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Biodegradable preparation, characterization and *In vitro* evaluation of stealth docetaxel lipid nanoemulsions for efficient cytotoxicity.

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Abstract

Docetaxel is currently used in treatment of breast and ovarian cancers, administered in the form of parenteral IV infusion. Lipid nanoemulsion (LNE) delivery system is biocompatible, biodegradable and easy to prepare. The aim of this study was to develop stable parenteral pegylated docetaxel lipid nanoemulsions (LNEs) for improving cytotoxicity. The O/W LNEs were prepared by homogenization and ultrasonication process. The globule sizes and zeta potentials were measured by using Malvern Zetasizer. The sizes of oil globules varied from 70.2 to 243.3 nm and zeta potential from 25.5 to 40.2mV. During in vitro drug release studies, the cumulative percentage drug released within 15 h from pegylated lipid nanoemulsions, PLNE-3 and PLNE-6 was 84.02±4.79 and 88.51±4.5 respectively. The total drug content and entrapment efficiencies for the prepared LNEs were determined by HPLC. The in vitro cell line studies were performed on two cell lines (MCF-7 and Hela cell lines). When compared to drug solution, PLNE-6 was found to have 1.55 and 1.6 folds more cytotoxic activity on MCF-7 and Hela cell lines respectively. The IC 50 values of pegylated LNEs on both the cell lines was statistically significant when compared to DS, $P<0.05$. In conclusion, stealth LNEs were prepared, characterized and found to be stable and superior in delivery of docetaxel to cancer cell lines in comparison to plain LNE and drug solution.

Keywords: Docetaxel, DSPE-PEG 5000, DPPE-PEG 5000, Stealth Lipid Nanoemulsions, cytotoxicity.

Introduction

The cancer is second leading cause of mortality after cardiovascular disease and accounts for one of every four deaths. Docetaxel is a clinically potent microtubule targeting agent for the treatment of cancer[1]. It is a semisynthetic derivative from10-deacetyl baccatin III, an inactive precursor extracted from the yew tree, *Taxus baccata*. The cytotoxic mechanism of docetaxel involves specific covalent binding to assembled tubulin, resulting in promotion and stabilization of persistent interphase microtubule complexes [2]. It has been approved for use in treatment of six different cancer indications, they are locally advanced and metastatic breast cancer, non-small cell lung cancer, androgen independent prostate cancer, advanced gastric cancer, head and neck cancer [3]. Each year, an estimated 3 million patients worldwide have been diagnosed with one of these types of cancer.

Nanomedicine approaches to drug formulation for the treatment of solid tumors are reaching clinical application, largely securing approval due to improved safety profiles. For example, Doxil® (pegylated liposome containing doxorubicin) is an approved alternative to conventional doxorubicin for treatment of Kaposi's sarcoma, and Abraxane® is an alternative to Taxol® for treatment of metastatic breast cancer [4]. Safety enhancements arise due to elimination of irritating excipients such as Polysorbate 80 or Cremophor (Taxotere and Taxol, respectively) and reduced non-specific distribution to sensitive tissues (e.g., reduced cardiotoxicity with Doxil®).

The enhanced permeability and retention (EPR) effect in solid tumors was first introduced by Matsumura and Maeda [5]. The EPR effect can enhance the diffusion of macromolecules or nanomedicines into the tumor via leaky tumor vasculature. The EPR effect causes increased retention of macromolecules within tumor tissues due to the poor tumor lymphatic drainage. EPR is a universal phenomenon in solid tumors.

Todate, various nanoparticles that exhibit the long blood-circulating property have been shown to preferentially accumulate in tumors due to the leakiness of microvasculature in solid tumors [6]. However, the uptake of nanoparticles by macrophages in the reticuloendothelial system (RES) should be avoided to achieve an efficient EPR effect-based passive targeting of drugs [7]. Various strategies, therefore, were developed including the modification of the surface of nanoparticles with ganglioside GM1, phosphatidylinositol or polyethylene glycol (PEG) [8,9]. Among them, the surface modification with PEG (pegylation) is known to be a successful strategy.

