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Research Article

Caprylate-Conjugated Cisplatin for the Development of Novel Liposomal Formulation

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Abstract. Cisplatin, first (platinum) compound to be evolved as an anticancer agent, has found its important place in cancer chemotherapy. However, the dose-dependent toxicities of cisplatin, namely nephrotoxicity, ototoxicity, peripheral neuropathy, and gastrointestinal toxicity hinder its widespread use. Liposomes can reduce the toxicity of cisplatin and provide a better therapeutic action, but the low lipid solubility of cisplatin hinders its high entrapment in such lipid carrier. In the present investigation, positively charged reactive aquated species of cisplatin were complexed with negatively charged caprylate ligands, resulting in enhanced interaction of cisplatin with lipid bilayer of liposomes and increase in its encapsulation in liposomal carrier. Prepared cisplatin liposomes were found to have a vesicular size of 107.9±6.2 nm and zeta potential of −3.99±3.45 mV. The optimized liposomal formulation had an encapsulation efficiency of 96.03±1.24% with unprecedented drug loading (0.21 mg cisplatin/mg of lipids). The *in vitro* release studies exhibited a pH-dependent release of cisplatin from liposomes with highest release (67.55±3.65%) at pH 5.5 indicating that a maximum release would occur inside cancer cells at endolysosomal pH. The prepared liposomes were found to be stable in the serum and showed a low hemolytic potential. *In vitro* cytotoxicity of cisplatin liposomes on A549 lung cancer cell line was comparable to that of cisplatin solution. The developed formulation also had a significantly higher median lethal dose (LD_{50}) of 23.79 mg/kg than that of the cisplatin solution (12 mg/kg). A promising liposomal formulation of cisplatin has been proposed that can overcome the disadvantages associated with conventional cisplatin therapy and provide a higher safety profile.

KEY WORDS: cisplatin; complexation; cytotoxicity; LD_{50}; liposome.

INTRODUCTION

Cisplatin is the first anticancer platinum compound to be used in cancer treatment and is still holding its position as an effective chemotherapeutic agent among many other agents available. The main disadvantage which hinders its widespread use is its broad, dose-dependent toxicity profile which includes nephrotoxicity, ototoxicity, neurotoxicity, and gastrointestinal toxicity (1–3). Improvements sought to reduce its toxicity profile led to many other platinum compounds to be evolved, which include USFDA approved carboplatin, oxaliplatin, and satraplatin and many others which are still under commercial development like picoplatin, miriplatin, prolindac, BP-C1, and aroplatin (4–6).

Looking at cisplatin's chemistry, in aqueous solution, it undergoes a reversible ligand exchange reaction with water molecules replacing chlorines of cisplatin to form monoaqua and diaqua species (7,8). Cisplatin's anticancer activity and its toxicity, both are attributed to these reactive aqua species which undergo reactions with ligands containing nitrogen, oxygen, and sulfur which in turn lead to its pharmacological actions. These species react with various negatively charged phospholipids of cell membrane (9), *i.e.*, phosphatidylserines, phosphatidic acids, and phosphatidylglycerols; and with negatively charged nitrogen bases of DNA leading to several replication and translation defects that ultimately cause cell death (3,10).

Various novel drug delivery carriers have been tried to enhance its anticancer potential and to reduce its toxicity. Such delivery vehicles include nanoparticles (11), liposomes (12,13), matrix gels (14), nanocapsules (15) *etc*. Liposomes offer an advantage of improving the anticancer activity along with reducing the toxicity of cisplatin. But the development of cisplatin liposomes is hindered by its low lipophilicity. In addition, its low aqueous solubility (~2 mg/mL) hampers its entrapment in sufficient quantities in aqueous compartment of liposomes. So the only formulation available to date is its solution form for intravenous injection.

Complexation of drugs with polymers has been used for various purposes like increasing drug entrapment in a carrier (16,17), sustaining drug release (18,19), and reducing the toxicity (19). Cisplatin's aquated species are reported to form coordination complexes with various ligands like carboxylic acids, negatively charged phospholipids, and other polymers. In addition, fatty acids can incorporate themselves into the

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lipid bilayer; such complexation of drugs with fatty acids may increase liposomal entrapment of drugs. Moreover, conjugation with caprylate has been reported to form a lipophilic derivative of tumor necrosis factor (TNF) and enhance its liposomal entrapment (16,17). If the complexation of cisplatin is carried out with such a fatty acid, it would result in the formation of a complex that might facilitate interaction with lipid bilayer and thereby improve the entrapment of cisplatin in liposomes. This would also double the advantage because as a liposomal formulation, it can be modified as a long circulating carrier, and this would facilitate the tumor delivery of cisplatin due to enhanced permeation and retention (EPR) effect.

Hence, in this investigation, the complexation of cisplatin was done with caprylate ligands to form a cisplatin-caprylate complex which in turn was used for liposome preparation. This led to an increased entrapment of cisplatin in liposomes along with improving the drug:lipid ratio.

EXPERIMENTAL

Materials and Chemicals

Cisplatin was obtained as a gift sample from Sun Pharma Advanced Research Centre (SPARC, Vadodara, India). Hydrogenated soybean phosphatidylcholine (HSPC), egg phosphatidylcholine (EggPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine [methoxy(polyethyleneglycol)-2,000] (DSPE-mPEG-2,000) were generous gifts from the Lipoid Company (Ludwigshafen, Germany). Caprylic acid, sodium salt, was purchased from S.D. Fine Chemicals Limited (Mumbai, India). *o*-Phenylenediamine (OPDA) and cholesterol were purchased from Merck (Germany). A549 lung carcinoma cell line was purchased from the National Culture Collection Society (NCCS, Pune, India). Dulbecco's Modified Eagle Medium (DMEM), antibiotic solution (penicillin G-streptomycin-amphotericin B), fetal bovine serum (FBS), L-glutamine, and 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Himedia (Mumbai, India). All the chemicals and solvents used were of analytical grade.

Preparation of Liposomes

Cisplatin (MW 300.1 g/mol) was complexed with caprylic acid (sodium salt) (MW 166.1 g/mol) in the molar ratios of 1:1, 1:2, and 1:3. Briefly, sodium caprylate was dissolved in water, and cisplatin was added gradually while stirring the solution at 60°C so as to have the maximum solubility of cisplatin (20). Heating was continued until the yellow color of cisplatin disappeared and white-colored complex formed. The resultant dispersion of complex was allowed to cool to room temperature before the preparation of liposomes.

Liposomes were prepared by emulsification solvent evaporation method (compositions of various liposomes shown in Tables I and II). For that, HSPC:Chol (1.5:1 molar ratio; formulations F1, F2, and F3) or HSPC:EggPC:Chol (1:0.5:1, 1:0.7:1, 1:0.9:1 molar ratios; formulations F4, F5, and F6, respectively) were dissolved in chloroform, and emulsification was done with the complex being dispersed by rapid stirring on a magnetic stirrer. The emulsion was stirred at room temperature to evaporate solvent and then heated to 60°C to produce multilamellar vesicles (MLVs). The resultant MLVs were sonicated (3 cycles of 60% amplitude×0.6 s exposure for 1 min) to get small unilamellar vesicles (SUVs) using a probe sonicator (LABSONIC® M, Sartorius, Germany). Unentrapped cisplatin complex was separated from the liposomal dispersion by centrifugation (15,000 rpm for 30 min) (CPR-30 Remi Compufuge, Remi, India). In case of the preparation of liposomes with cisplatin without complexation (HSPC:EggPC:Chol ratio of 1:0.9:1, formulation F7), emulsification was done with cisplatin solution (6 mg/mL) heated at 60°C to dissolve cisplatin. Prepared liposomes were further frozen at −70°C and thawed at room temperature. Freeze-thaw cycle was repeated 10 times. Liposomes, then, were size reduced and passed through a 0.2-μ membrane filter to remove large liposomal particles. Liposomes were separated from free cisplatin by passing through Sephadex G50 column.

