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Original Article

CARMUSTINE LOADED NANOSIZE LIPID VESICLES SHOWED PREFERENTIAL CYTOTOXICITY AND INTERNALIZATION IN U87MG CELL LINE ALONG WITH IMPROVED PHARMACOKINETIC PROFILE IN MICE: A STRATEGY FOR TREATMENT OF GLIOMA

BHABANI SANKAR SATAPATHY[1*], JNANRANJAN PANDA[2]

[1]School of Pharmaceutical Sciences, Siksha 'O' Anusandhan (Deemed to be University), Kalinga Nagar, Bhubaneswar, Odisha, [2]Department of Physics, Jadavpur University, Kolkata, West Bengal
Email: bbhabanisatapathy@yahoo.com

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ABSTRACT

Objective: Successful treatment of glioma still remains a tough challenge. The present study aims at the development and evaluation of carmustine loaded nanosize phospholipid vesicles (CNLVs) for the treatment of glioma.

Methods: The experimental NLVs were developed by conventional lipid layer hydration technique and were characterized by different *in vitro* tools such as diffraction light scattering (DLS), zeta potential, field emission scanning electron microscopy (FESEM), cryo-transmission electron microscopy (cryo-TEM), *in vitro* drug loading capacity, drug release study etc. *In vitro* cytotoxicity and cellular uptake of the optimized drug-loaded NLVs were carried out in U87MG human glioblastoma cell line. *In vivo* pharmacokinetic study was conducted in Swiss albino mice.

Results: DLS data showed an average vesicle diameter of 92 nm with narrow size distribution. Optimized CNLVs were spherical in shape with a smooth surface as depicted from FESEM data. Cryo-TEM study confirmed formation of unilamellar vesicles with intact outer bilayer. A reasonable drug loading of 7.8 % was reported for the optimized CNLVs along with a sustained release of CS over a 48 h study period. *In vitro* cytotoxicity assay revealed a considerable higher toxicity of CNLVs than free drugs in the U87MG cells. Confocal microscopy showed a satisfactory internalization of the optimized drug-loaded NLVs in the tested cell line. Pharmacokinetic data demonstrated an enhanced mean residence time of optimized CNLVs in blood than free drug.

Conclusion: Results depicted the potential of experimental CNLVs for the treatment of glioma after further *in vivo* tests.

Keywords: Carmustine, Nanosize lipid vesicles, Glioma, Cytotoxicity, Pharmacokinetic

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INTRODUCTION

Successful treatment of brain tumor still remains a herculean task for medical experts. Glioma is the most common type of primary brain tumor that develops in the glial cells of the brain [1]. Based on the type of glial cells, gliomas can be of astrocytomas, ependymomas, oligodendrogliomas etc. [1]. Glioma at its fourth stage is referred to as glioblastoma multiforme, which is the most dangerous stage with a median survival rate of 1-2 y. According to a report from the American Cancer Society, an estimated 23,890 adults in the United States have been diagnosed with primary brain tumor in the year 2019-20, along with about 3,540 children under the age of 15, which simply exposes the inefficiency of the present therapy [2]. The delicate and sensitive characteristics of brain tissue make surgery and radiotherapy very limited, whereas the presence of the blood-brain barrier (BBB) further restricts the usefulness of chemotherapy [3]. Many conventional chemotherapeutics drugs are though available in the market, but the majority of them fails to maintain the desired therapeutic concentration in the brain tissue due to their inability to pass effectively through BBB [4]. Again, severe dose-related toxic effects associated with conventional chemotherapy throw further challenges for the successful treatment of glioma. Novel drug delivery strategies like nanoliposomes, nanoparticles, polymeric micelles etc. have been investigated widely in past years to improve the efficacy of conventional chemotherapeutic agents [5-7]. However, till today, very few of them are available at the clinical stage. Among various types of nanocarrier platforms, nanosize lipid based vesicular carriers (NLVs) have been largely preferred for the successful delivery of toxic chemotherapeutic drugs to the brain [8]. Due to high lipophilic nature as well as ultra-small size, they fulfill the prime requisite criteria to overcome BBB to get into the brain.

NLVs, also referred as nanoliposomes, are the ultra-micron size phospholipid vesicles consisting of self-assembled lipid bilayers enclosing small aqueous phase in their core [9]. Due to this unique feature, they act as dual platforms for both hydrophobic and hydrophilic molecules. The hydrophobic/lipophilic agents get entrapped in the outer lipid bilayer, where as the hydrophilic agents remain encapsulated in the aqueous core [9]. NLVs owing to their biodegradability, biocompatibility, sustained drug release property, non-immunogenicity, ease of surface manipulation etc. have been the preferred drug delivery vehicle in nanomedicine based research [10]. Due to sustained delivery of the loaded cargo as well as site-specific delivery, the dose of the cytotoxic anticancer drugs is expected to be reduced, leading to better treatment outcomes and fewer side effects.

Carmustine (CS) is a cell-cycle phase nonspecific alkylating anticancer drug, which has been used in the treatment of brain tumors and other types of malignancies [11]. It causes cross-linking in the nucleic acids, leading to inhibition of protein synthesis. However, its short half-life and serious toxic effects like pulmonary fibrosis, bone marrow depression etc. limits its effective use in the treatment of glioma [11-13]. Thus, there is a need to develop novel strategies for the effective delivery of CS to the brain and thereby reducing the dose-related side effects associated with the conventional forms. Till now, no phospholipid based nanocarriers have been evaluated for the successful delivery of CS for the treatment of glioma.

In the lieu of this, the present study aims to investigate the anticancer potential of phospholipid based nanovesicular carrier systems loaded with CS for glioma therapy. The NLVs will be prepared by the conventional method with optimization of critical manufacturing conditions to achieve the desired size range. Preferably, we want to develop the formulation within 100 nm size range for effective tumor tissue permeation and retention as well as to escape from reticuloendothelial system. The selected drug-loaded NLVs will be tested for their *in vitro* effectiveness in U87MG human glioblastoma cell line. Further, *in vivo* pharmacokinetic (PK) study will also be carried out in experimental animal models, which is yet to be reported.

