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Original Article

IN VITRO EVALUATION OF LECTINIZED CISPLATIN BEARING LIPOSOMES SYSTEM

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ABSTRACT

Objective: The purpose of this study was to evaluate the extent and mechanism anti-cancer drug-loaded liposomes using *wheat germ agglutinin* as a guiding molecule.

Methods: For the drug-loaded liposome synthesis, the thin film hydration method was used and the drug cisplatin was loaded during the synthesis and followed by the surface modification using wheat germ agglutinin (WGA) lectin. The developed system was confirmed based on transmission electron microscopy (TEM), atomic force microscopy (AFM), particle size (PS) analyzer, polydispersity index and Zeta Potential analyzer.

Results: The results showed the surface modified by liposomes had the particle size 200±5 nm. The wheat germ agglutinin coated on the surface to liposome led to a reduction in zeta potential and drug entrapment efficiency while particle size increased. Plain liposomes containing cisplatin had less effect than WGA modified liposome on MCF-7 cell lines.

Conclusion: The MTT studies indicated that the drug molecules were initially get delivered to the inside the cell. This formulation offered new simple approach and effectively kill the cells via targeting the nucleus.

Keywords: Liposomes, Wheat germ agglutinin, Cisplatin, MCF-7 cells, Nucleus targeting

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INTRODUCTION

The cisplatin is the anticancer drug and acts by forming platinum-DNA adducts that hinder rapidly dividing cells from duplicating their DNA for mitosis, thus lead to activations and enhancement of apoptosis. Apart from its potential therapeutic effects, it suffers from some limitations like low aqueous solubility, low bioavailability, and drug resistance. The cisplatin resistances mechanisms contribute to the multifactorial nature of the problem and mainly limit the extent of DNA damage, increased DNA adduct repair, and reduced drug uptake [1]. The less cisplatin accumulation in cancer cells may be occurred due to defects in the uptake process, inhibition in drug uptake, an increase in drug efflux. In this regard, some drug delivery based approaches are developed but drug conjugation to nanocarriers, cisplatin provides limited choices. The presence of two free ammonia ligands with amine ligand is highly chelated to the platinum metal ion and reduced amide bond formation with a carboxylic acid [2]. It has been found that the drug encapsulated liposomal system has the potential to protect the stability and integrity of therapeutic molecules and deliver the therapeutic agent to the desired target site. Incorporation of this therapeutic molecule in the lipid bilayer is a self-assembly process and helps to reach drug molecule at the target site in control release manner because the local density of certain polymer residues on the liposome surface increases with the protonation of the polymer pendant groups, triggering the collapse of the liposomal membrane and release of its payloads [3].

In the present work, lectinized liposomal system i. e lectin-conjugated liposomal-system hypothesized as a drug carrier. In this system, WGA lectin was used as ligand because, it show a high transcytosis rate and binds to N-acetyl-D glucosamine and sialic acid of the plasma membrane of cancerous cells. WGA lectin recognized via specific glycosylation pattern i.e. O-glycans overexpressed at the surface of cancer cells, initiating their own uptake. This happens by forming plasma membrane invaginations, due to crosslinking properties of lectins with multivalent carbohydrates and glycoproteins expressed on the cancer cells [4]. In cancerous conditions, N-acetylgalactosamine linked by an alpha glycosidic bond to a serine or threonine (i.e. as an O-glycan), which are frequently overexpressed on the surface of cells. The mechanism involved GlcNAc glycosylation of O-serine (and O-threonine) that helps lectin to transverse and localize in the cytosol and nuclear receptors [5].

In this study, the film hydration method was adopted to develop the liposomal formulation to load anti-cancer drug, cisplatin. For the specific nucleus delivery, the lectin was immobilized on the liposomal surface using N-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC–NHS) chemistry. The lectin coupling to the liposomes enhanced the cellular uptake and also guided the intracellular routing because the lectin has the ability to bind the gangliosides receptors of cells. The nucleus targeting was confirmed through microscopy-based imaging and anticancer activity using MCF-7 cell lines.

MATERIALS AND METHODS

Materials

Cisplatin was obtained from Neon Laboratories Ltd., India. WGA lectin purchase from Medicago AB (Denmark). L-a-soya phosphatidylcholine (PC), stearylamine (SA), cholesterol (CHOL), Triton X-100, Sephadex G-25, N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC), Fluorescein isothiocyanate (FITC) were procured from Sigma Chemicals, St Louis. All other reagents and solvents used were of analytical grade.