Liposomal dispersions were analyzed for entrapment efficiency and vesicular size and observed over a period of 24 h for thickening and under optical microscope for the presence of cisplatin precipitates, if any. Optimized formulation containing HSPC:EggPC:Chol with 1:1 cisplatin:caprylate ratio was PEGylated (formulation F8) using DSPE-mPEG-2,000 by incorporating 5 mol% (based on total moles of lipids in liposomes) of the same in preparing a chloroform solution of lipids. This formulation was used for lyophilization and further analyses. Liposomes were lyophilized with 1:3, 1:5, and 1:10 sucrose levels (lipid to sucrose ratios by weight) used as a lyoprotectant. The liposomes were exposed to lyophilization cycle using Advantage Plus XL-70 Lyophilizer (Virtis, SP Scientific, USA). The lyophilized liposomes were stored in tight glass containers at 4°C and used for further analyses. Liposome integrity was ensured by vesicular size analysis and entrapment efficiency before and after lyophilization.

Characterization

Analysis of Complex

The complex formed between cisplatin and sodium caprylate (cisplatin:caprylate at 1:1 molar ratio) was analyzed by differential scanning calorimetry, infrared spectroscopy, and electron spray ionization-mass spectrometry. Cisplatin, sodium caprylate, physical mixture thereof, and the complex were subjected to DSC and FTIR studies. Differential scanning calorimetry (DSC) was performed on a differential scanning calorimeter (DSC-70, Shimadzu, JAPAN) at a heating rate of 10°C/min over the range of 35–300°C. For the Fourier transform infrared spectroscopy (FTIR) study, the materials were compressed with potassium bromide to form a disc, and FTIR spectra were recorded on FTIR spectrophotometer (Bruker, Japan). Electron spray ionization-mass spectrometry (ESI-MS) of complex was also performed on mass spectrometer (DSQ II, Thermo Scientific, USA).

Vesicular Size and ζ Potential

Mean vesicular size, vesicular size distribution, and ζ potential of PEGylated cisplatin liposomes were determined at 25°C using dynamic light scattering technique (Zeta-Sizer NanoZS, Malvern Instruments Ltd., UK) after reconstituting the lyophilized liposomes with distilled water and diluting up to 10 times with the

Table I. Vesicular Size and Entrapment Efficiency of Various Cisplatin Liposomes

Formulation code	Composition of liposome (mg)	Cisplatin:caprylate molar ratio	Entrapment efficiency (%)	Vesicular size (nm)
F1	HSPC:Chol (32:8)	1:1	95.88±0.41	498.1±23.3
F2	HSPC:Chol (32:8)	1:2	96.35±0.97	552.9±19.5
F3	HSPC:Chol (32:8)	1:3	96.12±1.12	568.3±23.5

HSPC hydrogenated soybean phosphatidylcholine, *Chol* cholesterol

same. Vesicular size and ζ potential of liposomes prepared with free cisplatin (without complexation) and ζ potentials of blank liposomes prepared without cisplatin-caprylate complex and DSPE-mPEG-2,000 were also measured.

Transmission Electron Microscopy

Morphology of prepared liposomes was visualized on cryo-transmission electron microscope (TECNAI G2 Spirit Bio TWIN, FEI-Netherlands) at ×750,000 magnification. The grid was evenly covered with optimized liposomal dispersion after the glow discharge of the grid to render it hydrophilic. It was then frozen in liquid ethane at −180°C. Cryo-frozen grid prepared so was transferred to cryo-holder maintained at −175°C using liquid nitrogen storage box. After inserting the cryo-holder inside the microscope, imaging of the sample was carried out.

Entrapment Efficiency and Drug Loading

Entrapment efficiency of liposomes was determined using the UV-visible spectrophotometric method of derivatizing cisplatin with *o*-phenylenediamine (OPDA) (21). In brief, liposomal formulations were heated at 90°C for 30 min with OPDA solution (OPDA concentration 600 times that of cisplatin on molar basis) in dimethylformamide (DMF). The dilutions were made with DMF-water mixture (7:3 *v*/*v*, pH 6.2 adjusted with 0.1 N HCl), and the reaction product was estimated at 705 nm on the UV-visible spectrophotometer (UV-1,800, Simadzu, Japan). Amount of cisplatin was calculated from the standard calibration plot. Percentage drug entrapment (%DE) and drug loading (DL) in total lipids (mg of cisplatin/mg of lipids) were determined using the following equations:

$$\%\,\mathrm{DE} = \quad = (W_E/W_T) \times 100 \tag{1}$$

$$\mathrm{DL} = (W_E/Wp) \tag{2}$$

where W_E, W_T, and W_P were the respective amounts of encapsulated cisplatin, total cisplatin, and total lipids used.

In Vitro Drug Release Study

Dialysis method was used to determine cisplatin release from liposomes at three different pH values (5.5, 6.6, and 7.4). The activated dialysis membrane (Dialysis Membrane-70, pore size 7,000 daltons, HiMedia Lab. Pvt. Ltd., India) bags were filled with 200 μL of liposomal dispersion diluted to 1 mL with distilled water. Each filled dialysis bag was put in 100 mL dialysis media. Three media, namely pH 7.4 phosphate buffer, pH 6.6 phosphate buffer, and pH 5.5 acetate buffer were used for the study. Constant stirring rate and temperature of 37°C were maintained throughout the study. At appropriate intervals, 500 μL aliquots of dialysis medium were withdrawn and replaced with equal quantity of fresh medium. Samples were stored in a at 2-8°C until analyzed. The amount of cisplatin released was determined using the UV-visible spectrophotometric method after derivatization of cisplatin with OPDA. The drug release was calculated using the following equation:

$$\%\,\mathrm{Drug\ Release} = (C_T/C_0) \times 100 \tag{3}$$

where C_T was the amount of cisplatin released from liposomal dispersion at time t, and C_0 was the total amount of drug in liposomal dispersion.

In Vitro Serum Stability Study

In vitro stability of cisplatin liposomes in the presence of serum was evaluated using a method described by Oku and Namba (22). Liposomal agglutinability in the presence of serum was evaluated by turbidity change. Specific volumes of liposomal dispersion were treated with FBS (so as to have 50% *v*/*v* concentration of serum) and diluted with distilled water. Appropriate volume of liposomal dispersion was taken to give 1 mM of lipid concentration. Control samples (no liposome treatment) were prepared by diluting FBS with distilled water. All samples, after final volume make-up, were incubated at 37°C for 30 min. After incubation, turbidity of

Table II. Effect of EggPC on Entrapment Efficiency and Vesicular Size of Cisplatin Liposomes

Formulation code[a]	Composition of liposome (mg)	Entrapment efficiency (%)	Vesicular size (nm)
F4	HSPC:EggPC:Chol (22:9.7:8)	95.90±0.70	206.0±11.5
F5	HSPC:EggPC:Chol (22:14.6:8)	96.80±1.20	97.5±7.6
F6	HSPC:EggPC:Chol (22:19.4:8)	96.70±1.00	85.8±8.1
F7	HSPC:EggPC:Chol (22:19.4:8)	29.13±3.69	145.8±8.9
F8	HSPC:EggPC:DSPE-mPEG-2,000:Chol (22:14.6:9.5:8)	96.03±1.24	107.9±6.2

HSPC hydrogenated soybean phosphatidylcholines, *EggPC* Egg phosphatidylcholine, *DSPE-mPEG-2,000* 1,2-distearoyl-sn-glycero-3-phosphoethanolamine [methoxy(polyethyleneglycol)-2,000], *Chol* cholesterol

[a] All formulations were prepared using cisplatin:caprylate molar ratio of 1:1 except for F7 where preparation involved use of free cisplatin

each sample was determined by measuring transmittance at 450 nm using transmittance of control sample as 100% (Eq. 4).

$$\text{Turbidity} = T_C - T_F \quad (4)$$

where T_C and T_F were transmittances with control and with formulation treatment, respectively.

