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Cisplatin loaded Nano Lipid Carriers for the Treatment of Skin Cancer (AbstractView.aspx?PID=2020-13-3-75)

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Cisplatin loaded Nano Lipid Carriers for the Treatment of Skin Cancer

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ABSTRACT:
The aim of the present study was to develop a Cisplatin loaded NLCS to enhanced and effective delivery of drug to the skin cancer. NLCS bearing Cisplatin were prepared by emulsification solvent diffusion method. The formulated NLCS were characterised for average particle size, polydispersity index (PDI) Zeta potential, entrapment efficiency and in vitro drug release study. The prepared formulations were studied for its in vitro cell line and cell uptake study. It was revealed that the average size of NLCS was found 172.2±3.2, PDI was 0.135, % entrapment efficiency was found 68.9±2.04 and Zeta potential was found -15.3±1.3. In-vitro release determined by Frenz diffusion cell was found 63.7±2.8% after 72 hr. MTT assay shows that Cisplatin loaded NLCS were giving more cytotoxity as compare to plain drug. The cell uptake study was found enhanced uptake of FITC loaded NLCS in comparison to plain FITC. Cisplatin and Cisplatin loaded NLCS showed 22.3±1.2 and 32.8±0.9 growth inhibition respectively after 48h upon incubation at 0.5 µg/mL concentration ($p<0.05$). The result of the studies was concluded that NLCS can be use as impending drug delivery system which may enhance the drug uptake and maintain the drug level for longer period of time and it is potential carrier system which can be use for the treatment of skin diseases like cancer.

KEYWORDS: Cisplatin, NLCS, Cytotoxicity, Skin cancer, Cell uptake.

INTRODUCTION:

The occurrence of melanoma skin cancers cases has been rising over the past decades. According to WHO, 2 and 3 million non-melanoma skin cancers and 132,000 melanoma skin cancers occur globally every year. One in every three who suffered from cancers diagnosed is a skin cancer[1,2,3]. Excessive UV exposure suppresses the expression of p53 suppressor gene which plays a key role in removal of sunburn cells and avoidance of basal cells transformation[4,5]. Cisplatin used to treat a number breast cancer, head and neck cancer, stomach cancer, prostate cancer and non-small-cell lung cancer. It is a alkalyting agent and works by interfering with DNA which stopping cell growth. Common side effects include hair bone marrow suppression, hearing problem, kidney problem, numbness, and heart disease. Unwanted

drug distributions or chemotherapeutic failure is the major reason behind these side effects[6,7] and it is necessary to design a suitable drug delivery system and select a route of administration which overcomes above side effect by preventing unwanted distribution of drug. A need for simple, safe and stable formulation is necessary[8,9,10]. Nano Liquid Crystals (NLCS) are a novel drug delivery systems have properties and advantages like enhanced drug entrapment and reduced drug expulsion on storage[11,12].NLCS are nontoxic and can be prepared without use of toxic materials[13]. They are capable to protect the drug molecules against degradation and provide sustained release pattern[14,15]. The topical administration of Cisplatin-loaded NLCS for skin cancer can be minimizing the side effects of Cisplatin and increase the therapeutic effectiveness. The nano size of NLCS

promotes drug absorption through the stratum corneum, it is the outermost barrier of epidermis that able to retard the drug penetration into skin[16]. This study was aim to investigate and assess the potential of Cisplatin-loaded NLCS for the treatment of skin cancer using skin cancer cell line.

MATERIALS AND METHODS:

Materials:

Cisplatin was obtained as gift sample from Sun Pharma Pvt Ltd. Ahmadabad, India. Stearic acid was purchased from Himedia, Mumbai, India. Oleic acid was purchased from Loba Chemical, Mumbai, India. Soya PC was purchased from Sigma Aldrich, USA. Other chemicals and solvents were used of analytical reagent grade.