Among the various colloidal drug delivery systems, parenteral lipid emulsions are commercially available in market for nutrition and medicinal purpose. The lipid emulsions (LE) are biocompatible, biodegradable and easy to prepare [10]. In LE the lipid core (oil) is stabilized by phospholipid monolayer and dispersed in continuous aqueous phase. These are, useful in delivering lipophilic drugs, mass-produced, easily sterilizable and stable even at room temperature for 1–2 years [11].

PEG is a water-soluble, nontoxic, non-antigenic and biocompatible polymer, that has been approved by the FDA for human intravenous, oral, and dermal applications [12,13]. Attachment of PEG (pegylation) to drugs, peptides, proteins, nanoparticles, micelles, and liposomes is a mature technology for enhancing the bioavailability, stability, safety, and efficacy of a wide range of therapeutic agents. Few of the pegylated products commercially available in the market or in clinical trails are Oncaspar (pegylated L- asparaginase), PEGASYS (pegylated alpha interferon), Cimzia (Certolizumab Pegol), Mircera (pegylated form of erythropoietin), Neulasta (pegylated recombinant methionyl human granulocyte colony-stimulating factor), Adagen: (PEG-adenosine deaminase etc. Docetaxel submicron lipid emulsions were reported [14]. There is no work regarding the docetaxel being incorporated in pegylated emulsions.

Hence, the objective of this study was to prepare, pegylated lipid nanoemulsions (PLNEs) containing docetaxel for parenteral intravenous application using biocompatible excipients. We investigated the influence of pegylated lipids i.e., 1, 2- distearoyl sn- glycero-3-phosphatidylethanolamine (DSPE)-PEG 5000 and 1, 2- dipalmitoyl sn- glycero-3-phosphatidylethanolamine (DPPE)-PEG 5000 on in vitro cell lines. Further, formulations were characterized, evaluated and cytotoxic activity on two different cell lines was compared with Docetaxel solution (DS) and Plain Lipid nanoemulsion (LNE).

Materials and Methods

Materials

Purified olive oil (Lipoid, Germany), egg phosphatidyl choline (EPC-80) (Lipoid, Germany), cholesterol & glycerol (Merck, Mumbai), -tocopherol (Sigma, Mumbai), oleic acid (S.D. Fine, Mumbai), docetaxel (A kind gift from Dr Reddy's Laboratories, Hyderabad), DSPE-PEG5000 and DPPE-PEG 5000 (A kind gift from Lipoid, Germany) sodium dihydrogen phosphate, disodium hydrogen phosphate (S.D fine Chemicals , Mumbai), centrisort tubes (Sartorius, Germany), dialysis membrane (Hi media, Mumbai), chloroform, acetonitrile (Merck., Mumbai). HeLa- Human cervical carcinoma cell line (NCCS, Pune), MCF-7 – Human breast carcinoma cell line (NCCS, Pune), RPMI-1640 (Himedia, Mumbai), dimethylsulphoxide (DMSO) (Merck India Ltd, Mumbai), SDS lysis buffer (Himedia, Mumbai), trypsin (Himedia, Mumbai,), MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (Himedia, Mumbai). All other chemicals used were of reagent grade.

Methods

Preparation of lipid nanoemulsions (LNE) containing docetaxel

The LNEs were prepared by hot homogenization and ultrasonication process and the compositions are shown in Table 1. Required quantities of ingredients were weighed. The oil soluble substances were added to the oily phase and the water soluble substances to the aqueous phase. Then both the phases were heated to 70º C. The aqueous phase was added to oily phase slowly drop wise with stirring and then homogenized for about 3 minutes at 15,000 rpm (Homogenizer Diax 900, Heidolph, Germany). The coarse emulsion was then sonicated (Vibra Sonics, Sonics and Materials Inc., USA) for about 20 minutes at 50% amplitude and characterized for size, polydispersity and zeta potential (Zp).