Hemolysis Study

Hemolytic potential of liposomes over the range of 0.1 to 5 mM lipid concentration was determined by a method described by Oku and Namba (22). Blood samples collected from Sprague Dawley rats were heparinized and washed three times with normal saline before use. All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department, The M.S. University of Baroda. Blood samples were centrifuged at 3,000 rpm to pellet the red blood cells (RBCs). RBC pellet was resuspended in normal saline containing specific amounts of liposomes (RBC concentration 0.5% *v*/*v*). Treated dispersions were incubated at 37°C for 30 min. After incubation, samples were centrifuged at a low speed (3,000 rpm) (Remi Centrifuge, Remi Laboratory Instruments, India) to separate the RBC mass, and supernatants were analyzed for UV absorbance at a 541 nm wavelength against normal saline as a measure of hemolysis. Positive control and negative control were made using Triton-X100 (0.5%) and normal saline (no treatment), respectively. Percentage of hemolysis occurring at different levels of liposome treatment was expressed according to the following equation:

$$\%\text{Hemolysis} = (A_F/A_C) \times 100 \quad (5)$$

where A_F and A_C were absorbance values with liposomal formulation and with positive control, respectively.

In Vitro Cell Cytotoxicity Study

In vitro cytotoxicity assay of cisplatin liposomes was performed on A549 lung cancer cells by MTT assay (23), and blank liposomes prepared without cisplatin were used as placebo. Cells with no treatment were used as control. Cells were cultured in DMEM supplemented with L-glutamine (2 mM), antibiotic solution (penicillin G, 100 units/mL; streptomycin,100 μg/mL; amphotericin, 50 μg/mL), and 10% FBS. Cells were seeded on a 96-well plate with a cell density of 5,000 cells/well and incubated in 200 μl of DMEM containing 10% FBS for 24 h. After incubation, culture medium was replaced with 200 μl of DMEM containing cisplatin liposomes and cisplatin solution (1 mg/mL) at serial concentrations (0.005–100 μg/mL). After the exposure period of 6 h, each well was replaced with fresh medium. Cells were allowed to grow for a period of 48 and 72 h replacing the medium at each 24 h interval until analyzed by MTT assay. Thereafter, cells were incubated for 4 h with 100 μl of MTT solution (1 mg/mL). The medium was removed, 200 μL of DMSO was added in each well, and absorbance (A) was measured at 570 nm using a microplate reader (BIO-Rad 680 XR, BIO-Rad, USA). Cell viability was calculated according to the following equation:

$$\%\ \text{Cell Viability} = (A_{\text{SAMPLE}}/A_{\text{CONTROL}}) \times 100 \quad (6)$$

where A_{SAMPLE} and A_{CONTROL} were mean absorbance values obtained in the presence and absence of cisplatin (either as a solution or as liposomes), respectively.

In Vivo Acute Toxicity Study

Up-and-down procedure (UDP) of the Organization for Economic Co-operation and Development (OECD) (24) with some modifications was used to determine *in vivo* acute toxicity of cisplatin liposomes. OECD Test Guideline 425 was followed for the study, and animal care was observed as per the guidelines of OECD and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, India). In brief, Swiss Albino female mice (obtained from Torrent Pharmaceuticals, Gujarat, India) were injected with cisplatin solution (1 mg/kg in normal saline) or cisplatin liposomes at specified doses intravenously. Dosing sequence was started from 3.8 mg/kg for cisplatin solution (based on the presumed LD_{50} of 12 mg/kg for cisplatin) and at 4.4 mg/kg for cisplatin liposomes. All the solutions and liposomal dispersions to be injected were sterilized by filtration through a 0.2 μ membrane filter (Pall, India). The test substances were administered in a restrained mouse *via* tail vein using sterile single-use disposable polystyrene syringes. In the circumstance that a single dose was not possible, the dose was given in smaller fractions over a period not exceeding 24 h. Dosing was continued depending on the fixed time interval of 48 h and recording outcomes of all the animals up to 14 days in terms of death. Initial close observation of animals was practiced for 6 h post-injection and then daily at regular intervals. If moribund status was found, animal was euthanized by intraperitoneal injection of phenobarbital (50 mg/mL). Animals were observed for weight loss after injection daily and at the time of euthanasia. Observations recorded were fed to AOT425StatPgm program (EPA, United States Environment Protection Agency), and the LD_{50} was calculated depending on the maximum likelihood method with 95% confidence interval.

Physical Stability Studies

The stability of liposomes on storage was determined by storing freeze-dried liposomes at room temperature (25–30°C) and in a refrigerator (2–8°C) for 3 months based on the guidelines laid by ICH for the stability testing of products to be stored in a refrigerator (25). After intervals of one month, liposomes were reconstituted with distilled water and analyzed for vesicular size and entrapment efficiency.

Statistical Analysis

All measurements were performed in triplicate, and data values presented here are as mean±standard deviation. Data analyses were carried out by Student *t* test and one-way

Table III. Complexation of Cisplatin with Caprylate at Different Molar Ratios

Cisplatin:caprylate molar ratio	Complex Formation	Remarks
1:0.5	−	Yellow cisplatin precipitates were observed on standing.
1:1	+	Uniform white dispersion of complex was obtained.
1:2	+	Aggregates of white cisplatin-caprylate complex observed.
1:3	+	Aggregates of white cisplatin-caprylate complex observed.

analysis of variance (ANOVA), and statistical significance was considered at p values of <0.05.

RESULTS

Preparation of Liposomes

Synthesized cisplatin-caprylate complex appeared as a white dispersion in contrast to the yellow cisplatin. At cisplatin:caprylate ratio of 1:0.5, yellow cisplatin precipitates were observed, while at ratios of 1:1 to 1:3, there were no cisplatin precipitates. Uniform white dispersion was observed at 1:1 ratio, while white aggregates were observed at higher caprylate amounts. Visual observation of cisplatin:caprylate complexes is shown in Table III. Cisplatin:caprylate ratios of 1:1, 1:2, and 1:3 were used for further optimization of liposomes.