Preparation of Cisplatin loaded NLCS:

The Cisplatin loaded NLCS were prepared solvent diffusion melt dispersion techniques according to which the lipid, stearic acid (3.0%w/v), oleic acid (0.5%w/v), and soya phosphatidylcholine (1.5%w/v) were dissolved in 10 mL of enthanol:acetone solvent system in a 50 mL of beaker[17]. The mixture was melted 70 °C to get clear lipid phase. Aqueous solution of Cisplatin (10%w/w of lipid phase) was added to above mixture and stirred at 10000 rpm to disperse the drug in lipid solution and then sonicate the lipid solution for 10 min using probe sonicator (Lark, BTI-48). In separate beaker 60 mL of double distilled water containing tween 80 was stirred under magnetic stirrer and then heated at 70°C with heating mental. The aqueous phase was maintained at 70°C and kept under the high speed homogenizer (IKA T 25 digital ULTRA-TURRAX®, Germany) at 10000 rpm to make microemulaion. The oil phase containing drug was added dropwise to the aqueous phase using preheated (at 70°C) hypodermic syringe. After 30 min of stirring, heating was stopped. Then 20 mL of cold water was added dropwise to reduce the temperature of the dispersion. The dispersion was continuously stirred for 2 hr to removal of ethanol and acetone from the NLCS. After separating the prepared formulation was lyophilized using mannitol as cryoprotectant in Labconco freeze dryer (Labconco, Cascade Freezone Plus 4.5 L Benchtop Freeze dryer, USA) and stored at 2-8C until further used for characterization.

Preparation of Gel Base:

Carbopol 934 (2%w/v) was accurately weighed and dispersed into double distilled water (80ml) in a beaker. This solution was stirred continuously at 800 rpm for 1 hour and then 10ml of propylene glycol was added to this solution. The obtained slightly acidic solution was neutralized by drop wise addition of 0.05 N sodium hydroxide solutions, and again mixing was continued until gel becomes transparent. Volume of gel was adjusted to 100 ml and then sonicated for 10 min on bath sonicator to remove air bubbles. Final pH of the gel base was adjusted to 6.5. Gel was also prepared with plain drug by adding 5%w/w (5mg of drug in 100mg of gel) dispersed properly by following same procedure that was given above. The same procedure was used to formulate NLCs containing gel in which previously prepared NLCs (equivalent to 5mg of drug in 100mg of gel) were added in place of plain drug.

CHARACTERIZATIONS OF CISPLATIN-LOADED NLCS:

Determination of particle size, PDI and Surface Charge:

Mean particle size distribution of Cisplatin-NLCS formulations and polydispersity index (PDI) of the NLCS were determined by photon correlation spectroscopy using a Zetasizer DTS, version 4.10 (Malvern Instrument, UK). Before analysis, the formulations were diluted with 1:9 v/v with deionized water. The particles size and PDI were represented by the average diameter of the Gaussian distribution function in the logarithmic axis mode. Surface charge measurement of the NLCS was based on the zeta potential (ε) that was calculated according to Helmholtz–Smoluchowsky from their electrophoretic mobility. Zeta potential was determined by a Zetasizer (Zetasizer Nano ZS, Malvern Instruments, UK) with field strength of 20 V/cm on a large bore measures cell. Before analysis, samples were diluted with 0.9 % NaCl to adjust conductivity of 50 μS/cm[18]. Particle size, size distribution and zeta potential the prepared formulation was performed at the SAIL, School of Pharmaceutical Sciences, Rajiv Gandhi Proudyogiki Viswavidyalaya, Bhopal (MP), India.

Shape and Surface Morphology:

The shape and surface morphology were examined by Transmission electron microscopy and scanning electron microscopy (SEM). For the SEM analysis, the prepared NLCS were sprinkling on a double adhesive tape which was stuck on an aluminum stub. The stubs were then coated with gold layer about 300 Å of thickness using a sputter coater. The photomicrographs of prepared NLCS were taken with SEM (FESEM, GeminiO, Giess, Neterlands).

The shape of the NLCS was determined by Transmission Electron Microscopy (TEM; Philips CM12 Electron Microscope, Eindhoven, Netherlands). For the TEM, the samples were prepared by taking one drop NLCS dispersion and a copper grid was placed on it. After 60 second the grid was removed and excess of liquid was soaked with filter paper. Then the grid was negatively stained with 2.0 % phosphotungstic acid solution at an 20 kV acceleration voltage. The TEM images of prepared NLCS were taken which was shown in fig. 2. Analysis of sample on SEM and TEM was carried out at IISER, Bhopal, India [18, 19].