Determination of globule size, polydispersity index and zeta potential

The mean size and polydispersity index of emulsion globules was determined by photon correlation spectroscopy using Zetasizer (Malvern Nano ZS90, Malvern, UK). Each sample was diluted to a suitable concentration (1 in 50) with filtered double distilled water. Analysis was performed at 25 ºC with an angle of detection of 90 º. During the measurements, average globule count rate was maintained between 50 and 500 kcps. Average of the three readings was noted.

The same sample which was used for size analysis was taken in to a dip cell, was then inserted and average of three readings was noted by using the instrument Zetasizer (Malvern Nano ZS90, Malvern, UK).

Effect of varying concentration of DSPE-PEG 5000 and DPPE-PEG 5000

LNEs were prepared as described above using different concentrations of DSPE-PEG 5000 and DPPE-PEG 5000 with 0.15%, 0.3%, and 0.45% w/v keeping the other ingredients constant.

In vitro drug release studies of LNE formulations

The drug release was studied by open tube method using Cellulose membrane (DM 60 Molecular weight cut off 12000-14000, Himedia, Mumbai). Initially, the dialysis membrane was hydrated overnight in phosphate buffer pH 7.4 at room temperature. The emulsions/solution (1ml) were placed in dialysis open tube and then suspended in 100 ml of 30%v/v ethanol phosphate buffer pH 7.4 mixture in 250 ml beakers as dialysis medium, which was stirred continuously on a magnetic stirrer (Remi Equipments, India). At different time intervals i.e., 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 hrs during dialysis, 1 ml of samples were withdrawn from the beaker and replaced by equal volume of fresh

medium for analyzing the drug content. The amount of drug in collected samples was calculated by using the linear calibration curve plotted with known concentrations of drug by measuring the absorbance at λ_{max} 227 nm on a UV-Visible spectrophotometer (ELICO SL 159, India). The release pattern of the drug from different formulations was calculated by plotting cumulative percentage drug release vs time.

Stability of LNEs: Effect of centrifugal stress, thermal stress, dilution stress and storage

The selected formulations (LNE, PLNE-3 and PLNE-6) were studied for the effects of centrifugal stress, thermal stress, dilution stress and stability on storage.

Effect of centrifugal stress on stability

LNEs of 1ml were filled in eppendorf tubes and were subjected to centrifugation (Heraeus Biofuge, Germany) at 13,000 for 10min. The creaming volume percentage for each emulsion was calculated by using the formula previously reported by Krishna et al., 1998 and formulations were compared.

Effect of autoclaving on the stability of LNEs

The LNEs were filled into vials and subjected to autoclaving at 121ºC for 15 minutes. The formulations were observed for the possible changes in particle size, PDI and zeta potentials.

Effect of Dilution (Desorption Stress) on Stability

The selected LNEs were diluted with double-distilled water 50 to 5000 times (1:50, 1:100, 1:200, 1:500, 1:1000 and 1:5000) and the effect of dilution on size and Zp was studied using Zeta Sizer.

Stability of LNEs during storage

About 1ml of emulsion was filled in eppendorf tubes and was stored at 4 ºC and 25 ºC for 6 months. At predetermined intervals i.e., 0, 1, 2, 3, 4 and 6 months, the samples were analyzed for size, polydispersity index and zeta potential.

Determination of total drug content and entrapment efficiency

High performance liquid chromatography (HPLC) system consisting of a LC-20AD solvent delivery system containing double reciprocating plunger pump (Shimadzu, Japan), a SPD-20A UV–Visible variable wavelength detector with deuterium lamp (Shimadzu), and a 250 x 4.6 mm, 5µ, C-18 reverse phase analytical column (Lichospher®, Merck, Mumbai, India) was used to determine total drug content and entrapment efficiency of the formulations. The mobile phase consisted of 65 parts of acetonitrile and 35 parts of double distilled water. The flow rate was 1 mL/min and the detection was performed at λ_{max} of 227nm.

Drug content

Each docetaxel loaded LNE (0.1 mL) was diluted to 1mL and further diluted twice (1:10) with methanol. The 20µl was injected and drug content was determined by the reported HPLC method [15].