The cisplatin level in liposomal dispersion was 6 mg/mL in contrast to the limited solubility of cisplatin in water (2 mg/mL). There were no traces of yellow cisplatin precipitates in liposomal dispersion when observed under an optical microscope. Vesicular size and entrapment efficiencies of various liposomes prepared are shown in Tables I and II. The entrapment efficiency of the prepared liposomes without complexation with caprylic ligands was only 29.13±3.69%, and its vesicular size was found to be 145.8±8.9 nm. Conjugation of cisplatin with caprylate ligands increased the entrapment efficiency, and all the batches showed a significantly higher (>95%) entrapment efficiency. For HSPC:Chol batches, there was no significant difference in the entrapment efficiency of different batches prepared using varying ratios. The vesicular size was found to be in the range of 498–568 nm. With an increase in the caprylate amount, there was an increase in the vesicular size. Moreover, a substantial thickening over a 24 h time period was observed in the batches prepared using 1:2 and 1:3 cisplatin:caprylate molar ratios. Hence, 1:1 ratio of cisplatin:caprylate was considered to be optimum. The batches prepared with HSPC:EggPC:Chol showed no precipitation of cisplatin or thickening over a 24 h observation period. The addition of EggPC resulted in reduction of vesicular size without affecting the entrapment efficiency. Substantially reduced size was obtained by replacing HSPC with EggPC by 1:1.5 molar ratio (30 mol% of HSPC replaced by 45 mol% of EggPC on total lipid basis). In addition, neither any traces of cisplatin precipitation nor any thickening was seen in the formulation over 24 h observation period. The same batch was surface-modified by incorporating DSPE-mPEG-2,000 during preparation and then utilized for further analyses and studies. Entrapment efficiency of optimized batch was compared with that prepared with free cisplatin (no complexation) which was found to be 29.13±3.69%.

Table IV. Effect of Lyophilization on Entrapment Efficiency and Vesicular Size of Liposomes

Lipid:Sucrose Ratio by weight	Entrapment Efficiency (%)	Vesicular size (nm)	PDI (polydispersity index)
Before lyophilization	96.03±1.24	107.9±6.2	0.160±0.026
1:3	94.36±2.35	260.7±8.6	0.428±0.089
1:5	95.22±1.20	206.0±7.9	0.266±0.086
1:10	95.65±0.95	115.3±5.6	0.186±0.046

Lyophilization

Effect of lyophilization on vesicular size distribution and entrapment efficiency is shown in Table IV. Increase in vesicular size along with increase in polydispersity of liposomal dispersion was found at all sucrose levels with the least change observed at lipid:sucrose level of 1:10 by weight. Entrapment efficiency was not affected significantly (p<0.05) on lyophilization. Sucrose to lipid level of 10 was found to be optimum to retain the vesicular size and entrapment characteristics of liposomes.

Analysis of Complex

DSC thermograms (Fig. 1) indicate a marked difference between the thermographs of cisplatin, sodium caprylate, and cisplatin-caprylate complex which confirms the complexation reaction between cisplatin and caprylate. Cisplatin-caprylate complex shows a melting point around 300°C which is significantly different from that of sodium caprylate and cisplatin.

The FTIR spectrum of cisplatin-caprylate complex shows a marked alteration in the characteristic peaks of cisplatin and

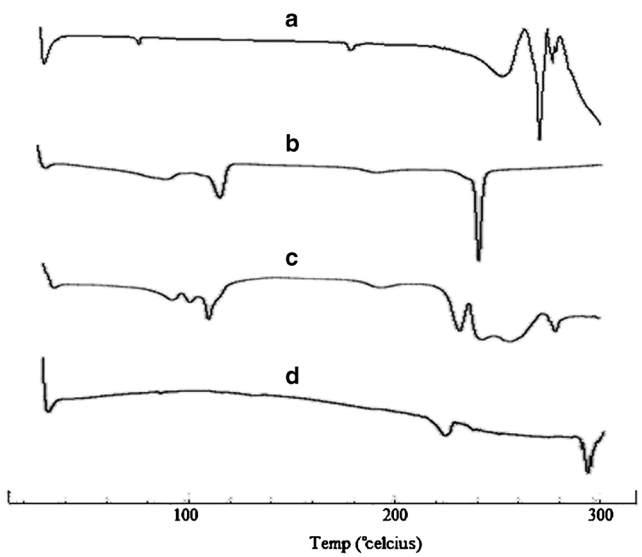

Fig. 1. DSC Thermograms of cisplatin (**a**), sodium caprylate (**b**), physical mixture (**c**), and complex thereof (**d**)

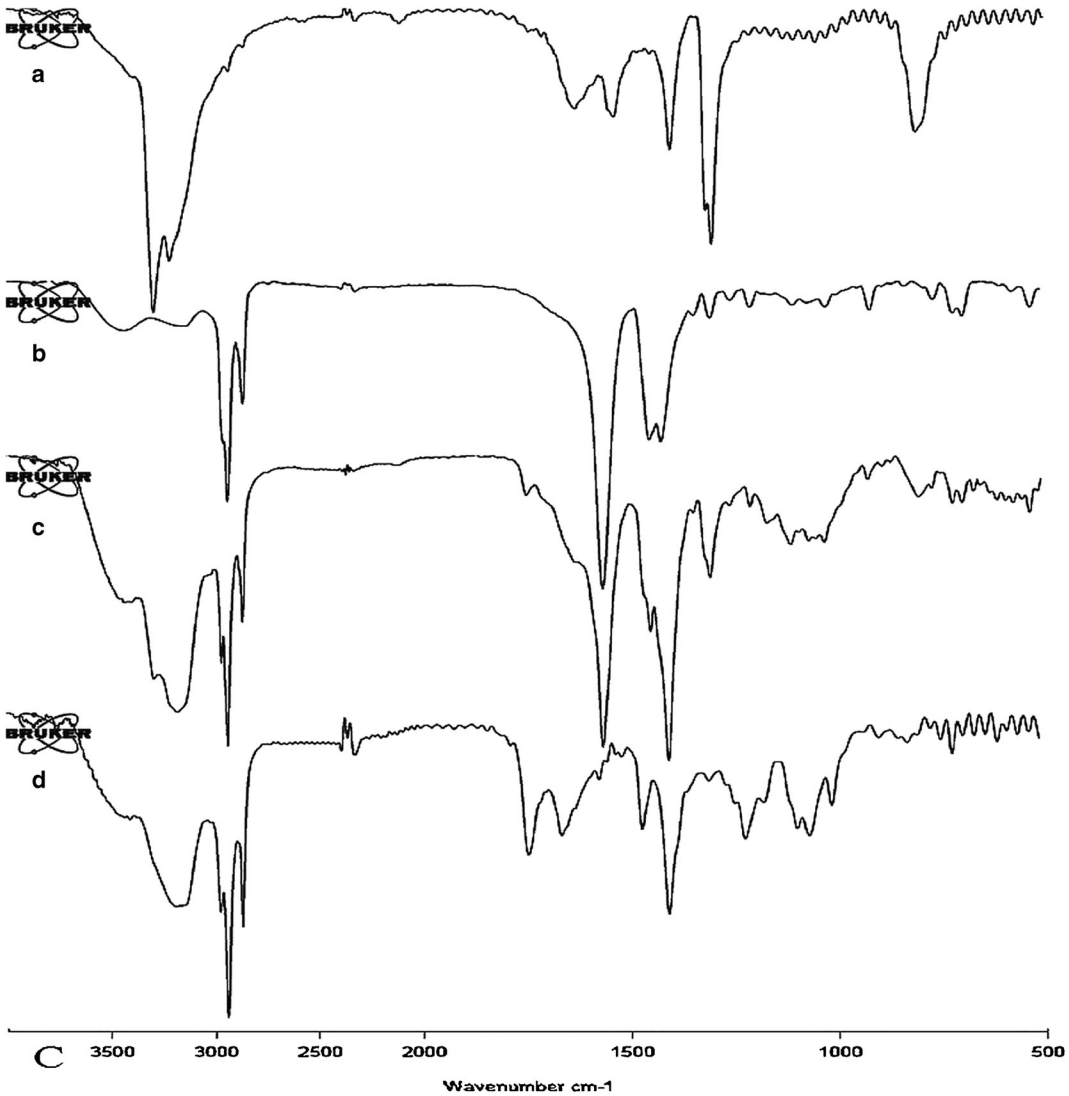

Fig. 2. FTIR Spectra of cisplatin (**a**), sodium caprylate (**b**), physical mixture (**c**), and complex thereof (**d**)