Preparation of the non-coupled liposomes (NCL)

Cisplatin loaded conventional liposomes consisting lipids were prepared by a film hydration method as reported by Bangham *et al.* [6]. Briefly, 7:3:1 molar ratio of the lipids (PC: CHOL: SA) were dissolved in a chloroform and methanol mixture (2:1), and evaporating the solvent under reduced pressure in a rotary evaporator (Buchi type, York Sci. Co., Mumbai, India). The dried lipid film was hydrated with phosphate buffer saline (PBS, pH 7.4) consisting 1 mg/ml cisplatin followed by continuous vortexing of the flask for further 1 h to get multilamellar vesicles, which were then sonicated (Ultrasonicator, Imeco Ultrasonic, India) at amplitude 60%, time 5 min and 2 pulse/s to get the unilamellar vesicles.

Preparation of the WGA lectin coupled liposomes (WCL)

WGA lectin was conjugated with liposomes by the method reported by Bogdanov *et al.*, [7] with minor modifications. Briefly, 20 µl of

0.25M EDC and 20 µl of 0.1M NHS were added to 200 µl of liposomes and incubated for 6 h. The unbound WGA lectin was removed by passing the liposomal suspension through the Sephadex G-25 spin column at 2000 rpm for 2 min. An aliquot of the clear supernatants obtained after centrifugation was lyophilized by using 0.5 M glucose as a cryoprotectant in an industrial freeze-drier (Genesis Pilot Lyophilizer) to achieve a preservable white powder.

Characterizations

The analysis of the particle size, PDI and zeta potential (ζ) of the liposomal formulation i. e NCL and WCL were carried out at 25 °C using a Zeta-sizer (NanoPlus AT zeta analyzer and Horiba instrument). The surface morphology of WCL was observed using TEM (TECNAI-12) by using the method previously reported [8]. The external morphology of WCL was further visualized by AFM (INTEGRA Prima, NT-MDT) in a semi-contact mode. Liposomes formulation as the suspension was diluted 10 times with distilled water. This diluted formulation was placed on a glass slide to form a dry film of suspension for observation [9]. The studying proteins and lipids, protein-lipids interactions by using Attenuated total reflection FTIR (ATR-FTIR) Bruker Tensor-37 using established methods [10].

% Entrapment efficiency (EE)

% EE of NCL and WCL were determined by using the method described by Fry *et al.*, 1978 [11]. The amount of drug unentrapped in the NCL and WCL was determined by passing the formulation through a Sephadex spin column, centrifuged at 3,000 rpm, and the eluent was separated and collected. Then collected NCL and WCL liposomes were lysed using 1 % Triton X-100. Mixture of 1.4 mg/ml of o-phenylenediamine (OPDA) solution, phosphate buffer pH 6.8, and dimethylformamide (DMF) (1 : 2 : 7) was added to each formulation i.e., NCL and WCL separately and heated at 100 °C for 10 min in order to get the formation of a light green color. The UV absorption at 706 nm was measured by a UV-Vis spectrophotometer (Thermo Scientific, Orion AquaMate 7000) to estimate the concentration of cisplatin. A solution of cisplatin (10 µg/ml) in phosphate buffer pH 6.8 treated with the same procedure as above was used to prepare a calibration curve of cisplatin [12].

$$\text{Entrapment efficiency (EE)} = \frac{\text{Amount of free drug}}{\text{The total amount of drug}} \times 100 \ldots \text{(eq.1)}$$

***In vitro* drug release**

The *In vitro* drug release rates of cisplatin from both NCL and WCL formulations were investigated by using the dialysis tube (Sigma membrane, MW cut off 13 kDa). The dialysis membrane was soaked in double distilled water for 24 h before being utilized. First, both formulations were separately centrifuged (Remi, Mumbai, India) at 3000 rpm for 2 min through Sephadex G-25 spin-column to remove the unentrapped drug. Subsequently, 1 ml of liposomal suspension of each formulation was taken into a dialysis tube and placed in a separate beaker containing 20 ml of PBS (pH 6.8). The beaker was placed over a magnetic stirrer, and release medium was maintained at 37 °C with constant stirring at 100 rpm throughout the study. Samples were withdrawn at predetermined time intervals and replaced with the same volume of PBS (pH 6.8). The accumulated release of cisplatin was calculated (n=3) by determining the drug quantity.