Entrapment efficiency

Entrapment efficiency (EE) was determined by measuring the concentration of free drug (unentrapped) in aqueous medium [16]. The aqueous medium was separated by ultra-filtration using centrisort tubes (Sartorius, USA), which consisted of filter membrane (M.Wt. cut off 20,000 Da) at the base of the sample receiver chamber. About 2ml of the formulation was placed in the donor chamber and centrifuged at 6500 rpm for 15 min (Refrigerated Biofuge). The LNEs along with encapsulated drug remained in the donor chamber and aqueous phase moved into the receiver chamber through filter membrane. The amount of docetaxel in the aqueous phase was estimated by HPLC method and the entrapment efficiency was calculated.

In vitro cytotoxic activity by MTT Assay

The adherent cells (Hela and MCF-7) were trypsinized and were resuspended in fresh medium after centrifugation [17]. Cell suspension was mixed thoroughly by pippetting several times to get a uniform single cell suspension. Different dilutions of drug solutions and formulations were made in medium (RPMI-1640) with final DMSO concentration in the well to be less than 1%. About 100µl of cell suspension was transferred aseptically to each well of a 96 well plate and to it 100µl of 1% media/ drug solution (n=6) in media was added. The plate was then incubated at 37ºC for 48 hours in CO_2 incubator. After 48 hours of incubation, 20µl of MTT was added to each well. The plate was again incubated for 2 hours. After incubation, 80µl of lysis buffer was added to each well, the plate was wrapped in aluminum foil to prevent the oxidation of the dye and the plate was placed on a shaker for overnight. The absorbances were recorded on the ELISA reader at 562nm wavelength. The absorbance of the test was compared with that of DMSO control to get the % inhibition.

Results and Discussion

Formulation and *In vitro* release studies

The lipid nanoemulsions (LNEs) were prepared by hot homogenization and ultrasonication process. The initial typical formula was selected based on previous literature on parenteral lipid emulsions [18]. Plain emulsion LNE consisted of 10% (w/v) olive oil as the oil core, 1.2% (w/v) EPC-80 as a phospholipid emulsifier, oleic acid (0.3%) as a negative charge inducer, -tocopherol (0.25%) as antioxidant and glycerol (2.25%) (Table 1) was added to maintain the isotonicity of the formulation for IV administration.

Table1: Compositions of the prepared Docetaxel Formulations.

Formulation ingredients	Plain emulsion	Drug Solution	Varied concentrations of DSPE/DPPE-PEG 5000					
	LNE	DS	PLNE-1	PLNE -2	PLNE -3	PLNE -4	PLNE -5	PLNE -6
Docetaxel (mg)	10	10	10	10	10	10	10	10
Olive oil (mg)	1000	0	1000	1000	1000	1000	1000	1000
Egg lecithin (mg)	120	0	120	120	120	120	120	120
Cholesterol (mg)	30	0	30	30	30	30	30	30
-tocopherol (mg)	25	0	25	25	25	25	25	25
Oleic acid (mg)	30	0	30	30	30	30	30	30
DSPE-PEG 5000 (mg)	0	0	15	30	45	0	0	0
DPPE-PEG 5000(mg)	0	0	0	0	0	15	30	45
Glycerol (mg)	225	0	225	225	225	225	225	225
Ethanol (ml)	0	10	0	0	0	0	0	0
Double distilled water to make (ml)	10		10	10	10	10	10	10

For preparation of pegylated LNEs, DSPE-PEG 5000 or DPPE-PEG 5000 was incorporated in LNE formulation during the preparation. In this study, two pegylated lipids were used at different concentrations of 0.15%, 0.3%, 0.45% w/v. The pegylated lipids could decrease the size of globules considerably when compared to plain emulsion LNE (Table 2). The average size of the globules of DSPE-PEG 5000 was slightly less when compared to that of DPPE-PEG 5000 and probably this might be due to the difference in packing of surfactant molecules in globular surface.

The percentage drug entrapment of the prepared LNEs was found to be excellent and in the range of 97-100% (Table 2). Whereas, the drug content was found to be in the range of 9.7 to 10.3 mg (Table 2). The drug content was estimated by reported HPLC method by using isocratic Shimadzu HPLC.

