{100}$$

$$\textbf{Percent drug loading} = \frac{\boldsymbol{W}\ (\textbf{Amount of drug in SLNs})}{\boldsymbol{W}\ (\textbf{Total amout of lipid, emulsifier and drug added})} \times \mathbf{100}$$

The above formula was used to calculate the percentage of drug encapsulation efficiency (% EE) and drug loading (%DL) of the prepared Nanostructured Lipid Carriers (NLCs). The free drug containing supernatant was collected through dispersing prepared NLCs and centrifugation. The measurement of the absorbance of free drug by using UV spectrophotometer denotes the percent entrapment and drug loading efficiency [18].

***In-vitro* drug release**
The *in-vitro* drug release of entrapped paclitaxel from NLCs formulation was determined using dialysis tube. The NLCs formulation was first separated from free drug by passing through Sephadex column and then centrifugation. Separated NLCs formulation (5ml) was taken in to the dialysis tube (molecular weight cut off 12,000 Da, Hi Media, India) and placed in a beaker containing 50 ml of PBS (pH 7.4). The beaker was placed over a magnetic stirrer and the temperature was maintained at 37±2˚C throughout the procedure. Samples were withdrawn at definite time intervals and replaced with same volume of phosphate buffer. It was then spectrophotometric analyzed for drug content by measuring absorbance at 236.0 nm against blank [19].

***In-vitro* cell line studies**
The purpose of a targeted drug delivery system is to deliver the drug at the desired site. The developed NLCs formulations were evaluated for their toxicity, storage conditions, leakage and drug release and were found to be stable enough with desired drug release profile, for further evaluation in-vitro cell line study. However, developed formulations were designed to target drugs to tumor. In the present section invitro assessment of the formulations was performed to ascertain cytotoxicity and cellular uptake against cancer cell line (A549). However, prior to in-vivo studies, the formulations should be evaluated for their efficacy through ex-vivo by checking for various interactions. Hence the ex-vivo studies were taken up prior to direct in-vivo efficacy studies in animals and were performed in a view to explore the target ability of the prepared formulation against human adenocarcinoma cell line A549. Cell line studies were performed in a view to explore the target

ability of the prepared formulations against cancer cell lines. A549, a human adenocarcinoma cell line, was selected for the study as it over expresses folate receptors. Two different studies cell inhibition cytotoxicity assay and cell uptake assay were performed to assess the target ability of the formulations. Choosing a cell viability or cytotoxicity assay from amongst the many different options available can be a challenging task. Picking the best assay format to suit particular needs requires an understanding of what each assay is measuring as an endpoint, of how the measurement correlates with cell viability, and of what the limitation of the assay [20-22]**.**

IV. RESULTS AND DISCUSSION

Formulation development

The Nanostructured Lipid Carrier (P-NLCs and FC-NLCs) was prepared by solvent injection method, which involves the rapid diffusion of solvent across the solvent-lipid phase into the aqueous phase. Tristearin, FA-PEG-DSPE, Oleic acid and drug were
dissolved in ethanol, maintained at an elevated temperature of 70°C and was injected into an aqueous solution (Poloxamer 188 and distilled water) maintained at the similar temperature and kept under continuous stirring for 45 min. at 3000 rpm to form lipid suspension and sonicate to form FC-NLCs. The prepared formulation was optimized for various parameters like lipid ratio, drug-lipid ratio, surfactant ratio, stirring time, stirring speed and sonication time to obtain nano-size NLCs with maximum drug entrapment. Particles of optimum size 193.4 ± 2.1 nm with maximum drug entrapment (84.8±0.7) were obtained at 1% surfactant concentration. However, on further increasing surfactant concentration, although the particle size decreases because of formation of micelles but the entrapment efficiency also decreases because of the leaching out of the drug. Drug entrapment was determined using Sephadex G50 column and was found to be 80.19±1.8%. FC-NLCs was further, subjected to various characterization parameters i.e. surface morphology, particle size and polydispersity index [23].

Characterization

NLCs formulation was characterized on the basis of surface morphology, particle size, polydispersity index and zeta potential. Surface morphology of the particles was studied by SEM and TEM analysis. Particle size, polydispersity index and zeta potential
studies were carried out by Laser Light Scattering technique (Malvern Instrument U.K). PDI of D2V2S2R3T3P3 (FC-NLCs) was found to be 1.000 and average particle size of 190.1±4.6 nm was recorded and PDI of FC-NLCs was found to be 0.821 and average
particle size of 231.3±2.3 nm. The average particle size of the P-NLCs was found to be 231.3 nm with a PDI of 0.821. The drug entrapment efficiency of Paclitaxel loaded Folate conjugated NLCs was measured to be 79.4±1.6%.