MTT assay

In a 96-well plate (Corning, USA), 1 × 10 5cells were seeded per well in 100 µL of Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (fetal bovine serum) and allowed to adhere for 24 h. Then, media from the cells was aspirated and various concentrations of formulations were added. The DMEM with FBS was used as a control in each experiment and incubated for 24 h then [13]. Cells were then incubated with 3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT assay) in sterile PBS (5 mg ml−1) for 4 h at 37 °C. After incubation, they were dissolved in 100% DMSO [14] and absorbance(Abs) was measured using a microplate reader at 570 nm (Infinite Pro200, TECAN, Switzerland). The values thus obtained for the untreated control samples were equated to 100% and relative percentage values for DS, NCL and WCL liposomes were calculated accordingly.

$$\%\ \text{Cell viable cells} = \frac{\text{Abs treated cells} - \text{Abs blank(only media)}}{\text{Abs untreated cells} - \text{Abs blank (only media)}} \times 100 \text{ (eq.2)}$$

RESULTS AND DISCUSSION

In the present work cisplatin bearing liposomal drug delivery system was prepared by thin-film hydrations method using PC, CHOL and SA in the ratio of 7:3:1. An optimum ratio of cholesterol with lipid is for required to maintain integrity and rigidity of vesicles [15]. In addition, platinum compounds are present in the core of cisplatin, carries a 2+or 4+charges, two NH3 groups and two C1 groups that allow efficient liposome encapsulation [16, 17].

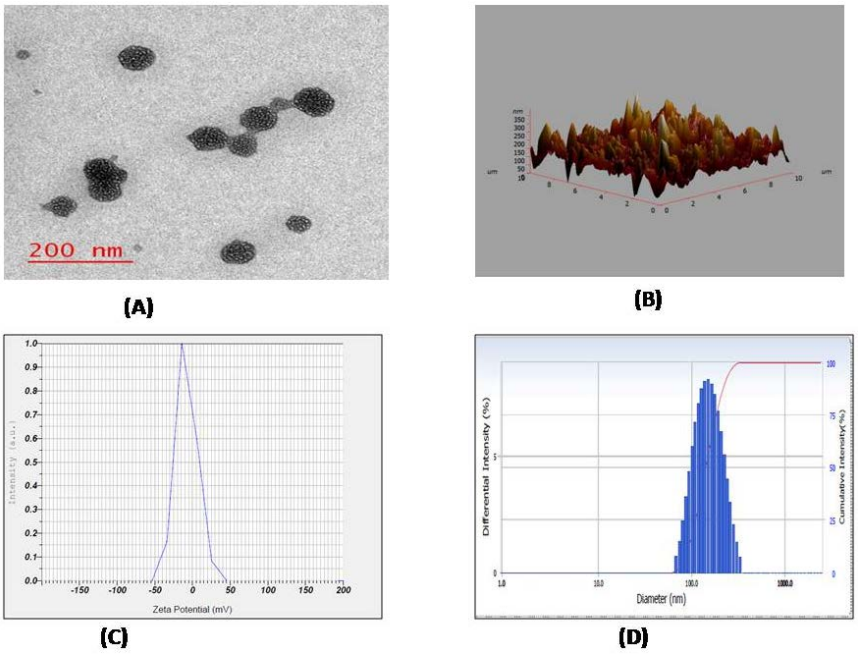

Fig. 1: Results of physico chemical characterizations (A) TEM analysis (B) atomic force microscopy: 3D views (C) Zeta potential (D) Particle size analysis (183.5±0.024 nm)