Table 2: Size, PDI, Zeta potentials, Assay Percentage drug entrapment of the prepared LNEs (n=3).

Formulation code	Size (nm)	PDI	Zeta potential(mv)	Assay (mg)	%Drug entrapment
LNE	200.4±2.3	0.035±0.21	-35.5±2.3	10.1±0.3	97±0.2
PLNE-1	115.2±2.7	0.12±0.14	-29.9±1.9	9.8±0.2	98±0.1
PLNE -2	93.4±1.3	0.051±0.11	-35±1.3	9.9±0.21	99±0.3
PLNE -3	70.2±3.1	0.034±0.19	-40.2±1.8	9.8±0.1	100±0.1
PLNE -4	125.4±1.6	0.017±0.18	-28.3±3.2	9.7±0.21	97±0.1
PLNE -5	101.2±1.9	0.031±0.11	-29.5±2.1	9.78±0.15	98±0.4
PLNE -6	83.5±1.3	0.077±0.11	-32.4±1.7	10.2±0.2	101±0.3

The *In vitro* release of LNE after 12 hrs was found to be 96.56± 1.79, whereas the in vitro release of the pegylated formulations i.e. PLNE-1, PLNE-2, PLNE-3, PLNE -4, PLNE-5 and PLNE-6 after 15 hrs was found to be 93.99±1.55, 90.81±1.53, 84.02±4.79, 95.21±1.45, 91.32±1.57 and 88.51±4.5 respectively (fig 1 and 2). The in vitro release of the pegylated LNEs was slower than the plain emulsion i.e. LNE might be due to the rigidization of the membrane at the surface of globules [19]. The *In vitro* release of drug from DSPE-PEG 5000 containing emulsions was relatively less than that of the DPPE-PEG 5000 containing emulsions in various concentrations. This is probably due to the DSPE moiety

that might have produced rigid packing [20] than DPPE chain at the interface.

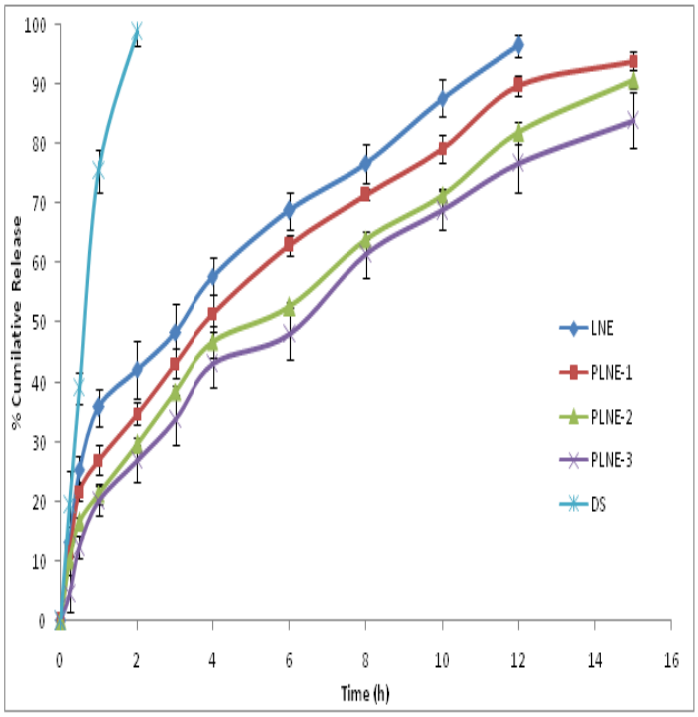

Figure 1: Cumulative amount of drug released from DS, LNE, PLNE-1, PLNE-2 and PLNE-3 formulations