Determination of entrapment efficiency (EE) and drug loading efficiency:

Entrapment efficiency of prepared NLCS was determined according to [20] The lyophilized formulation equivalent to 10 mg of drug was taken and dissolved in 50 mL of beaker containing 10 mL of ethanol. The solution was than centrifuged at 10000 rpm for 10 min. The 1.0 mL of supernatant solution was withdrawal with the help of micropipette and transfer into a 10 mL of volumetric flask. The volume was made upto 10mL with ethanol and analyze spectroscopically for drug content using Shimadzu 1700 UV-spectrophotometer at 207nm and it was calculated according to the formula given below.

$$\% \text{ Drug entrapment} = \frac{(\text{Theroretical drug content} - \text{Practical drug content})}{(\text{Theoretical drug content})} \times 100$$

$$\% \text{ Drug loading efficiency} = \frac{(W\alpha - Ws)}{(W\alpha - Ws + Wl)} \times 100$$

Wα stands for the weight of drug added to the formulation and Ws is the analyzed weight of drug in supernatant and Wl is weight of lipid.

CHARACTERIZATION OF NLCS CONTAINING GEL:

Measurement of viscosity:

Viscosity measurements of prepared topical NLCS based gel were measured by Brookfield viscometer using spindle no. 63 with the optimum speed of 10rpm; viscosity was found to be 3059cps.

Drug content:

Accurately weighed equivalent to 1.0 g of topical NLCs gel was taken in beaker and added 20 ml of 0.01N HCl. This solution was mixed thoroughly and filtered using Whatman filter paper no.1. Then 1.0 mL of filtered solution was taken in 10 mL capacity of volumetric flask and volume was made upto 10 mL with 0.01 N HCl. This solution was analyzed using UV-Spectroscope at λmax 300 nm.

Extrudability study:

Extrudability was based upon the quantity of the gel extruded from collapsible tube on application of certain load. More the quantity of gel extruded shows better extrudability. It was determine by applying the weight on gel filled collapsible tube and recorded the weight on which gel was extruded from tube. Extrudability of gel required 170 grams of weight to extrude a 0.6cm ribbon of gel in 6 seconds.

Spreadibility:

Spreadibility of formulation is necessary to provide sufficient dose available to absorb from skin to get good therapeutic response. It was determined by method reported by an apparatus in which a slide fixed on wooded block and upper slide has movable and one end of movable slide tied with weight pan. To determine spreadibility, placing 2-5 g of gel between two slide and gradually weight was increased by adding it on the weight pan and time required by the top plate to cover a distance of 10 cm upon adding 80g of weight was noted. Good spreadibility show lesser time to spread[21].

$$\text{Spreadibility (g.cm/sec)} = \frac{\text{Weight taken to upper slide X Length moved on the glass slide}}{\text{Time taken to slide}}$$

***IN VITRO* DRUG DIFFUSION STUDY:**

The *in-vitro* diffusion study is carried by using Franz Diffusion Cell. Egg membrane is taken as semi permeable membrane for diffusion[22,23]. The Franz diffusion cell has receptor compartment with an effective volume approximately 60 mL and effective surface area of permeation 3.14sq.cms. The egg membrane is mounted between the donor and the receptor compartment. Gel equivalent to 10 mg drug is applied on one side of membrane facing donor compartment. The receptor medium is phosphate buffer saline pH 7.4. The receptor compartment is surrounded by water jacket so as to maintain the temperature at 37 ± 0.5°C. Heat is provided using a thermostatic hot plate with a magnetic stirrer. The receptor fluid is stirred by Teflon coated magnetic bead which is placed in the diffusion cell[13]. During each sampling interval, samples were withdrawn and replaced by equal volumes of fresh receptor fluid on each sampling. The samples withdrawn were analyzed spectrophotometrically at wavelength of drug (Fig. 2)

STABILITY STUDIES:

Stability study was carried out for drug loaded NLCS at two different temperatures i.e. refrigeration temperature (4.0 ±0. 2°C) and at room temperature (25-28±2°C) for 3 weeks. The formulation subjected for stability study was stored in borosilicate container to avoid any interaction between the formulation and glass of container. The formulations were analyzed for any physical changes, average particle size and drug content (Table 2).