sodium caprylate spectra supporting the complexation of cisplatin with caprylate ligands *via* carboxylate groups of caprylate as shown in Fig. 2. Such alteration includes shift of peak at 1,560 cm^{-} of caprylate carbonyl group to 1,738 cm^{-} and 1,628 cm^{-} and 1,535 cm^{-} peaks of cisplatin to 1,738 cm^{-} and 1,658 cm^{-}, respectively. It is noteworthy that characteristic peaks of amino groups of cisplatin (3,200–3,300 cm^{-}) were intact in complex.

ESI-MS of caprylate complex showed no molecular ion peak; however, characteristic peak corresponding to caprylate complex *viz*. m/z of 372.05 $[(NH_2)_2Pt^{+}(Caprylate)]$ was observed in addition to the m/z of 266.22 and 265 $[(NH_2)2Pt^{+}Cl]$ and 347.02 $[(NH_2)Pt^{+}(Cl)]$.

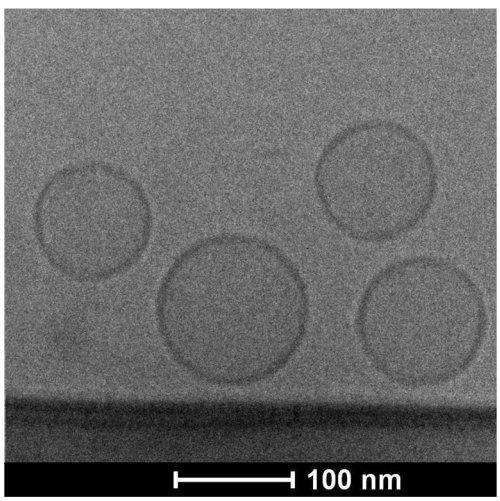

Fig. 3. TEM micrograph of developed liposomes

Vesicular Size and ζ Potential

Vesicular size analysis before and after lyophilization is shown in Table IV. Liposomes showed a slight increase in vesicular size after lyophilization. The increase was very low at 1:10 lipid-to-sucrose level than at lower sucrose amounts (1:3 and 1:5 lipid-to-sucrose by weight). The polydispersity of liposomes was also found to increase after lyophilization, with the least increase occurring at the highest sucrose level used.

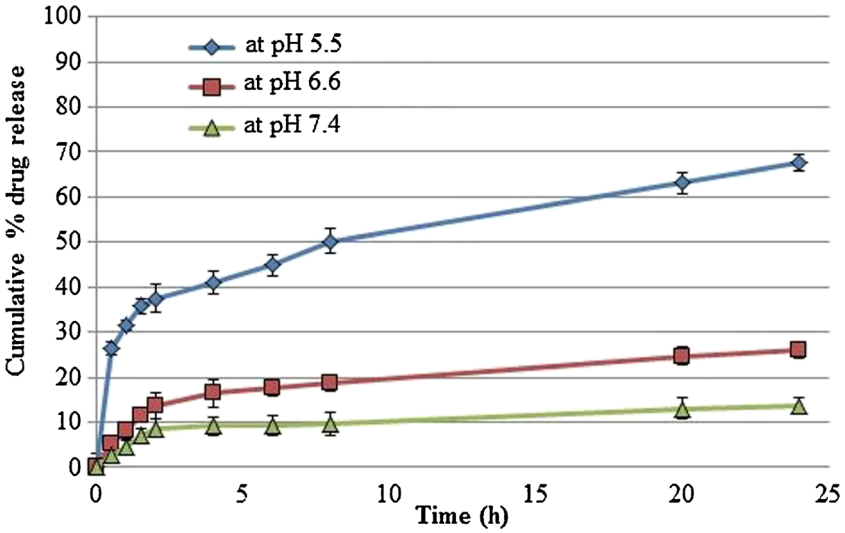

Fig. 4. Cisplatin release from liposomes at different pH

Vesicular size of prepared HSPC:EggPC:DSPE-mPEG-2,000:Chol liposomes was found to be ~115 nm with a polydispersity index (PDI) of 0.186.

ζ potential of prepared liposomes was found to be slightly negative to neutral (−3.99±3.45 mV), while that of non-PEGylated liposomes and non-PEGylated blank liposomes (without cisplatin-caprylate complex) were found to be −12.4 ±4.98 and 3.15±3.41 mV, respectively.

Transmission Electron Microscopy

TEM micrograph (Fig. 3) of developed cisplatin liposomes revealed a spherical small unilamellar structure of vesicles and excludes the formation of micellar or any other structures during liposome preparation. The size of vesicles obtained by TEM was around 110 to 120 nm which was in concordance with the vesicle size results obtained through dynamic light scattering analysis.

Entrapment Efficiency and Drug Loading

Entrapment efficiency of cisplatin-caprylate complex in liposomes is tabulated here (Tables I and II). It was found to be very high, around 94–96%. Entrapment efficiency was not affected to a great extent after lyophilization. Loading of cisplatin in liposomes per milliliter of dispersion was 5.74± 0.06 mg of cisplatin, and cisplatin loaded per milligram of lipids used was 0.26 mg. In contrast, the entrapment of cisplatin in liposomes without any complexation was only 29.13± 3.69%, and cisplatin loaded per milligram of lipids used was 0.08 mg.

Table V. Serum Agglutinability of Liposomes

Formulation (mean ζ Potential, mV)	Turbidity (%)
Control	0.0
HSPC:Chol blank (+3.15)	14.7±1.2
HSPC:EggPC:Chol blank (+4.12)	10.7±0.6
Cisplatin-caprylate non-PEGylated (−12.4)	5.7±0.6
Cisplatin-caprylate-PEGylated (−3.99)	2.3±0.6

HSPC hydrogenated soybean phosphatidylcholines, *PEG* polyethyleneglycol, *PC* phosphatidylcholine, *Chol* cholesterol

In Vitro Drug Release

Release of cisplatin from cisplatin solution and prepared liposomes is shown graphically in Fig. 4. Cisplatin release from cisplatin liposomes was found to be very slow at pH 7.4, showing only 10% release within initial 8 h reaching to a maximum of 13% at 24 h, while liposomes were found to release 19% and 50% after 8 h and up to 25% and 68% of the drug in the span of 24 h at pH 6.6 and 5.5, respectively.

In Vitro Serum Stability

In vitro serum agglutinability of blank non-PEGylated liposomes, non-PEGylated cisplatin-caprylate liposomes, and PEGylated cisplatin-caprylate liposomes against that of control is shown in Table V. There was a drastic decrease in the serum agglutinability of liposomes with PEGylation. The average surface potential of various liposomes is also shown in the parentheses indicating the effect of charge on serum agglutinability. Turbidity change observed in the liposomes' presence was only 2.3±0.6% as compared to the control sample.