MATERIALS AND METHODS

Materials

CS was obtained as a gift sample from Fresenius-Kabi Oncology Ltd. (West Bengal, India). Cholesterol (CHL), soya-L-α-lecithin (SL), 1,2-distearoyl-sn-glycero-3-phosphotidylethanolamine (DSPE) were procured from Merck (Mumbai, India). Chloroform, butylated hydroxyl anisole (BHA), fluorescein isothiocyanate (FITC) were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai, India). 4',6-Diamidino-2-phenylindole) (DAPI) and tetrazolium dye 3-(4,5-dimers dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Banglore, India). U87MG human glioma cells were procured from National Center for Cell Science (Pune, India). All other chemicals used in the experiment were of analytical grade.

Animals

For PK studies, healthy Swiss albino mice of either sex (male: female ratio 1:1) were used. Animals were purchased from Indian Institute of Chemical Biology (IICB), Kolkata. All animal-related experiments were in accordance with CPSCEA guidelines. Animals were kept in polypropylene cages and maintained in the Jadavpur university animal house at normal room temperature, 55 % relative humidity environment with normal day and night cycle. The protocol of the animal study was approved by the Institutional Animal Ethical Committee, protocol number: SVCP/IAEC/Ph. d/02/2017. Before experiments, animals were properly fed standard diet and drinking water *ad libitum*. The guidelines of Animal Ethical Committee, Jadavpur University were followed strictly during the entire study period. The animals were kept for three weeks in the animal house environment before the study.

Method of development of experimental NLVs

The experimental drug-loaded NLVs were prepared by a conventional thin-film hydration method with necessary modification of process parameters [14]. For the preparation of CS loaded NLVs (CNLVs), SL was used as the main phospholipid. Along with that, we used DSPE and CHL. Briefly, weighed amount of CS, SL, CHL along with DSPE were dissolved in a required volume of chloroform taken in a 250 ml round bottom flask. To this mixture, BHA (2 % w/v) was added as an antioxidant, since all phospholipids are generally sensitive to oxidation. The prepared mixture was then subjected to gentle rotation along with the evaporation of the solvent in a rotary vacuum evaporator (Rotavap, PBU-6, Superfit, Mumbai, India), connected with a water bath. The temperature of the water bath was kept at 40 °C. After, evaporation of chloroform, a thin film was formed along the inner wall of the round bottom flask. The flask was then kept in a dessicator overnight, which caused further removal of any residues of organic solvent still left in the thin film. On day 2, the formed thin film was hydrated with phosphate buffer saline (PBS), pH 7.4 for 1 h at a rotation of 130 rpm. During this period, the formed film was completely dispersed in the PBS. Following hydration, the mixture was subjected to sonication in a bath-type sonicator (Trans-o-sonic, Mumbai, India). Sonication helps the reduction of large size vesicles into the desired ultra-small size range. After sonication, the formulation was allowed to stand for 1 h at room temperature followed by storage in a refrigerator overnight at 4 °C. On day 3, the sample was subjected to cold centrifugation at 15 000 rpm for 45 min (Sigma Lab Centrifuge, UK). After centrifugation, the supernatant was discarded and the sediments were collected, which was stored at-20 °C overnight. On next day, the sample was dried for 12 h in a lyophilizer (laboratory lyophilizer, Kolkata, India).

Development of fluorescent NLVs

For cellular uptake study, fluorescent CNLVs were prepared with FITC. For this, FITC at a concentration of 0.4 % w/v was dissolved in a required volume of chloroform and ethanol mixture. From this stock preparation, about 50 µl was added during the first step of preparation of CNLVs. All other steps mentioned above remained unchanged [15].

Characterization of CS loaded NLVs

Determination of average vesicle diameter (Z-average) and surface potential

For the determination of mean vesicle diameter (Z-average), polydispersity index (PDI) and overall surface charge (zeta potential) of the experimental formulations, a weighed amount of the formulation was dispersed in milli Q water, sonicated for 5 min and observed under a dynamic light scattering (DLS) instrument (DLS-nano ZS, Zetasizer, Malvern Instrument Ltd, UK) [16]. The data was interpretated by the instrument software.

Percentage of drug loading and loading efficiency

For the calculation of the amount of CS loaded in the experimental NLVs, about 2 mg of the lyophilized formulation was dissolved in required volume of acetonitrile. The sample was then sonicated in a bath sonicator for 10 min. After that, it was vortexed for another 3 min followed by centrifugation at 15 000 rpm. After centrifugation, the sediments were discarded and the absorbance of the collected supernatant was measured at 230 nm in UV-visible spectrophotometer (Advanced Microprocessor UV-Visible single beam, Intech 295, India) [15, 16].

The amount of CS loaded in the experimental NLVs was calculated by applying the following formula

$$\% \text{ CS loading} = \frac{\text{Amount of CS in NLVs}}{\text{Amount of NLVs obtained}} \times 100 \quad \ldots\ldots\ldots (1)$$

$$\% \text{ CS loading efficiency} = \frac{\text{Practical \% CS loading}}{\text{Theoretical \% CS loading}} \ldots\ldots (2)$$

Yield percentage

To determine the % yield of each formulation batch, the fully dried CNLVs obtained after lyophilization was weighed after each batch run [15]. The % yield was calculated by applying following equation.

$$\% \text{ Yield} = \frac{\text{Amount of NLVs obtained after lyophilization}}{\text{Total amount of all components used in the formulation batch}} \times 100 \quad \ldots (3)$$

Surface morphology study by field emission scanning electron microscopy (FESEM)

To obtain surface morphology of the optimized CNLVs (CNLV-2), electron microscope was used (JSM 6100, JEOL, Japan). For the experiment, lyophilized CNLV-2 was spread on carbon tape, fixed over a stub. Platinum coating was applied on the tested sample for 5 min with a voltage of 10 kV by means of a platinum coater [14]. Finally, the samples were observed under FESEM under liquid nitrogen conditions.