CELL CULTURE AND MTT ASSAY:

CELL CULTURE AND MTT ASSAY:

Cell cytoxicity assay (MTT assay) for the optimized formulation was performed using B16F10 skin cancer (melanoma) cells lines. Cancer cells were cultured in RPMI 1640 medium and added 10% of FBS, and 100 g/ml of streptomycin at 37 ºC into CO_2 incubator. After reaching 70% convergence cells were seeded in a 96 well microplate with 1000 µL of growth medium. The prepared samples of plain drug, and optimized formulation of NLCS was added in the 96 well plate in order to increasing concentration of Cisplatin then cells were incubating for 24 hr. After incubation, 200 µl of fresh media containing 20 µl of MTT solution was added and then incubated it for 4 hr at 37 ºC. After incubation, media was replaced with 200 µL of DMSO to dissolve Formazon crystals. The 96 well plate was analyze using microplate reader by taking absorbance at 570nm.

CELL UPTAKE ASSAY:

Preparation of Fluorescein Isothiocyanate Loaded NLCs:

NLCS formulation containing fluorescent label (with fluorescein isothiocyanate (FITC)) were prepared adding 0.04% of FITC in place of drug to the lipid. The fluorescent NLCS were prepared according to method used for the preparation of Cisplatin-loaded NLCs.

Fluorescence Microscopy:

Qualitative Cellular uptake of conjugates was determined with the following procedure. Briefly, the optimized formulation was previously labeled with fluorescein isothiocyanate (FITC) solution. 2.5 x 10^5 cells/well were seeded in a six-well plate and incubated for 24 h at 37°C with 5% CO_2, and then the medium in each well was replaced with 2 mL of medium containing labeled formulation and plain FITC. The fluorescence due to uptake of fluorescent labeled formulation was analyzed under inverted fluorescent microscope (Labomed, Germany) after 4, 12, 24 and 48 hrs[13].

RESULT AND DISCUSSION:

Prepared formulations of NLCS were optimized on basis of particle size, shape, surface charge and entrapment efficiency. Particle size of NLCS determined by light scattering method (Malvern Zetasizer, ZEM 5002, and UK) and found that average particle size of optimized formulation was 172.2±3.21 nm. The PDI was found 0.135 and Zeta potential was -15.3±1.3 (Table 1). It was observed that the particles size of NLCS was increase with increasing the concentration of stearic acid and similarly particle size was decrease with increasing the concentration of tween 80, stirring speed and increasing the sonication time. The size reduction may be due to surfactant action of surfactant, mechanical force and sonication wave force was responsible respectively to reduce the size of particles. There was no significant difference in average particle size was observed with increasing the drug concentration. But in increasing the sonication time the size particle was decrease from. The low PDI value represents the uniformity of formulation in which there is no major difference in size of particles. The PDI of optimized formulation was found 0.135. PDI of formulation was varied with increasing or decreasing the concentration ratio of lipid and surfactant and sonication time. It was observed that when lipid ratio in formulations was decreased and surfactant concentration was increased then the PDI was found decrease. It was due to the high mechanical forced of sonication waves which was resultant in heat generation which leads to agglomerates or denaturing the lipid molecules after breaking the particles.