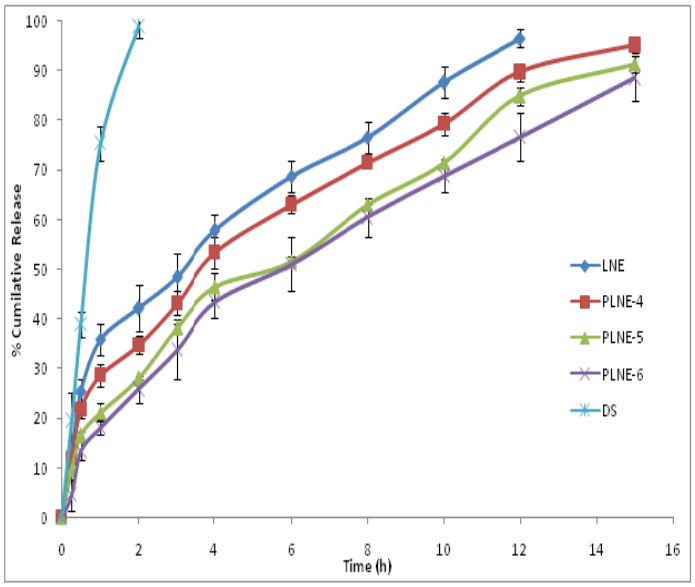

Figure 2: Cumulative amount of drug released from DS, LNE, PLNE-4, PLNE-5 and PLNE-6 formulations

Stablity Studies

Basing on the size and release profiles, the PLNE-3 and PLNE-6 were used for further studies i.e., the effect of centrifugal stress, thermal stress, dilution stress and storage on stability was determined. In centrifugal studies, the higher the creaming values the better the stability of emulsions [21]. The selected formulations (PLNE-3 and PLNE-6) showed higher creaming values (Table 3)

Table 3: Effect of centrifugation (% creaming volume) on physical stability of LNEs (n=3).

Formulation code	Creaming volume (%) (n=3)			
	I	II	III	Avg ± SD
LNE	96.0	97.0	95.0	96.0 ± 1.0
PLNE-3	98.0	97.5	98.0	97.8 ± 0.3
PLNE-6	99.0	98.5	99.5	99.0 ± 0.5

indicating the stability of the LNEs. The effect of thermal stress on stability is evaluated by autoclaving technique. As these LNEs are given by IV route they need to be sterilized before injection. Groves and Herman, 1993 reported that the phospholipids rapidly relocate during autoclaving from aqueous to oily phase, forming a cubic liquid crystalline structure, the bulk of oil which is converted to a lamellar phase on cooling and this orientation of interfacial materials accounts for the enhanced stability of phospholipid emulsions after sterilization. There were no considerable changes in size and zeta potential values after autoclaving in selected LNEs indicating the stability of LNEs (Table 4).

The selected formulations LNE, PLNE-3 and PLNE-6 were subjected to dilution (50 to 5000 times) stress (Table 5). The dilution of an emulsion disturbs the rigidity of the surfactant layers at the interface leading to instability of system. The extent of stability of an emulsion can be rapidly checked by measuring the changes in zeta potential due to dilution. Stable emulsions withstand higher dilution effect and do not undergo changes in zeta potential due to dilution [22]. There was no significant difference found in size and zeta potentials of all the tested formulations during the dilution studies with some changes jn PDI. This indicated that all the emulsions were relatively stable.

The effect of storage on stability were determined by incubation of the LNEs at 4 C and room temperature for 6 months and evaluated for size, PDI, Zp, total drug content and entrapment efficiencies. The globule sizes increased by 5-15% of initial size. However, Zp changes are not much different, when compared to LNEs at 0 day (Table 6). Thus, these LNEs were found to be stable at 4 C and room temperature for 6 months period.

Table 4: Effect of autoclaving on size, PDI and zeta potential of selected formulations (n=3).

Formulation code	Before Sterilization			After Sterilization		
	Size (nm)	PDI	Z.P(mV)	Size (nm)	PDI	Z.P(mV)
LNE	243.30±5.17	0.05±0.01	-35.7±1.91	232.30±8.07	0.12±0.03	-33.3±1.11
PLNE -3	77.2±5.05	0.13±0.05	-39.83±0.45	70.7±1.39	0.243±0.14	-36.04±0.4
PLNE -6	91.5±2.36	0.135±0.1	-32.16±0.35	85.80±7.36	0.12±0.11	-27.8±1.64

Table 5: Effect of Dilution (desorption) on size, PDI and zeta potentials (n=3).