Cryo-transmission electron microscopy (Cryo-TEM)

For Cryo-TEM analysis, the weighed amount of lyophilized CNLV-2 was dispersed in Milli-Q water. The dispersion was vortexed in a cyclomixture for 5 min and a little quantity of the dispersed CNLV-2 (4 µl) was taken on a clean grid. The sample was then immediately vitrified in liquid ethane followed by storage in liquid nitrogen condition until imaging [15]. Images of the sample were taken with the help of a electron microscope (Tecnai Polora, version 4.6, Netherlands) equipped with an FEI Eagle 4K x 4K charge-coupled device (CCD) camera. During imaging, vitreous grids were transferred into the electron microscope with the help of a cryostage. Throughout the experiment, the temperature of the samples was maintained at -170 °C to observe the NLVs in their native form without any degradation to the bilayer structure.

In vitro drug release study

For the *in vitro* drug release study of the selected formulation (CNLV-2), the conventional dialysis method was employed [16, 17]. For the experiment, a weighed amount of lyophilized CNLV-2 was dispersed in PBS pH 7.4 containing sodium lauryl sulfate (SLS) as a solubilizing agent (release medium). The dispersion was put inside a dialysis bag. The two ends of the dialysis bag were tied with the thread and the whole system was immersed in a beaker containing 100 ml of the above release medium. After that, the beaker was placed on a magnetic stirrer at a rotation of 300 rpm using a magnetic bead. At various time intervals for 24 h, 1 ml of sample was withdrawn from the beaker with the simultaneous replacement of the fresh release medium to maintain the sink condition. The experiment was carried out for 24 h. The samples after collection were filtered with the help of membrane filter followed by measurement of the absorbance at 229 nm with the help of High-

Performance Liquid Chromatography system. PBS containing SLS was taken as the blank during measurement.

Estimation of drug release kinetics

Release kinetics helps to predict the mechanism of drug release from the tested NLVs. For this, the data obtained from the *in vitro* drug release studies were fitted in various kinetic models. We determined the release pattern in five different models such as zero-order (cumulative amount of drug released Vs time), first-order (logarithmic value of cumulative amount of drug remained to be released Vs time), Higuchi (cumulative amount of drug released Vs square root of time), Korsmeyer–Peppas (logarithmic value of the cumulative amount of drug released Vs logarithmic value of time), Hixson–Crowell (cube root of percentage drug remained to be released Vs time) [18]. The linearity of the plots was assessed from the calculated R^2 values.

Assessment of *in vitro* cytotoxicity

The cytototxic effect of the CNLV-2 was tested on U87MG human glioblastoma cell line and the effect was compared to that of free CS suspension at equivalent drug concentrations. For this, MTT assay was used [19]. For the experiment, the tested cell line was cultured in Dulbecco's modified eagle's medium containing 10 % fetal bovine serum in a 96 well culture plate and maintained inside a CO_2-incubator at 37 °C. After attaining the required density of cells in the plates (~5 000 cells per well), the cells were treated with varying concentrations of CNLV-2, free drug suspension and blank NLVs (without drug). As a negative control, few of the wells were treated with equivalent volumes of pure culture medium. After 48 h, the media in each well was discarded and about 100 µl of MTT solution (1 mg/ml) was added to each well. The plate was kept inside CO_2-incubator for another 4 h. After incubation, MTT solution was removed out of the well followed by addition of dimethyl sulfoxide (100 µl) in each well. Addition of dimethyl sulfoxide caused solubilization of formazan crystals to produce a purple color. The intensity of the color is related to the number of viable cells after treatment in the well. The optical density was measured at 560 nm by micro plate reader (Spectra Max, Molecular Devices Corporation, Sunnyvale, USA). Percentage viability of the tested formulation and free drug suspension was evaluated by the following formula

$$\% \text{ Cell viability} = \frac{\text{Optical density of the sample at 560 nm of treated cells}}{\text{Optical density of the sample at 560 nm of untreated cells}} \times 100 \quad \ldots (4)$$

Assessment of internalization efficiency

The internalization capacity of the selected fluorescent formulation (FITC-CNLV-2) was tested on the U87MG cells with the help of confocal microscopy [19]. For the experiment, the cells were seeded in six-well culture plates and allowed to grow on cover slips at a density of 10^4 cells per well. The volume of cell culture was taken as 3 ml per well and incubated at 37 °C in CO_2-incubator for 24 h. FITC CNLV-2 was then added to the culture wells at a concentration of 100 µg/ml. After 0.5 h, coverslips were carefully washed with PBS and the treated cells were fixed with 4 % paraformaldehyde solution. Following fixation, the cells were washed twice with fresh PBS and stained with DAPI. Coverslips were dried and mounted on glass slide for imaging by confocal laser scanning microscope (Andor Spinning Disc Confocal Microscope, Andor Technology, UK).

PK study

PK study was carried out on Swiss albino mice of body weight 20-25 g. The animals were divided into three groups. Each group was having 6 animals. Group I animals were intravenously administered CS suspension (10 mg/kg body weight). Group II animals were intravenously administered CNLV-2, containing CS equivalent to 10 mg/kg. Group III animals received saline (control group). For the study, post intravenous dosing, blood samples were collected from each animal at 0.5, 1, 3, 5, 8, 12, 18, 24 h intervals by heart puncture in pre-heparinized tubes. The blood samples were centrifuged using cold centrifuge at 5000 rpm for 10 min. Plasma was collected and stored at-40 °C till analysis.