The developed formulations were further characterized for the size of the vesicles, Zeta potential, polydispersive index, % EE and surface morphology by TEM and AFM. The vesicle size (nm) of NCL and WCL formulations was found to be 154±.045 nm and 183.5±0.024 nm, respectively**.** Importantly, size and composition of the liposome, play a significant role in reticuloendothelial system (RES) uptake and remove the liposomes with a diameter larger than 400 nm, whereas a liposome with a diameter around 200 nm can remain in circulation for a long time [18]**.** The PDI value was found to be in less than 0.2 in both the cases i. e NCL and WCL, which indicates homogenous size distribution of liposomal formulation. The zeta potential of NCL was recorded as-21.26±0.2.5 mV, while in the case of WCL it was-7.3±3.0 mV. The negative charge on NCL and WCL helps to prevent aggregation on storage and, thus, imparts stability to the liposomal dispersion. The % EE of liposomal preparation was 31.5±0.11% and 27.2±0.07 % for NCL and WCL, respectively. Less encapsulation of cisplatin may be due to its water solubility. The surface morphology of WCL liposomal formulation was observed by TEM and AFM microscopy. The TEM images captured reveals the morphology of prepared liposomes. Fig. 1 A, shows the TEM image of the vesicles. In the images, the liposomes were shown in a spherical shape with a smooth surface. The AFM topographical imaging of the optimized formulations is depicted in fig. 1 B. AFM in the semi-contact mode allows the observation of the liposomal surface morphology without staining, labeling, and fixation. AFM also provides the measurement of the height, diameter and surface properties of the formulations as compared to TEM. The fig. clearly shows that the height and the diameter as appeared on the image are range wherein vesicle size was around 200 nm.

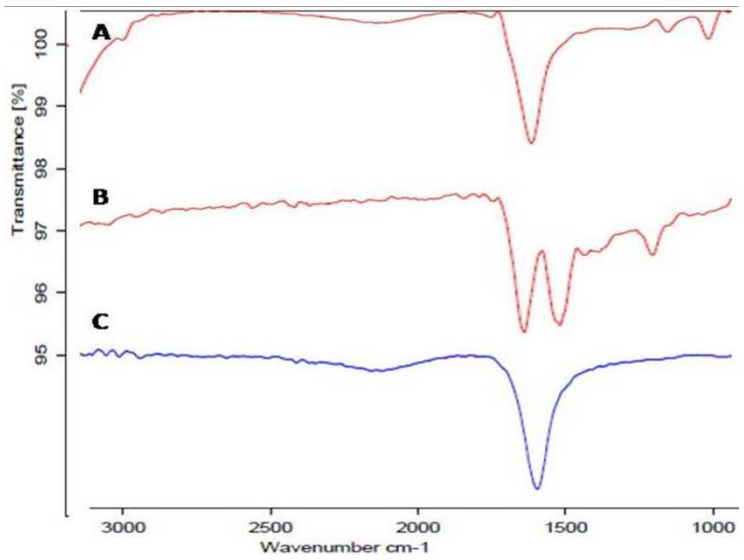

Fig. 2: Fourier-transform infrared spectroscopy (FTIR) spectra results of (A) WGA coupled liposomes (B) WGA lectin (C) Plain liposome

The FTIR analysis was performed to confirm the WGA lectin binding on the liposomes. The results were shown in fig. 2. Characteristics peaks of liposomal formulation at 2920 cm^{-1} and 3369 cm^{-1} were observed, which may be due to the presence of alkyl group and ester group in phosphatidylcholine, respectively**.** Wang *et al.* and Mudakavi *et al.* reported a similar peak pattern for the plain liposomal formulations[19, 20]. Fig. 2 B, shows the characteristics peaks of WGA lectin. According to Bonnin *et al.*, two characteristics peaks of WGA, which correspond to Amide I bands, at 1700-1610 cm^{-1}, are principally due to C=O stretching vibrations with some N-H bending and C-H stretching vibrations while amide II bands, at 1575-1480 cm^{-1}, are principally due to N-H bending vibrations with some C≡N stretching vibrations [21]. The presence of peaks at 1640 cm^{-1} and 1527 cm^{-1} (fig. 2 B) confirms the structure of WGA lectin. Fig. 2 A showed an intense peak at 1639 cm^{-1} due to amide C=O stretch. The disappearance of the WGA peaks i.e., 1640 cm^{-1} and 1527 cm^{-1} (due to Amide I and Amide II) and the appearance of a new peak at 1639 cm^{-1} confirmed the formation of amide linkages between the NH_2 group present on the liposomal surface and–COOH group of the WGA lectin.