Formulation code	Dilution factor	Size (nm)	PDI	Zp (mV)
LNE	1:50	243.30±5.17	0.05±0.01	-35.7±1.91
	1:100	249.5±1.63	0.17±0.2	-34.30±0.43
	1:200	253.5±4.85	0.25±0.41	-33.53 ±2.62
	1:500	259.47±5.17	0.29±0.12	-30.46±2.41
	1:1000	263.2±3.65	0.30±0.04	-29.5±1.53
	1:5000	269.5±1.65	0.29±0.1	-27.1±3.34
PLNE -3	1:50	77.2±5.05	0.13±0.05	-39.83±0.45
	1:100	78.32±2.68	0.19±0.04	-38.33±1.34
	1:200	80.43±3.33	0.13±0.03	-37.23 ±2.22
	1:500	81.24±4.46	0.17±0.06	-36.8±0.15
	1:1000	83.3±3.43	0.21±0.03	-35.1±3.54
	1:5000	84.6±2.21	0.23±0.04	-34.5±4.34
PLNE -6	1:50	91.5±2.36	0.13±0.1	-32.16±0.35
	1:100	92.03±4.34	0.12±0.02	-31.70±2.05
	1:200	95.17±3.90	0.19±0.07	-30.17±4.31
	1:500	96.64±3.99	0.23±0.07	-29.04±3.52
	1:1000	97.52±6.45	0.26±0.04	-28.30±4.22
	1:5000	99.87±3.65	0.30±0.05	-26.0±5.72

Stability of parenteral emulsion was mainly studied in terms of maintaining of its physical integrity, namely the dispersed phase particle size, charge and size distribution on storage at 4 C and RT. [23]. measured the zeta potential as the indicator for the surface potential to evaluate the stability of lipid emulsions. There were no appreciable changes in size, Zp and PDI indicating that the LNEs were stable on storage (Table 6).

Cytotoxicity

Basing on the results of stability testing and release studies the following formulations were selected (LNE, PLNE-3, PLNE-6) for the cytotoxicity study on MCF-7 and Hela cell lines. The IC 50 values were calculated by using the GraphPad Prism software (version 5).The IC 50 values on MCF-7 cell lines of LNE, PLNE-3, PLNE-6 and DS were found to be 32.31±1.2 nM , 26.91±0.8 nM, 22.97±1.3 nM and 35.68±1.9 nM respectively (Table 7) . The IC 50 values on Hela cell lines of LNE, PLNE-3, PLNE-6, and DS were found to be 37.79±1.12 nM, 31.7±1.3 nM, 26.13±1.2 nM and 41.71±1.67 nM respectively (Table 7). The IC 50 values of PLNE-3 when compared to DS, was found to have 1.32 and 1.31 folds more cytotoxic activity on MCF-7 and Hela cell lines. Similarly, when compared to LNE, PLNE-3 was found to have 1.2 and 1.19 folds more cytotoxic activity on MCF7 and Hela cell lines respectively. The statistical results of one way ANOVA by Newman–Keuls Multiple comparison test when applied it was found that PLNE-3 was statistically significant with DS and non-significant with LNE $P<0.05$.

When compared to DS, PLNE-6 was found to have 1.55 and 1.6 folds more cytotoxic activity on MCF-7 and Hela cell lines. Similarly, when compared to LNE, PLNE-6 was found to have 1.4 and 1.45 folds more cytotoxic activity on MCF-7 and Hela cell lines respectively. The statistical results of one way ANOVA by Newman–Keuls Multiple comparison test when applied, it was found that PLNE-6 was statistically significant with DS and LNE $P<0.05$.

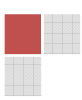

Table 6: Effect of storage (4ºC and RT) on stability of LNEs (n=3).