For the determination of CS concentration in plasma samples, a LCMS/MS technique was employed [20]. The LCMS/MS Agilent C_{18} column was used. The mobile phase for the analysis was composed of acetonitrile, milli Q water along with formic acid (0.1 % v/v). The flow rate of mobile phase was kept as 0.4 ml/min. Sample volume for injection into the chromatographic column was 20 µl. The analyte was monitored using mass spectrometer equipped with a double quadruple along with an electrospray ionization interface, operated in a positive mode (ESI+). For the analysis, samples were mixed with about three-volume of methyl-tert-butyl ether. The mixture was then vortexed for 5 min followed by centrifugation at 3000 rpm for 10 min. After the process, the extracted CS present in the supernatant was collected. The organic solvent was allowed to dry under nitrogen atmosphere. For LCMS/MS analysis, the dried samples were solubilized in 100 ml of the mobile phase. From the prepared stock, about 50 ml of reference standard solution was added in each sample. From the mixture, 20 µl sample was injected into the LCMS/MS column (Agilent 6410, Triple Quad MS-MS, USA). The important PK parameters i.e. area under the curve (AUC), area under the first moment curve (AUMC), the volume of distribution (Vd), mean residence time (MRT), total body clearance (Clt) etc. were determined using non-compartmental PK Solver software (Version 2.0)

Statistical analysis

All the experiments were carried out triplicate for accuracy and reproducibility. Data was expressed as the mean±standard deviation (SD). One-way ANOVA was used to evaluate statistical followed by Tukey *post hoc* test with the help of Origin Pro 8 software. Differences were considered statistically significant when $p<0.05$ at 95 % confidence level

RESULTS

Formulation optimization

Several formulations were prepared by varying concentrations of drug and lipids along with the variation of specific manufacturing parameters. All the formulations were characterized by different *in vitro* techniques. Out of several formulations, here we report three formulations having desired properties (table 1). We basically compared the formulations based on their drug loading capacity, Z-average, as well as % yield. Out of these three, based on the physicochemical characteristics, we finally selected CNLV-2 as the optimized one for further works.

Determination of % drug loading, loading efficiency and yield percentage

The % drug loading for CNLV-2 was 7.8±0.5%, whereas for CNLV-1 and CNLV-3, the values were 3.2±1.4 % and 4.7±0.8%, respectively. CNLV-2 also showed higher loading efficiency and yield percentage (table 1).

Table 1: **Formulation ingredients, % yield, % drug loading and % drug loading efficiency of selected experimental formulations[b]**

Formulation code	SL: CHL: DSPE ratio (w/w)	% yield[a]	Practical % drug loading[a]	% drug loading efficiency[a]
CNLV-1	100:100:10	48.3±0.2	3.2±1.4	59.8±2.1
CNLV-2	150:50:10	73.6±1.1	7.8±0.5	86.4±1.3
CNLV-3	165:33:10	61.5±0.4	4.7±0.8	72.3±2.4

[a]Data show mean±SD (n =3). [b]Abbreviations: CNLV, carmustine loaded nanosize lipid vesicles

Determination of Z-average and surface potential

DLS data revealed that the experimental formulations were well within the desired nanosize range (fig. 1). The optimized formulation (CNLV-2) showed an average vesicle size of 92.31±1.3 nm (table 2). The PDI value of CNLV-2 was 0.315±0.07 and zeta potential was found to be-69.6 mV (fig. 2). The lower PDI value suggested a narrow size distribution pattern of the experimental

formulation. Higher negative value of zeta potential was reported for the optimized formulation, which indicates the formulation would be stable in the suspension stage due to strong repulsive force between the vesicles.

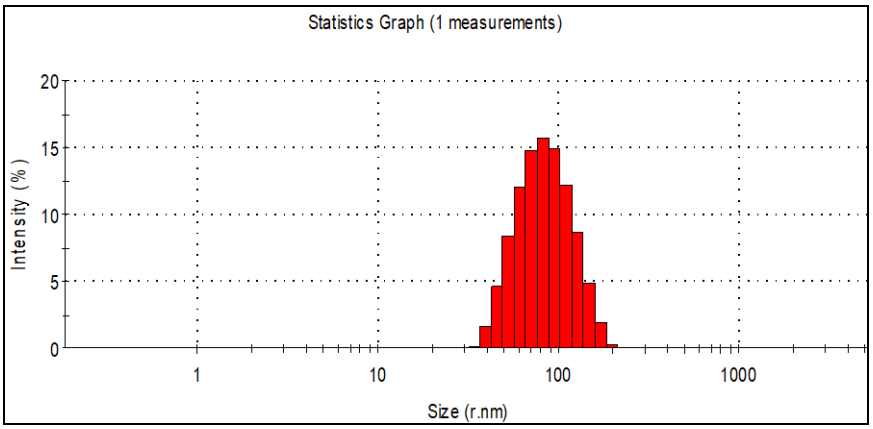

Fig. 1: Average size distributions of selected carmustine-loaded nanosize lipid vesicles (CNLV-2)

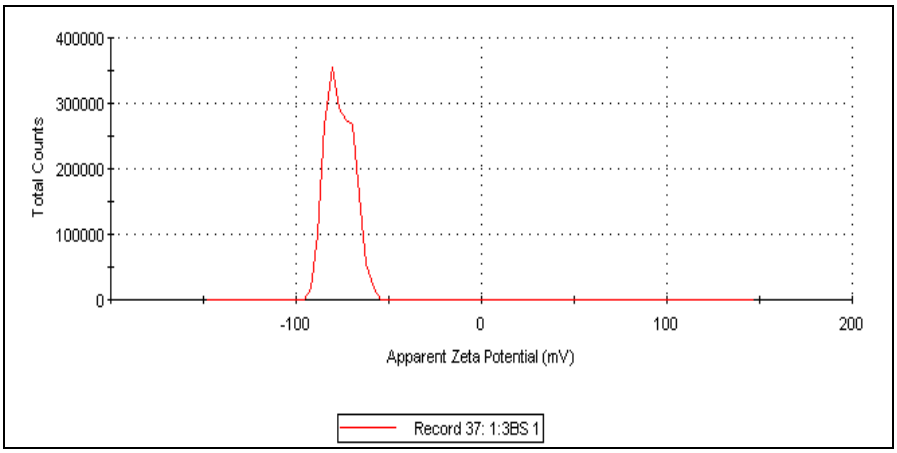

Fig. 2: Zeta potential of selected carmustine-loaded nanosize lipid vesicles (CNLV-2)