Table 1: Characterization of Optimized formulation of NLCs

Characterization	Average vesicle size (nm)	% Entrapment efficiency	% Loading Efficiency	PDI	Zeta Potential (mV)
Cisplatin-NLCS	172.2± 3.21	68.9± 2.04	13.7± 0.79	0.135	-15.3 ±1.3

The shape and surface morphology of prepared NLCs was determined by using TEM and SEM. The photomicrograph was taken which shown in figure 1 and was found that they were spherical in shape with smooth in the surface. % Entrapment efficiency of optimized NLCs formulation was found 68.9±2.049%.

a)

b)

Fig. 1: SEM (a) and TEM (b) photomicrographs of optimized formulation of Cisplatin loaded NLCS at 1000KX

It was observed that the percent drug entrapment was decreased with increasing the concentration of surfactant and on increasing the time of sonication. It is due to the extract out the drug from particles on increasing the mechanical force by sonication and size reduction of size NLCS on increasing the concentration of surfactant due to their surfactant action. Stability study data was revealed that the optimized formulation was stable after 3 month of storage at 4°C while at 25-28±2°C, the formulation was found unstable. Stability of formulation was observed on the basis of % drug remain, average particles size and and physical appearance. The average particle size of NLCS were found 172.2±3.2, 173.9±1.2 and 178.9±3.5 nm after 1, 2 and 3 month of storage at 4.0 ±0.2°C while at 25-28±2°C the average particle size were found 182.3±2.3, 196.5±3.7 and 238.3±3.4nm after 1, 2

and 3 moonth of storage. Drug remaining in NLCS formulation were 48.62±1.39, 35.29±1.08 and 23.83±2.11 % after 1, 2 and 3 month of storage at 25-28±2°C while there was no significant changes in % drug remain and physical appearance in NLCS formulation was observed after 3 month of storage at 4°C. (table 2).

Table 2: Stability study of optimized formulation of NLCS

Characteristic	Time (month)					
	1 month		2 month		3 month	
Temp.	4.0 ± 0. 2°C	25-28 ± 2°C	4.0 ± 0. 2 °C	25-28 ± 2°C	4.0 ± 0. 2 °C	25-28± 2°C
Aver. particle size (nm)	172.2 ± 3.2	182.3 ± 2.3	173.9 ± 1.2	196.5 ± 3.7	178.9 ± 3.5	238.3 ± 3.4
% EE	68.9 ± 2.4	67.5 ± 2.5	68.2 ± 1.34	63.5 ± 2.5	67.2 ± 3.15	58.3± 1.09

Prepared gel was evaluated for viscosity, drug content, extrudability, spreadability and drug release study. It was found that viscosity of prepared gel was 3059 cps, drug content was 1mg/20g of gel, Extrudability was 168g and Spreadibility (g.cm/sec) was found that 5.56 (g.cm/sec) respectively (Table 2).

Table 3: Characterization of gel based formulation of NLCS

Characterization	Viscosity (cps)	Release after 72 hr	Extrudability (g)	Spreadibility (g.cm/sec)
Optimized formulation (CIS-NLCS)	3059	63.7±2.8	168	5.56

In vitro drug release from NLCS was carried out using Franze diffusion cell method and found 63.7±3.2% in 72 hr. In first 30 min it was 11.5±2.5% drug release which slightly high. It was due to the release of free drug present in bag after leaching from NLCS. Drug release from NLCS formulation was found in very sustained and controlled manner and follow Higuchi and Korsemeyer Peppas relase kinetic (Fig. 2).

In vitro cell line study data shows that, Cisplatin loaded NLCS was entered by the cancer cells and retard the growth by killing them in very low concentrations. *In vitro* cytotoxicity using B16F10 was evaluated for plain Cisplatin[24] and Cisplatin loaded NLCS formulations in the concentration range of 0.05-0.5 µg of Cisplatin. However, Cisplatin and Cisplatin loaded NLCS showed 22.3±1.2 and 32.8±0.9 growth inhibition respectively after 48h upon incubation at 0.5 µg/mL concentration. This was due to the high penetration of Cisplatin loaded NLCS into cancer cells. The value of IC_{50} for Cisplatin and Cisplatin loaded NLCS was found to be 0.2 µg/mL and 0.1µg/mL following 24 hr and 48 hr incubation, respectively which was more than 2-fold lower as compared to free Cisplatin (Fig. 3).