In Vitro Hemolysis

Hemolytic potential of liposomes as compared to that occurring with the positive control (Triton X-100, 0.5%) is shown graphically in Fig. 5. Relative hemolysis occurring with different levels of liposomes on lipid basis (0.1–5 mM of lipids) ranges from 3 to 6%. The negative control also showed 3.1% of hemolysis as compared to the positive control. Liposomes were having only 0.03–0.06% probability to affect hemolysis as compared to the positive control. The hemolytic potentials of the negative control and that of liposome-treated samples indicate that the hemolysis occurred mostly due to the

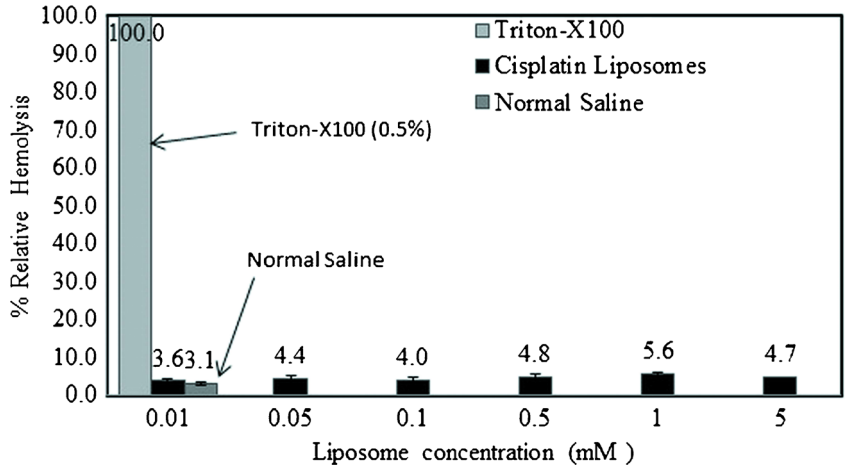

Fig. 5. Percentage of relative hemolysis occurring with developed liposomes

temperature treatment and mechanical damage on shaker rather than due to the presence of liposomal components.

In Vitro Cell Cytotoxicity

In vitro cell cytotoxicity study has revealed that cisplatin liposomes exhibited toxicity equivalent to free cisplatin on A549 cell line. Negligible toxicity was exerted on cells by blank liposomes prepared without cisplatin indicating that the cytotoxicity is solely due to cisplatin. Figures 6 and 7 represent the percentage-inhibition curves of cisplatin solution and cisplatin liposomes at 48 and 72 h, respectively. IC_{50} values of cisplatin solution and cisplatin liposomes at exposure of 48 and 72 h have been shown in Table VI. Decrease in IC_{50} value of liposomes was found at 72 h indicating higher release of cisplatin-caprylate complex from liposomes. The cytotoxic action of complex might either be due to direct ligand exchange reaction between the complex and DNA nitrogen bases or due to the formation of aqua species from complex, which in turn would be reacting with the nitrogen bases of DNA.

In Vivo Acute Toxicity in Mice

Acute toxicity on intravenous administration in mice showed a lethal dose of free cisplatin similar to that can be found in literature (*i.e.*, 12 mg/kg). While liposomes prepared with cisplatin caprylate complex were found to be lethal at a level ~2 times that of the free cisplatin, with LD_{50} value of 23.79 mg/kg of cisplatin based on the encapsulation efficiency of cisplatin in liposomes. Notable reduction in the toxicity of cisplatin was found by encapsulating complexed cisplatin in liposomal formulation.

Physical Stability

Figures 8 and 9 represent the change in vesicular size and cisplatin entrapment efficiency, respectively, of cisplatin liposomes on storage at room temperature (25–30°C) and in a refrigerator (2–8°C). The effect of storage conditions was more pronounced on vesicular size than that on entrapment efficiency. There was neither a significant decrease in entrapment efficiency nor a significant increase in vesicular size

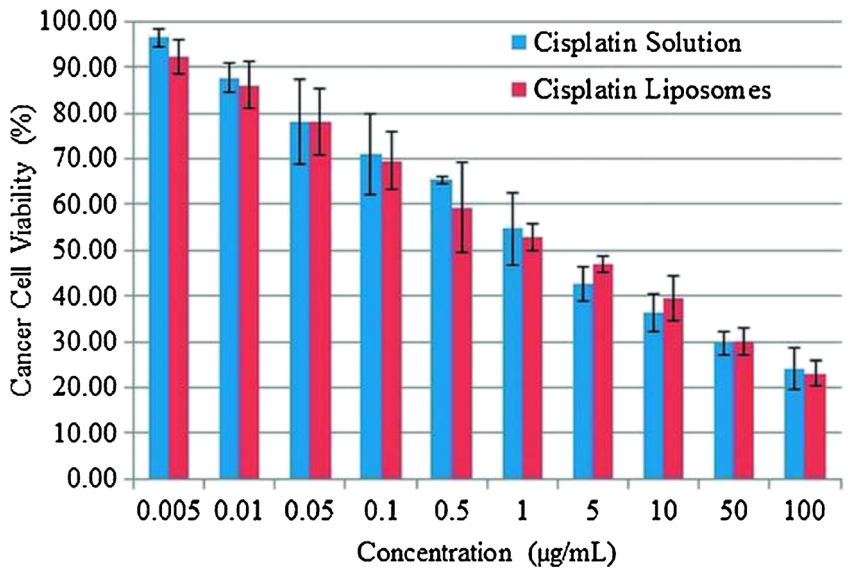

Fig. 6. Viability of A549 cells at 48 h

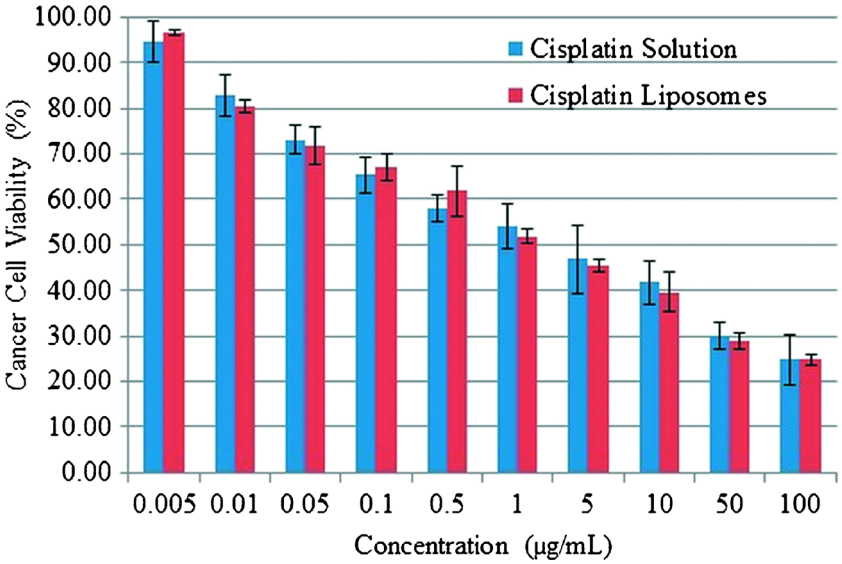

Fig. 7. Viability of A549 cells at 72 h

when lyophilized products were stored in a refrigerator. On storage at room temperature, vesicular size was much more deteriorating as compared to entrapment efficiency. Though low, decrease in entrapment efficiency was more on storage at room conditions than at refrigerated conditions.