In-vitro drug release

The *In-vitro* drug release analyses on different pH environment were performed to check the efficiency of the prepared NLCs formulation. Four different buffer medium is utilized for drug release study i.e., Phosphate buffer saline pH (4.0, 6.4, 7.4 and 7.8).
In-vitro drug release profile of different formulation suggests that the release was in a sustained manner. In-vitro drug release pattern from P-NLCs and F-NLCs formulations at different pH (4.0, 6.4, 7.4, 8.0) exhibited a non-linear release profile characterized by a relatively faster initial release during the first 3-4 h, followed by slower and continuous release in later part. In-vitro drug release profiles of both the formulations were carried out using dialysis tube. Formulation FC-NLCs showed a drug release profile of 83.70% up to 72 hrs. in PBS 7.4. P-NLCs showed a drug release profile of 86.44 % up to 30 hours in PBS 7.4. This data shows that folate conjugation slows down the release of drug from the formulation. Release of paclitaxel from F-NLCs was found to be slower as compared to P-NLCs in all drug-release conditions. Moreover, at pH 7.4 and 8.0, the release patterns were sustained by FC-NLCs resulting in about 68.80%, 58.88% drug release in 24 hrs, respectively. The reason attributed to surface engineering of folic acid as it led to more sealing at the nanoparticle periphery and hydrophobic interactions which delayed the drug release. As the pH of the drug-release media was increased, a slowed paclitaxel release pattern was observed. The possible reason might be that at acidic pH, the interior tertiary amine groups were protonated, leading to a repulsion of charges. In-vitro release studies at pH 6.4 were carried out in the view that the tumor vicinity is relatively acidic in nature. The rapid release behavior of the drug from NLCs formulations at pH 6.4 supports the hypothesis that the NLCs formulation will release the drug in higher concentration, whenever it will reach the target site i.e. tumor; as the pH in such environment is generally below 7. The P-NLCs formulation has shown the different release pattern on different pH medium i.e. at pH 4.0 show 96.17% drug release after12hrs; at pH 6.4 show 88.76%

of drug release after 16 hrs, at pH 7.4 shown 86.44% drug release after 30 hrs, at pH 8.0 shown 83.66% drug release after 72 hrs. These types of release behavior indicate that the P-NLCs have shown the better release of drug at lower pH range 4 and 6.4. This result is favoring our hypothesis that NLCs formulation release the drug in higher concentration in lesser times when it reaches to tumor sites, where the pH is always be lesser than 7. The F-NLCs formulation has shown the different release pattern on different pH medium i.e. at pH 4.0 show 91.63% drug release after 16hrs; at pH 6.4 show 86.34% of drug release after 16 hrs, at pH 7.4 shown 83.70% drug release after 30 hrs., at pH 8.0 shown 76.96% drug release after 72 hrs. These types of release behavior indicates that the F-NLCs has shown the better release of drug at lower pH range 4 and 6.4. This result is favoring our hypothesis that F-NLCs formulation release the drug in higher concentration in lesser times when it reaches to tumor sites, where the pH is always be lesser than 7. The release data indicates that Moreover, at pH 7.4 and 8.0, the release patterns were sustained by F-NLCs resulting in about 65.78%, 52.18% drug release in 16 hrs, respectively. The reason attributed to surface engineering of folic acid as it led to more sealing at the nanoparticle periphery and hydrophobic interactions which delayed the drug release. As the pH of the drug-release media was increased, a slowed paclitaxel release pattern was observed. The possible reason might be that at acidic pH, the interior tertiary amine groups were protonated, leading to a repulsion of charges [24].

In-vitro Cell line study

A549 cells were treated with six equivalent doses ranging from 0.05 to 0.5 µg/mL. The result of the cell inhibition assay exhibited significant differences with paclitaxel and NLCs formulations. FC-NLCs exhibited highest percent cell growth inhibition compared to P-NLCs formulations as well as drug itself. However, all the formulations showed dose-dependent inhibition of A549 adenocarcinoma cells. Higher uptake in case of the FC-NLCs was possibly due to the receptor specific targeting of NLCs due to surface conjugation with folic acid. GI50 values for Paclitaxel, P-NLCs, and F-NLCs were found to be 1.0, 0.296 and 0.144 µg/ml, respectively. The difference between IC50 values of FC-NLCs and P-NLCs were found to be statistically significant ($p<0.05$). The observations clearly indicated that the FC-NLCs exhibited higher cell inhibition compared to P-NLCs. At 0.5µg/ml concentration FC-NLCs has showed 70.8±2.3% percent cytotoxicity while 58.0±0.8% and 35.6±1.4% in case of P-NLCs and Drug (Paclitaxel) respectively, at similar concentration. Fluorescence study of NLCs formulations and Paclitaxel was performed with a view to assess the ability of different drug loaded formulations to target human adenocarcinoma cells, A549 cells. Similar to the results of the percent cell growth inhibition assay fluorescence studies also displayed higher uptake of folic acid conjugated formulations. The higher uptake was possibly due to the folate residue on the surface of the NLCs as compared to P-NLCs and Drug. Among the free Paclitaxel, the higher uptake was observed with the P-NLCs, which might be due to higher drug loading, resulting in a higher release. The results again support the strategy that ligand mediated anticancer delivery can provide higher uptake of drug to the A549 Adenocarcinoma cell line. This study is in agreement that the folic acid anchored NLCs entered into the cancer cell by receptor mediated endocytosis.

IC_{50} and GI_{50} Determination

Nonlinear regression analysis was applied to the dose-response data using a four-parameter logistic model to calculate IC_{50} and GI_{50} values.

Treatment	IC_{50} (µg/ml)	GI_{50} (µg/ml)
Paclitaxel	0.108	1.000
P-NLCs	0.141	0.296
FC-NLCs	0.185	0.144

The IC_{50} represents the concentration required to inhibit 50% of the maximum response, while GI_{50} denotes the concentration at which 50% of cell growth is inhibited relative to untreated controls. The data reveal that FC-NLCs achieved GI_{50} at the lowest concentration, indicating superior cytotoxic potency. This enhanced efficacy is likely due to folic acid conjugation, which facilitates targeted delivery via receptor-mediated endocytosis a mechanism commonly upregulated in cancer cells. P-NLCs also demonstrated improved performance over free Paclitaxel, suggesting that nanoencapsulation enhances cellular uptake and drug retention.

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