Table 2: Determination of Z-average, PDI and zeta potential of the selected formulation

Formulation code	Z-average (dnm.)[a]	PDI[a]	Zeta potential (mV)[a]
CNLV-2	92.31±1.3	0.315±0.07	-69.6

[a]Data show mean±SD (n =3)

Surface morphology study by FESEM

The FESEM image of the CNLV-2 was reported here, which was taken at 75 000 magnification scale (fig. 3a). The FESEM data demonstrated the smooth surface morphology of CNLV-2. All vesicles were found spherical in shape and within 30-40 nm size range. Though vesicles were found placed closely with each other, but there were no signs of any lumps or formation of big agglomerates throughout the formulation, which justifies the good formulation characteristics.

Cryo-TEM study

Cryo-TEM images showed the formation of unilamellar vesicles with intact lamellarity. The size of the vesicles was below 50 nm as depicted from the photograph (fig. 3b). This was in good agreement with the data obtained from FESEM study. All the vesicles were found distinctively spread through the sample without any damage to their native structure.

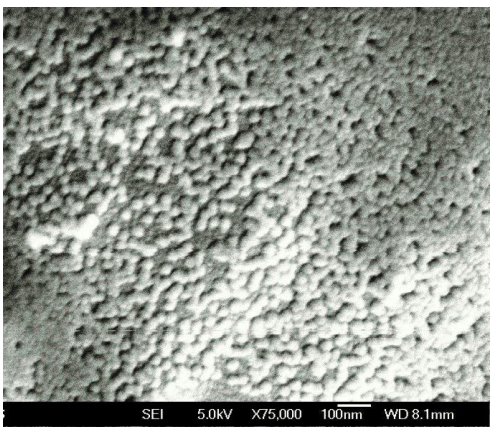

Fig. 3a: FESEM image of optimized formulation (CNLV-2)

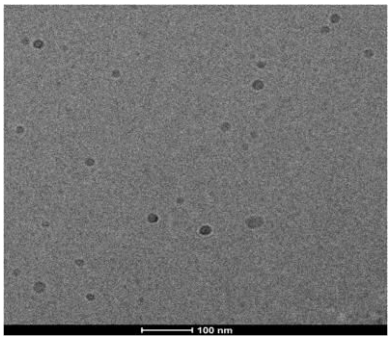

Fig. 3b: Cryo-TEM image of optimized formulation (CNLV-2)

In vitro drug release study and analysis of drug release kinetics

For the *in vitro* drug release study of the optimized formulation (CNLV-2), dialysis method was employed. Release study showed a sustained drug release pattern over a 48 h experimental time length (fig. 4). Initially, the drug release increased with time, but after 12 h, a more sustained release property was observed for the experimental formulation. A cumulative amount of 82.34±3.76 % CS was released from the formulation over the study period. To determine the nature of drug release from the experimental formulation, release data were fitted in different kinetic equations. From the respective graphs, corresponding R^2 values were calculated (table 3). For CNLV-2, among all the tested models, Koresmeyer–Peppas model demonstrated good linearity (R^2 = 0.988).

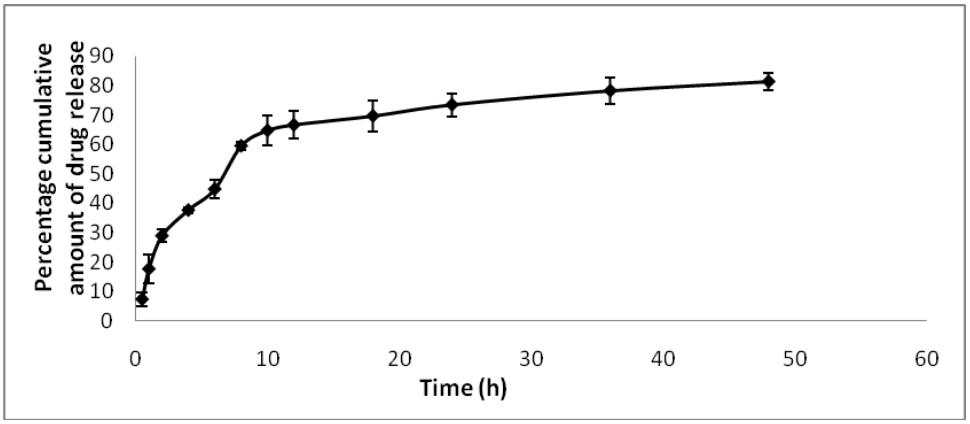

Fig. 4: *In vitro* drug release optimized formulation (CNLV-2). Data show mean±SD (n =3)

Table 3: *In vitro* drug release kinetics with R^2 values for a selected formulation

Kinetic model	CNLV-2
Zero Order Kinetics	y = 2.620x+7.436 R^2 = 0.856
First Order Kinetics	y =-0.018x+2.378 R^2 = 0.933
Koresmeyer Peppas	y = 0.942x+0.434 R^2 = 0.988
Higuchi	y = 16.71x-11.65 R^2 = 0.964
Hixon Crowell Kinetics	y =-0.051x+3.541 R^2 = 0.893