Value represent as mean ± SD (n=6)
Fig. 2: In vitro drug release of gel based NLCS

Value represent as mean ± SD (n=6)
Fig. 3: *In vitro* cytotoxicity of CISPLATIN and CISPLATIN-NLCS formulations (Percent Cell Growth Inhibition Assay) on skin cancer cell line

Qualitative (Fluorescence) uptake study of FITC, and FITC loaded NLCS was performed to assess the capability of NLCS to penetrate skin cancer cell lines. Similar to the results of the percent cell growth inhibition assay, uptake studies also displayed higher uptake of NLCS in comparison to plain FITC (Fig. 4). Uptake of FITC loaded NLCS by cancer cells showed an enhanced uptake of the fluorescent marker after 4 hr of incubation. A slight uptake was observed with the cell treated with free FITC.

Fig. 4: Cell uptake (fluorescence microscopy) *image of plain drug (A-D) and Cisplatin loaded NLCS (E-H)* after 4, 12, 24 and 28 hr of incubation respectively

STATISTICAL ANALYSIS:

Data are expressed as the mean ± SD and statistical analysis was carried out using one-way ANOVA test using the Graph Pad PRISM software. A value of $P < 0.005$ was measured which was statistically significant.

CONCLUSION:

We have demonstrated that NLCS are promising colloidal Nanosystems for dermal delivery of Cisplatin through the in vitro and in vivo studies. NLCS suspension enhances Cisplatin permeability and picks it up to cancer cells and decreases cell viability at different concentration. According to cell uptake study, a substantial improvement in the restriction of cancer cell was found. Further studies are necessary for the determination of long-term Cisplatin-NLCS stability and the lack of cytotoxicity on the various untargeted organs to exploit this carrier can be used for the treatment of skin cancer.

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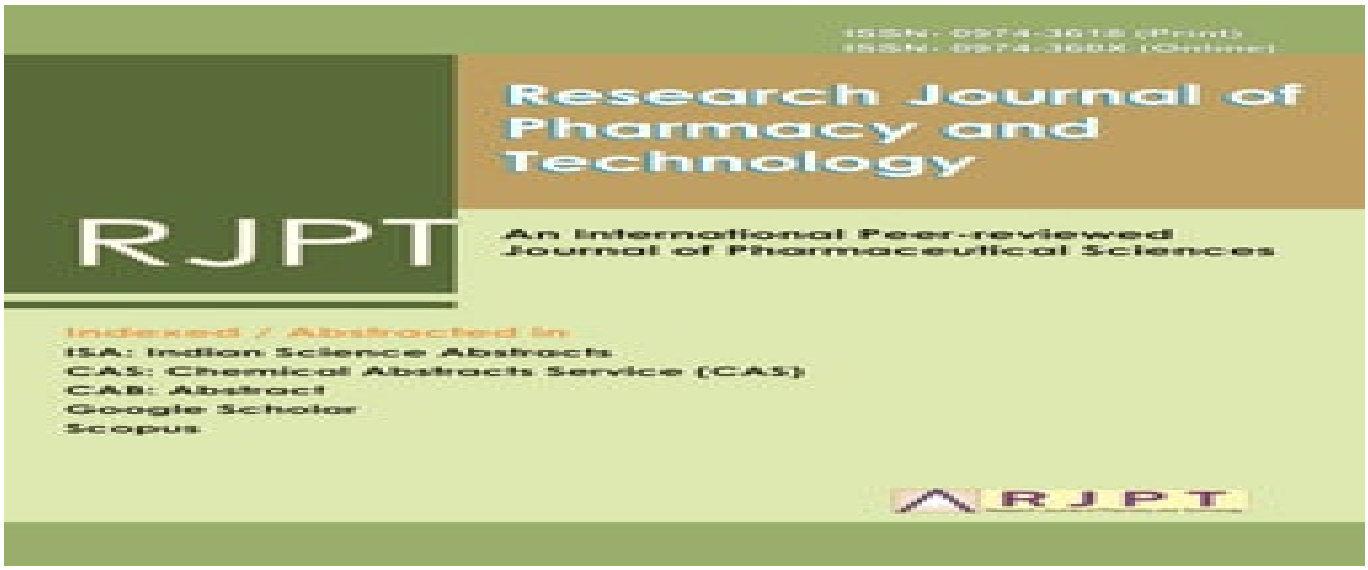

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