DISCUSSION

Complexation with negatively charged fatty acids seems to be a very promising approach for increasing the encapsulation efficiency of cisplatin and other positively charged drugs with very low lipid solubility, in nano-sized formulation. Though classified as a water-soluble anticancer compound, cisplatin is having a very low solubility in water (2 mg/mL). Heating of cisplatin solution at 60°C increases the solubility of cisplatin 3–4 folds (20), and adjusting the pH of the solution to the pKa of aquated forms (pH 6) will increase the aqua species in solution. Complexation of so formed positively charged aquated cisplatin with negatively charged fatty acids will increase the entrapment of cisplatin in the lipid bilayer of liposomes. Aquated species will undergo ligand exchange reaction with caprylate ligands replacing one water molecule from aqua-cisplatin and the formation of coordination bond between carboxyl groups of caprylate ligand with platinum. The proposed complexation mechanism between cisplatin and caprylate ligands and the liposomal incorporation of complex in liposomal bilayer is depicted in the Fig. 10. DSC, FTIR, and ESI-MS studies confirmed the reaction between the cisplatin and caprylate ligands supporting the view of the aforesaid coordination complex formation. These caprylate complexes of cisplatin will push their carbon chains in the liposomal bilayer. Four different cisplatin:caprylate molar levels (1:0.5, 1:1, 1:2, and 1:3) were evaluated to optimize the complexation and liposomal entrapment based on the stoichiometric reaction between the monoaqua and diaqua cisplatin and monovalent caprylate ligand. The complexation of cisplatin was found to be optimum at the level of 1:1 molar level indicating a prominent formation of 1:1 complex (I and II) between cisplatin and caprylate. Higher levels of caprylate ligands yielded aggregates of complex. In addition, higher levels did not affect the entrapment of cisplatin in liposomes significantly. Higher vesicular size observed with HSPC:Chol liposomes could be due to lesser fluidity provided by the saturated fatty chains of HSPC and higher cholesterol content, hindering significant size reduction on sonication. In addition, complex itself would have affected the particle size of liposomes. Incorporation of EggPC which contains various unsaturated phosphatidylcholines provided better surfactant activity to emulsify complex dispersion and also better reduction in vesicular size without reducing the entrapment of cisplatin-caprylate complex.

Table VI. IC_{50} of Cisplatin Solution and Cisplatin Liposomes at Different Time Intervals

	IC_{50} (μg/mL)[a] at	
Formulation	48 h	72 h
Cisplatin Solution	2.34±0.06	2.36±0.12
Cisplatin Liposomes	2.40±0.13	2.01±0.05

[a] IC_{50} is the concentration of cisplatin on exposure of which there would be 50% reduction in the viability of cancer cells

Liposomes are prepared as an aqueous dispersion, so they may undergo various chemical and physical changes, most important of which include vesicular size change (especially with SUVs), drug leakage, and degradation of lipids of liposomes (25–27). To prevent such changes, liposomes were stabilized by lyophilization using sucrose as a lyoprotectant. Sucrose, though required in higher amounts, provides advantages of low cost and low moisture sorption as compared to lactose, mannitol, and other generally used lyoprotectants and bulking agents (26,28). For the cisplatin liposomes, sucrose at the amount of 10 mg/mg of lipid (see Table IV) was found to provide a better stability to liposomes maintaining its vesicular size distribution and entrapment efficiency.

Better delivery of liposomes to tumor on intravenous administration is very much dependent on the size of

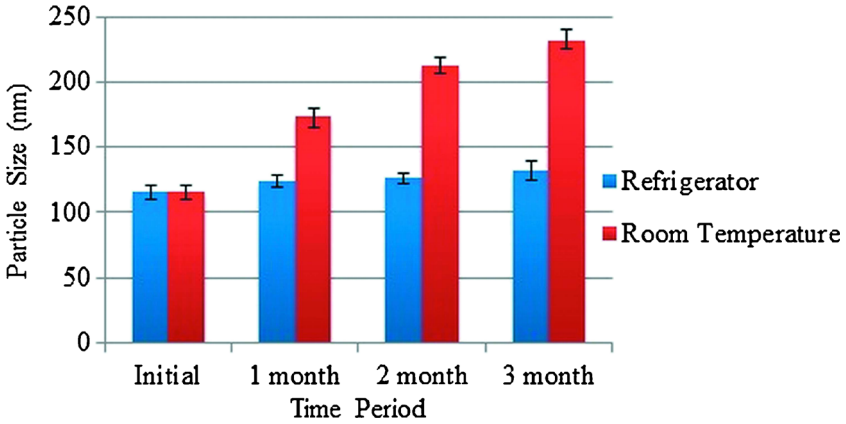

Fig. 8. Effect of storage conditions on vesicular size of lyophilized liposomes

liposomes, as lower vesicular size (<200 nm) enhances the accumulation of liposomes inside tumor tissue by enhanced permeation and retention (EPR) effect (29). It has been reported that such tumor-tissue localization of liposomes is dependent on their ability to cross the blood-vessel pores of 100–1,200 nm (30). Vesicular size of the optimized liposomes on reconstitution was around 115 nm that lies within an optimum size range that can effectively cross the tumor vessels and thus passively target the tumor sites.

Considering the ζ potential of liposomes, negative to neutral ζ potential reduces the clearance of liposomes from circulation by opsonization and subsequent RES uptake of liposomes (31,32). Slightly negative to neutral surface charge of liposomes and the incorporation of DSPE-mPEG-2,000 in liposomes indicate that the liposomes would be able to bypass the uptake by macrophages and thus will provide long circulation characteristics to the liposomes. Incorporation of DSPE-mPEG-2,000 reduces the surface charge, and this can be explained by masking of the surface and hence the surface charge by PEG chains.

Being a water-soluble agent with a maximum aqueous solubility of 2 mg/mL, cisplatin is very difficult to entrap effectively in liposomes' water compartment. This in turn also requires a very high lipid composition. The complexation of liposomes with caprylate ligands has provided a high entrapment of cisplatin along with reducing the lipid required to entrap cisplatin to a significant level as compared to two clinically evaluated cisplatin liposomes (SIP-077 and Lipoplatin); 1 mg/7.77 mg of prepared cisplatin liposomes against 1 mg/70 mg of SPI-077 (33), and 1 mg/10.2 mg of Lipoplatin (34).

Drug release studies at three different pH (7.4, 6.6, and 5.5) were carried out to imitate *in vivo* conditions which liposomes ought to face on intravenous administration. Drug release remained less than 15% and 30% at pH 7.4 and 6.6, respectively, while at pH 5.5, the released amount of drug exceeded 65% over a 24 h period. This deduces that the release of cisplatin from liposomes is very much pH-dependent. Very less cisplatin would get leaked from liposomes in blood and normal tissues, while a modest release of cisplatin would take place at tumor interstitium where the pH is slightly acidic around pH 6.5. When liposomes will get endocytosed, liposomes will be exposed to the acidic environment inside the endolysosomes where liposomes will rapidly release most of their drug content. This concludes the controlled release character of developed cisplatin liposomes.