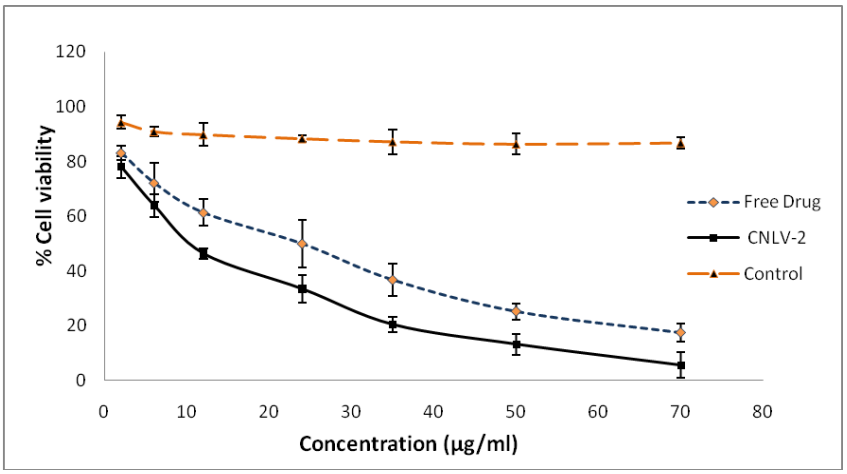

Fig. 5: Comparison of U87MG cell viability (%) upon treatment with optimized formulation (CNLV-2), free drug, and blank NLVs. Data show mean±SD (n =3)

Assessment of *in vitro* cytotoxicity

In vitro cytotoxic or anti-proliferative effect of CNLV-2 was evaluated in U87MG human glioblastoma cells. MTT assay results demonstrated a lower IC_{50} (inhibitory concentration causing 50% of cell death) for CNLV-2 as compared to free CS (fig. 5). The plot of % cell viability against the tested dose (µg/ml) showed that with an increase in the concentration of both CNLV-2 and free drug, the death rate of U87MG cells increased. However, CNLV-2 was found more effective (IC_{50} 12.4±2.1µg/ml) as compared to free CS (IC_{50} 23.8±1.6 µg/ml) at equivalent drug concentration. The results further showed that blank NLVs (without CS) were almost non-toxic to the U87MG cell line even at the highest tested concentration (fig. 5). Percentage of viable cells treated with blank NLVs was much higher compared to free CS, CNLV-2, justifying non-toxic nature of components used for the formulation.

Assessment of internalization efficiency

To estimate whether the optimized formulation possesses the ability to permeate into the cancer cells or not, we tested *in vitro* internalization capacity of the fluorescent optimized formulation (FITC-CNLV-2) in U87MG glioblastoma cells by confocal microscopy (fig. 6). Confocal images of the cell line clearly showed preferential internalization of FITC-CNLV-2 into the cells. We have provided two sets of images at the same time point (0.5 h), the second set of images were taken at higher magnification just to get a clear understanding of the internalization capacity. The fluorescent formulations are distributed throughout the cytoplasm. The nuclei of the cells were stained by DAPI, which distinctively visualized in the image and further confirmed that FITC-CNLV-2 could not cross the nucleus.

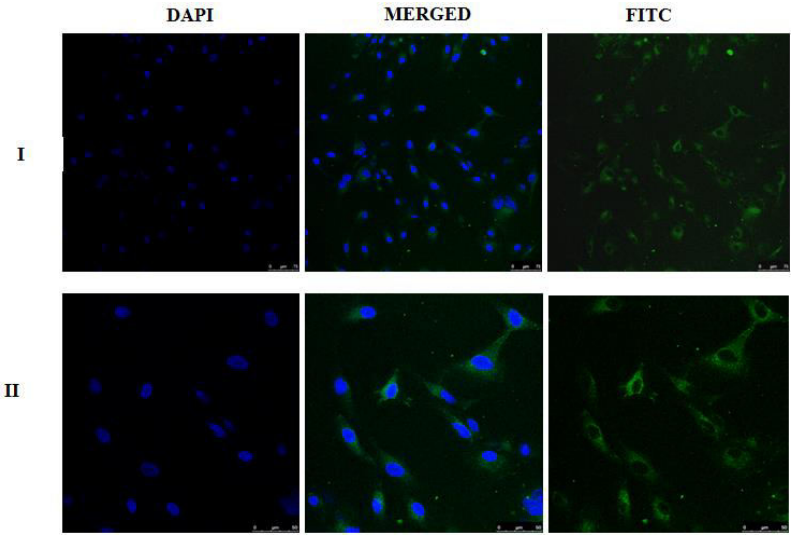

Fig. 6: I. Confocal laser microscopic images of U87MG human glioblastoma cells treated with FITC-CNLV-2 (0.5 h incubation); II. Images were taken at higher magnification

PK study

From the study, a reasonable difference was observed in the important plasma PK parameters between CNLV-2 and free CS treatments. The results were depicted in table 4. From the graph between plasma drug concentration Vs time, a prolonged blood residence time for CNLV-2 than free CS was clearly observed. After 12 h, the concentration of CS was not detectable. However, CS encapsulated in NLVs showed a much-sustained release of the drug and even at 24 h, a well detectable concentration (29.56 ng/ml) was found (fig. 7). $AUC_{0-\infty}$ value was 5348.3±211.4 ng h ml^{-1} for free CS administration, whereas it was 14656.1±1328.6 ng h ml^{-1} for CNLV-2 administration. Similarly, other important parameters like AUMC, MRT also showed preferential enhancements for CNLV-2 as compared to free CS injection. MRT was increased almost 4.5 fold for CNLV-2 treated animals (9.31 h) than the animals treated with free CS (2.24 h). The results thus suggest improved pharmacokinetic parameters of CNLV-2 as compared to the free CS.