Formulation code	Time (in Months)	Storage at 4◦C			Storage at RT		
		Size (nm)	PDI	Zeta(mV)	Size (nm)	PDI	Zeta(mV)
LNE	0 month 1	243.30±5.17	0.05±0.01	-35.7±1.91	243.30±5.17	0.05±0.01	-35.7±1.91
	month 2 month	247.23±3.10	0.13±0.07	-34.5±0.85 -	250.21±3.10	0.16±0.08	-33.5±0.85
	4 month 6	252.7±7.24	0.21±0.06	33.60±1.06 -	258.7±7.64	0.18±0.05	-32.80±1.06
	month	259.7±6.95	0.32±0.04	30.93±1.50 -	265.7±6.65	0.31±0.06	-29.93±1.50
		265.1±7.6	0.45±0.04	31.43±1.30	272.1±8.65	0.41±0.16	- 26.43±1.30
PLNE-3	0 month 1	77.2±5.05	0.13±0.05	-39.83±0.45	77.2±5.05	0.13±0.05	-39.83±0.45
	month 2 month	78.03±6.99	0.17±0.07	-37.50±2.01	79.03±6.91	0.21±0.05	-36.50±2.81
	4 month 6	81.37±5.34	0.19±0.04	-36.80±1.77 -	83.37±4.44	0.21±0.02	-35.80±1.57
	month	82.57±7.04	0.21±0.03	35.20±0.62	85.57±7.4	0.16±0.05	-33.20±2.62
		85.27±7.98	0.27±0.04	-34.80±0.26	87.27±7.8	0.27±0.04	-31.80±1.26
PLNE-6	0 month 1	91.5±2.36	0.13±0.1	-32.16±0.35	91.5±2.36	0.13±0.1	-32.16±0.35
	month 2 month	92.03±3.34	0.18±0.03	-31.70±1.05	93.83±3.34	0.2±0.02	- 30.70±1.05
	4 month 6	93.57±2.23	0.2±0.02	-29.17±2.41 -	94.97±2.23	0.21±0.03	-28.17±2.51 -
	month	96.77±2.39	0.21±0.03	28.07±1.42	97.27±2.39	0.21±0.02	27.73±1.42
		98.43±5.84	0.21±0.02	-26.03±2.72	100.33±5.84	0.14±0.01	-25.93±2.02

The improved cytotoxicity of pegylated docetaxel LNEs exhibited in MTT assay could be attributed to its enhanced uptake because of the lowest size and sustained release of the drug. Various studies showed that pegylation of the low molecular weight drugs provided a sustained release of the drug in the cells due to prolonged retention and higher cytotoxicity [24,25].Thus,thein vitro cytotoxicity study proved that the pegylated docetaxel LNEs acted more effectively in comparison to drug solution in both the cancer cell lines, as evident from the lower IC50 values shown in (Table 7).

Table 7: IC 50 values of different optimized docetaxel formulations on MCF-7 and Hela cell lines (n=6).

S. No	Formulation code	MCF 7 (nM)	Hela (nM)
1	LNE	32.31±1.2	37.79±1.12
2	PLNE-3	26.91± 0.8**	31.70±1.3**
3	PLNE-6	22.97± 1.3 **,a	26.13±1.2 **,a
4	DS	35.68±1.9	41.71±1.67

**Statistically significant with DS (Docetaxel Solution), P<0.05[a] Statistically significant with LNE (Docetaxel Lipid Nanoemulsion), P<0.05

Further, when DSPE-PEG 5000 and DPPE-PEG 5000 are compared, DPPE-PEG 5000 is showing relatively greater effect than DSPE-PEG 5000.

Conclusion

Docetaxel lipid nanoemulsions were prepared, characterized and the influence of various pegylated lipids was investigated. The pegylated lipid nanoemulsions were found to have excellent globule size, entrapment efficiency and retarded release of about 15hrs. Further, in vitro studies on two cell lines clearly indicated to have significant cytotoxic activity. In nut shell, these pegylated LNEs would become useful delivery systems in treating cancer.

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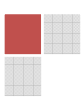

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