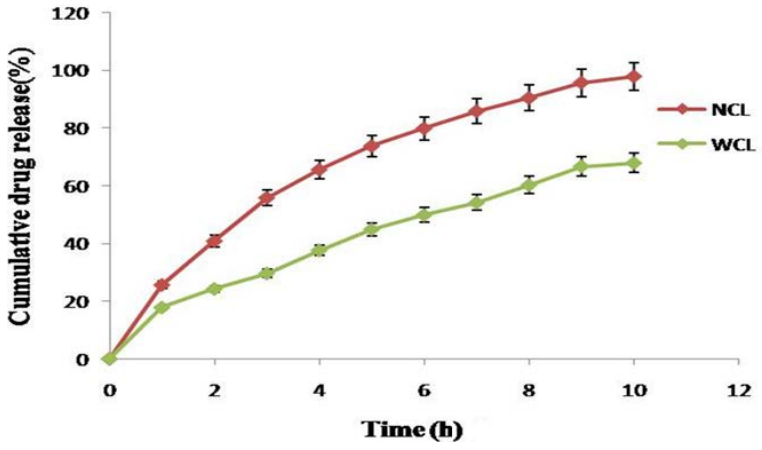

Fig. 3: *In vitro* drug release result of uncoupled liposomes (NCL) and lectin coupled liposomes (WCL)

In vitro drug release study was performed on two different formulations i. e NLC and WCL in phosphate buffer at pH 6.8 at room temperature. The NCL showed the fast releases of the drug as compared to WCL formulations, as shown in fig. 3. The amount of cisplatin released after 1 h was 25.6±1.73 % from NCL while it was 16.8±1.63 % from WCL. After 8 h the cumulative drug release was found to be 90.40±0.53 % from NCL while it was 65.27±0.53 % from WCL. The maximum amount of drug was released within eight hours from the NCL, while in WCL formulation, slow and sustained release pattern was observed. This may be due to the secondary barrier effect because of the WGA coat over the surface of the liposomal formulations. This pattern of drug release clearly indicated that the drug was available over a protracted time in circulation and will provide an enhanced drug effect after administration.

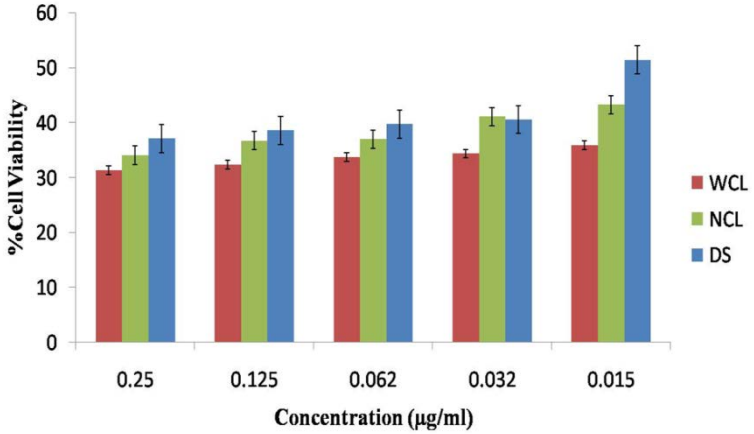

Fig. 4: Cell Cytotoxicity study result of (A) DS (B) NCL (D) WCL

The MTT assay was performed using MCF-7 cell lines. The % cell viability formula of DS, NCL, and WCL, and individual dose-response curves were calculated using the % cell viability formula as shown in eq 2. The % cell viability of plain drug solutions was found to be more as compared to the lectin-modified formulation. The concentration range of 0.015–0.25 µg/ml was used for the calculation of % cell viability. The % control growth at 0.015 mg/ml was found to be 51.39±1.6% for DS, 43.27±1.3% for NCL and 35.82±1.1% for WCL, which decreases as the concentration increases and found to be 37.06±0.7% for free DS, 34.04±0.5% NCL and 31.06±0.1% for WCL at 0.25 µg/ml (fig. 4). The results showed that the formulations were less toxic at 0.015 µg/ml. This may be due to low cellular absorption and internalizations of free drugs. In the case of lectinized formulations, the % cell viability was found to be less as compared to the plain DS and NCL formulation. This may be due to the anchoring of lectin over the liposomal surface that may facilitate their uptake via GlcNAc and sialic-acid receptors as well as due to avoidance of drug resistance (MDR). The reason for the cell death may be due to their transportation through endocytic compartments where they get coated with caveolin-1 protein, travels to the endoplasmic reticulum (ER), and finally reaching to the nucleus.

CONCLUSION

In this work, the lectin conjugated liposomal system was developed and their uptake path way was studied successfully. The studies confirmed the WCL lectin conjugated liposomes bind to the GlcNAc and sialic-acid receptors and it induce the drug uptake. The MTT assay suggested that the anti-cancer drug specifically caused the cell death and the death rates were mainly based on drug concentrations. The cell uptake and internalization studies indicated that the drug molecules were delivered to the cancerous cells.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

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