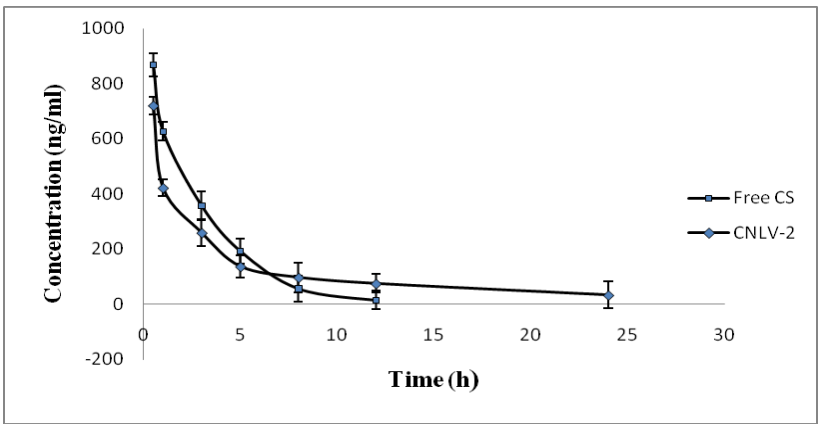

Fig. 7: Plasma concentration-time profiles of CS in Swiss albino mice after i. v. administration of CNLV-2 and free CS suspension (10 mg/kg), data show mean±SD (n=6)

Table 4: Estimation of pharmacokinetic parameters of CS after i. v. bolus administration of free CS and CNLV-2 suspensions

Pharmacokinetic parameters	free CS[a]	CNLV-2[a]
AUC $_{0-\infty}$ (ng h ml^{-1})	5348.3±211.4	14 656.1±1328.6*
AUMC $_{0-\infty}$ (ng h^2 ml^{-1})	29 162.1±2322.1*	81 471.4±131.5
Clt (L h^{-1})	0.008±0.121	0.004±0.023
MRT $_{0-\infty}$ (h)	2.64±0.32	8.31±0.22*
V_d (L)	0.04±0.31	0.16±0.03

[a]Data show mean±SD (n=6). [b]Abbreviations: AUC, area under the plasma concentration-time curve; AUMC, area under the first moment curve; Clt, total body clearance; MRT, mean residence time; t1/2, plasma half life; V_d, apparent volume of distribution, *Data were significantly different ($p<0.05$) where free CS and CNLV-2 were compared. It was assessed by one-way analysis of variance (ANOVA) through *Tukey–Kramer's* multiple comparisons test.

DISCUSSION

The present study was intended to develop an optimized method for formulation of NLVs encapsulating CS, as the model anticancer drug. CS is a FDA approved drug used for the treatment of brain tumor, however, it is associated with severe side effects. Further, presence of BBB restricts its successful application in glioma therapy. Thus, it is hypothesized that phospholipid based ultra micron size carriers may improve the delivery of CS to glioma cells and can maintain the desired therapeutic concentration by enhanced permeability and retention effect. During formulation development, we found that specific composition and process parameters have a significant impact on the formation of nanosize NLVs and to produce desired *in vitro* properties. Initially, we varied amount of SL, CHL (at a fixed CS concentration) to make different batches of formulations. However, we decided to keep the DSPE amount fixed in each batches. DSPE was not the primary phospholipid for the formation of NLVs in our work; rather, it has been incorporated in the formulation to increase the enhanced permeability and retention at the brain-microenvironment. Among different phospholipids present in brain and central nervous system, DSPE constitutes almost 45% of all lipids. Thus, presence of DSPE is expected to impart a brain tissue-mimicking property to the formulation, which would be helpful for its future *in vivo* studies. Reports from our previously published work have already demonstrated the potential of DSPE in improving brain delivery of drug-loaded lipid nanocarriers [15].

In the present work, percentage drug loading was initially increased with an increase in the amount of drug. However, beyond certain amounts, percentage loading was not increased proportionally with the amount drug. This finding was also in good agreement with the previous reports [14, 15]. Further, different process parameters, such as duration of hydration, temperature, sonication time, speed and time of centrifugation etc. affected the morphology, average vesicle size as well as drug loading capacity of the formulations. In our work, we have therefore standardized critical in-process parameters to obtain NLVs with desired physicochemical properties. At a fixed ratio of 1:5 drug: lipid (w/w) along with specific manufacturing parameters such as 45 min hydration in PBS at 140 rpm, 40 min sonication in a bath type sonicator, 45 min ultracentrifugation at 15 000 rpm etc. the obtained formulation showed satisfactory characteristics in terms of % yield, % drug loading and loading efficiency and thus taken for further studies throughout the work.

The selected formulation (CNLV-2) showed a satisfactory percentage of drug loading. For nanosize vesicular carriers, it has always been a problem to achieve higher drug loading. However, in our case, a reasonable drug loading of 7.8 % was reported, which may be attributed to the standardized formulation and in-process parameters.

CNLV-2 had a nanosize range as depicted from DLS study with a narrow distribution pattern. PDI value of CNLV-2 (0.315) was much less than 1, which signifies a homogenous distribution of vesicles in the formulation. It is known that smaller size drug carriers remain suspended for a longer period of time as compared to larger size carriers, since the rate of sedimentation of suspended particles is mostly governed by stoke's law. According to stoke's law, rate of sedimentation of suspended particles are directly proportional to the diameter of the particle. Again, a higher surface charge on the experimental formulations (-69.6 mV) also helps them to remain separated from each other due to higher repulsive force between individual vesicles. It has been reported that zeta potential of more negative than -30 mV or more positive than +30 mV is taken as critical to form stable suspensions [15]. Thus, in our case, ultra micron size (below 100 nm) and a higher negative charge of selected formulation would help to form stable suspension.