Serum stability of liposomes is considered an important parameter as serum protein binding would increase the aggregation of liposomes. And both of this, binding to serum proteins and binding-induced aggregation would promote RES uptake and so clearance of liposomes from the bloodstream. Such kind of serum binding is dependent on the lipid-bilayer composition. Presence of PEG chains on surface sterically hinders binding to plasma proteins by increasing the hydrophilicity of the surface of liposomes (32,35). Negative to neutral surface charge also reduces such binding as compared to

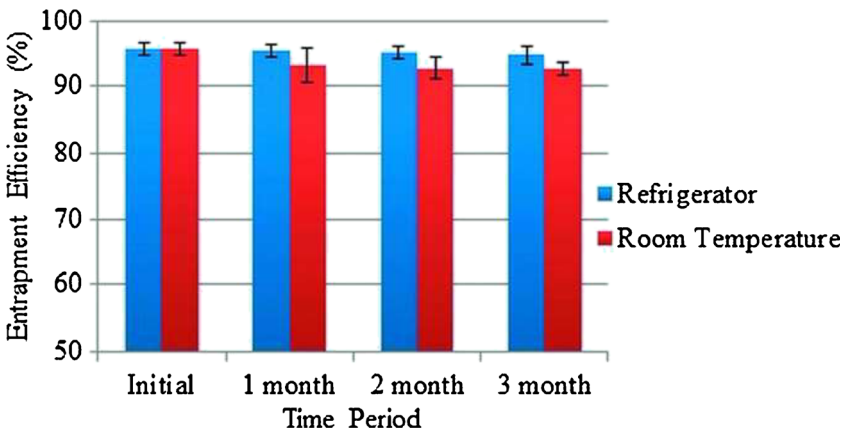

Fig. 9. Effect of storage conditions on entrapment efficiency of lyophilized liposomes

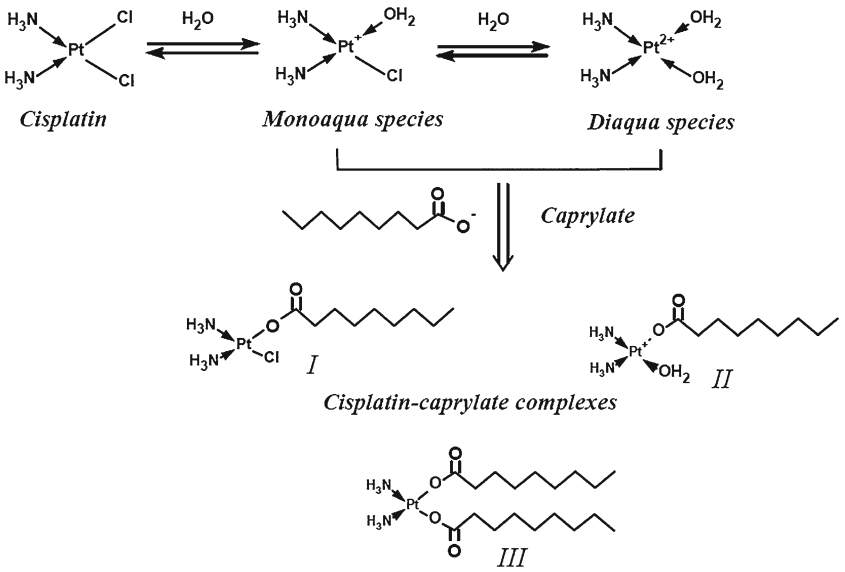

Fig. 10. Aquation of cisplatin and complexation of cisplatin with caprylate

positive surface potential. Results of serum stability studies show that negative ζ potential is effective in reducing the serum agglutinability of liposomes, but negative potential along with PEGylation is more effective in doing so. Cisplatin liposomes were PEGylated and were bearing a surface potential that would increase the serum stability of liposomes in the bloodstream.

Due to the resemblance of liposomes with cellular membranes, liposomes are essentially non-toxic to erythrocytes. But sometimes, free phospholipid components of liposomes and hydrolysis products of phospholipids can lead to hemolysis (36). Formulated cisplatin liposomes were found to cause very less hemolysis up to an exposure level of 5 mM of lipids. This in turn suggests that even if liposomes administered intravenously at a dose of 100–200 mg of cisplatin, liposomes would not be causing hemolysis.

Cisplatin is active as its aquated species that can form intrastrand and interstrand adducts with DNA, which in turn are responsible for cell death (3,37). Formation of these aquated species is dependent on the pH and chloride ion concentration of the environment (38,39). At acidic pH, positively charged monoaqua and diaqua species predominate. In extracellular sites, cisplatin will be in non-aquated form predominantly. Cisplatin gets inside the cells by diffusion and by active transport (40). Once inside the cells where chloride concentration is very low, cisplatin will form the aforementioned aquated species which will diffuse to nucleus and bind with DNA bases.

It has also been reported that endocytosis is the prime transport mechanism of the cellular uptake of PEGylated liposomes. From this, it can be speculated that cisplatin liposomes will enhance the delivery of cisplatin in its active form inside the cells as compared to cisplatin solution. But the formation of aquated species will require ligand exchange reaction between the chloride and caprylate ligands of cisplatin and water molecules. And this would be facilitated by the exposure of complexed cisplatin with the acidic environment of endosomes. But almost similar cytotoxicity of cisplatin solution and cisplatin liposomes against A549 cells indicate that although higher cisplatin load would be available inside the cells as explained earlier, formation of aquated species would be taking place at a slower rate as compared to that with free cisplatin, which would make the same amount of active cisplatin available to bind with DNA from cisplatin liposomes and cisplatin solution.

There is a broad side-effect profile associated with cisplatin. By preparing a novel liposomal formulation of cisplatin, its side effects can be minimized (41). As described earlier, more release of cisplatin at acidic pH and hindered aquation of the cisplatin-caprylate complex even at acidic pH reasons out that liposomes prepared with complexed cisplatin would show minimal toxicity to normal tissues as well as increase its safety. This was in concordance with the results of acute toxicity studies on mice. Liposomal cisplatin formulation almost doubled the LD_{50} of free cisplatin. This infers that proposed cisplatin liposomal formulation is a good replacement for its conventional solution form in its improved safety while retaining its cancer cell cytotoxicity.

On storage, liposomes are susceptible to various physical and chemical changes as phospholipids are susceptible to storage conditions. Liposomal integrity in terms of vesicular size and entrapment efficiency was much more retained when stored in a refrigerator. It has been reported that lipids are more stable at refrigerated conditions and liposomes retain their integrity in refrigerated conditions. The stability data on prepared cisplatin liposomes also conform to this, deducing refrigeration conditions (2–8°C) should be used for storage of cisplatin liposomes.

CONCLUSION

A novel way to increase the entrapment of cisplatin in liposomal formulation by complexation to a negatively charged lipid has been devised that will provide a better drug:lipid ratio as well as high drug loading. Formulated cisplatin liposomes were evaluated *in vitro* and *in vivo*. Higher cisplatin entrapment and lower vesicular size ensure better delivery to cancer cells. Liposomal formulation was stable in

the presence of serum proteins as well as showed minimal hemolytic potential. Liposomes showed a pH-dependent drug release profile providing a highest release at pH 5.5 indicating cancer cell-specific delivery of cisplatin. Liposomes of complexed cisplatin retained the cytotoxicity of free cisplatin in human lung cancer cells as well as increased the therapeutic index of cisplatin by raising the lethal dose in mice to almost twice to that of the free cisplatin. This will also allow for the high dose treatment as well as reduce the cost of therapy by minimizing the need for use of extra-therapy measures to be taken to reduce the side effects associated with cisplatin use. Further research involving the biodistribution and pharmacokinetic studies in suitable tumor-bearing animal models would provide a clear picture of the behavior of the developed cisplatin liposomal formulation *in vivo*.

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