FESEM images demonstrated smooth surface morphology, nanosize range (30-40 nm) and clear homogenous nature of the as-formed vesicles. It was observed that size of lyophilized CNLV-2 found in the FESEM image was less than those detected by DLS. It is because the DLS method actually measures an average hydrodynamic diameter of the vesicles in aqueous phase. The formed vesicles while dispersed long time in milli Q water during sample preparation for DLS measurement, might swell and increased in size than their native form. We found similar observations in other reports also [14, 15]. However, size of the sample observed by cryo-TEM was in good agreement with that of FESEM. Cryo-TEM method actually maintains the NLVs in their native form where they are observed under liquid nitrogen environment. Thus, the delicate nature of lipid vesicles is well maintained in cryo-TEM method than normal TEM. Cryo-TEM images showed intact bilayer of the vesicles without any damage to the native structure. We have taken experimental NLVs at a much diluted state so that the vesicles could be distinctively visualized. In FESEM, lyophilized solid samples are taken unlike Cryo-TEM, which shows a relatively compact arrangement of vesicles. Cryo-TEM clearly confirmed the satisfactory production of NLVs in the adopted process as well as maintenance of their internal architecture.

In vitro drug release study was carried out at physiological pH of blood (i.e. pH 7.4) to simulate the *in vivo* environment. As the NLVs are intended for administration through i. v. route only, testing of drug release pattern in physiological pH of blood is considered the ideal simulated condition. The study showed a sustained release of CS from the selected formulation. Initially, though the amount of drug release was increased with time, but after 12 h, a more sustained release pattern was observed. The sustained release property of the selected formulation would favor reduction of dose, dosing frequency and thus would lead to sharp reduction in dose related toxicity during *in vivo* application. The pattern of drug release when fitted to different kinetics models, the formulation was best fitted with the Korsmeyer–Peppas kinetics model. It signifies that the drug release from CNLV-2 might follow complex mechanisms with involvement of both diffusion and erosion. In case of the Korsmeyer–Peppas model, the fraction of drug released with time is generally represented as $Mt/M\infty = Ktn$, where the release mechanism is governed by 'n'. When $n \leq 0.45$, the drug release is said to follow Fickian diffusion mechanism. When value of n lies in between 0.45–0.89, the drug release is considered to follow non-Fickian. When $n \geq 0.89$, drug release is considered to follow Case II (relaxational) transport [19]. In our case, 'n' value was found as 0.793, which suggests that the drug release might follow non-Fickian diffusion.

The *in vitro* cytotoxicity data demonstrated a higher death rate of U87MG cells treated with CNLV-2 as compared to free CS suspension at equivalent drug concentration. Data clearly revealed better antitumor efficacy of the tested formulation than the free drug. CS delivered through NLVs was more cytotoxic to the glioma cells with lower IC_{50} value (12.4 µg/ml) as compared to free CS (23.8 µg/ml). The blank formulation (without drug) showed no significant impact on the cell death rate even at the highest tested concentrations. Clearly, it suggests the biocompatible and non-toxic nature of

excipients of the formulation, which is a good sign. Higher cytotoxicity of the optimized formulation against U87MG cells is very crucial since it would favor for future *in vivo* applications.

In order to visualize the internalization of CNLV-2, FITC was used as a fluorescent marker. The FITC-CNLV-2 produced green fluorescence while visualized under confocal microsope. Further, DAPI was used to stain nucleus, which produces blue fluorescence. Confocal images depicted a preferential uptake of FITC-CNLV-2 by U87MG cells. The formulation was predominantly spread throughout the cytoplasm around the nucleus. Higher cellular internalization of the optimized formulation could be attributed to the ultra micron size range. It may be possible that due to a much smaller size as well as higher lipophilic property, the experimental NLVs could sufficiently permeate through the cancer cells, which is again a good finding towards successful application of the experimental formulation for the treatment of glioma.

The PK parameters of CNV-2 treated animals demonstrated a higher value of AUC, AUMC, V_d, MRT in comparison to animals treated with free CS suspension. The plasma drug concentration for CNLV-2 treated group at 24 h was reasonably higher than that of CS treated groups. The drug concentration for free CS treated groups was not detectable after 12 h, since it dropped beyond the minimum detectable limit (10 ng/ml) of our LCMS/MS system. The data justifies the potential of the optimized formulation for prolonged blood circulation, thus more bioavailability. Owing to its extreme small size, the experimental NLVs might escape from the trap of reticuloendothelial cells successfully and maintained a longer presence in blood.

Harikiran *et al.*, 2018 prepared CS loaded lactoferrin nanoparticles and tested their *in vitro* efficacy against human glioblastoma cell line [21]. Zena Hasan *et al.*, 2019 prepared HER2 antibody functionalized nanosphere carrier for a combined CS-busulfan and trastuzumab and assessed the anticancer efficacy in lung carcinoma cell line [22]. Zhong *et al.*, 2012 prepared the complex of anionic liposomes and adenovirus and encapsulated CS as a chemotherapeutic agent. The formulations were tested on Lewis lung carcinoma and B16 melanoma cells both *in vitro* and *in vivo* [23].

However, our work will be quite different from all such reported works, since till now CS loaded NLVs have not been evaluated for their *in vitro* efficacy on U87MG cells. Further, no study has been reported so far the PK profile of CS encapsulated in NLVs. The formulation development steps were quite simple and optimized. Further all other data including FESEM, cryo-TEM, confocal microscopy, PK profile etc. were quite satisfactory and unique.

CONCLUSION

The study reported an optimized and easily controllable method for the development of a phospholipid based nanovesicular system for sustained delivery of CS for the treatment of glioma. The selected formulation (CNLV-2) showed a preferable nanosize (within 100 nm) as depicted from DLS, FESEM and cryo-TEM study. A reasonable drug loading (7.8%) was reported for CNLV-2 along with a sustained drug release property in a 48 h study period. Owing to its ultra small size and high lipophilic nature, CNLV-2 showed preferential internalization in U87MG human glioblastoma cells. MTT assay showed a higher toxicity of CNL-2 on the tested cancer cells than free CS. The formulation showed improved PK profile in experimental mice models than free drug. Significant increase in important PK parameters like AUC and MRT for CNLV-2 justified its higher bioavailability and *in vivo* stability. Future plan of work includes testing of the optimized CNLV-2 in brain tumor bearing xenograft models to gather data for future clinical applications.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors of this article have no conflict of interest to declare.

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