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Original Research Article

Cytotoxicity of Nanoliposomal Cisplatin Coated with Synthesized Methoxypolyethylene Glycol Propionaldehyde in Human Ovarian Cancer Cell Line A2780CP

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Abstract

Purpose: *To evaluate the cytotoxicity of pegylated nanoliposomal cisplatin on human ovarian cancer cell line A2780CP.*
Methods: *Synthesized methoxypolyethylene glycol (mPEG) propionaldehyde was characterized by 1H-nuclear magnetic resonance (^{1}H-NMR) and Fourier transform infrared spectroscopy (FTIR) and used as coating agent for the preparation of liposomal nanodrug formulation by reverse phase evaporation method. The characteristics of the nanoparticles were evaluated by dynamic light scattering (DLS) and scanning electron microscopy (SEM). Encapsulation efficiency was determined spectrometrically at 214.42 nm by inductively coupled plasma spectroscopy (ICP-OES). The cytotoxicity of both pegylated nanoliposomal and free cisplatin were evaluated by 3- [4, 5-dimethyl-2-thiazolyl] -2, 5-diphenyltetrazolium bromide (MTT) assay and expressed as half-maximal inhibitory concentration (IC50).*
Results: *The mean diameter and zeta potential of drug-loaded liposomal particles and empty nanoliposomes were 125 ± 2.9 nm and -16.6 mV, 108 ± 2.2 nm and -27.2 mV, respectively, while the cytotoxicity (IC_{50}) of free cisplatin and nanodrug formulation were 93.6 ± 3.1 µg/mL and 67.8 ± 2.3 µg/mL, respectively. In vitro toxicological results indicate that the formulation exhibited approximately 1.4-fold cytotoxicity compared with the free drug. Drug encapsulation efficiency of the nanoliposomes was approximately 98 ± 1 %.*
Conclusion: *The findings show that the cytotoxicity of pegylated nanoliposomal cisplatin is higher than that of free cisplatin in human ovarian cancer cell line A2780CP. In vivo studies are, however, required to ascertain its therapeutic potentials.*

Keywords: *Liposome, Nanodrug, Ovarian cancer, Polyethylene glycol, Cisplatin, Drug delivery, Cytotoxicity*

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INTRODUCTION

One of the most important classes of chemotherapeutic agents are platinum drugs such as cisplatin, carboplatin and oxaliplatin for treatment of various malignancies including ovarian cancer. Cisplatin is a neutral inorganic, square planar complex that reacts with DNA to induce apoptosis [1]. Along with anticancer activity, the drug has severe toxicity, particularly

nephrotoxicity [2]. It has been demonstrated that the high binding affinity of cisplatin to plasma proteins and enzymes induces this toxicity [3,16]. Nanoparticle-based drug delivery systems such as liposomes, have been widely used as anticancer drug carriers and simultaneously increase the efficacy of chemotherapeutic drugs [4]. Liposomes are actually vesicles consisting of a spherical lipid bilayer and an aqueous inner compartment [5,20].

However conventional liposomes have suffered from poor bioavailability and short blood-circulation time, and are easily removed from the circulation by reticuloendothelial system (RES) [6,14,19]. Coating the surface of the liposomes with polyethylene glycol (PEG) leads to increase the efficacy of anticancer drugs [7-9,17]. PEG derivatives are synthesized polymers which have several advantages for pharmaceutical applications such as high water solubility, lack of toxicity and immunogenicity and rapid clearance from the body [10-12].

The purpose of the present study was to evaluate the cytotoxicity and physicochemical properties of PEG-coated nanoliposomal cisplatin.

EXPERIMENTAL

Materials and equipment

Cisplatin, polyethylene glycol methylether (mPEG, 20KDa), 3-chloropropionaldehyde diethyl acetal (CPADA), sodium hydrogen phosphate, sodium dihydrogen phosphate, anhydrous sodium sulfate (Na_2SO_4), phosphatidylcoline (PC), isopropyl alcohol, cholesterol and tetrazolium compound (3- [4, 5-dimethyl-2-thiazolyl] -2, 5-diphenyl tetrazolium bromide) were purchased from Sigma-Aldrich Chemie (USA). Chloroform, sodium hydroxide, hydrochloric acid, sodium chloride and sodium hydrogen carbonate were obtained from Merck Chemical Co. (Germany). RPMI-1640 culture medium was purchased from Invitrogen (USA). All of the chemicals were of analytical grade. Recommended storage temperature for mPEG was -20 °C under inert gas stream [12]. The human ovarian cancer cell lines A2780CP were obtained from cell bank of Pasteur Institute of Iran. The cell lines were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 37 °C in a 5 % CO_2 incubator.

For synthesis and characterization of the samples, rotary evaporator (Heidolph, Germany), scanning electron microscope (S-4160, Hitachi, Japan), inductively coupled plasma spectrometer (ICP-OES) (Varian, Vista-MPX, Japan), dynamic light scattering (Malvern, Zen 3600, UK), Elisa microplate reader (BioTek, VT, USA), ultracentrifuge (Beckman Coulter, Optima L-90K, USA), ^{1}H-NMR spectrometer (Bruker, 500 MHz, Switzerland) and FTIR spectrometer (Thermo, Scientific Nicolet iS50R, Germany) were used.

NMR spectroscopy

^{1}H-NMR spectra were recorded on a Bruker spectrometer (500 MHZ, Switzerland) with deuteriated chloroform ($CDCl_3$) as the solvent in sterile tubes. Tetramethylsilane was used as the internal reference. Chemical shifts (δ) were reported in ppm relative to the residual proton signal of the $CDCl_3$ at 7.23 ppm.

FTIR spectroscopy

FTIR spectra were recorded on a Thermo spectrometer (Scientific Nicolet iS50R, Germany) and were scanned using potassium bromide pellets in the range of wavenumber 4000 -500 cm^{-1}. The pellets were prepared using 150 mg of potassium bromide and 1.5 mg of samples were mixed homogenously and pressed by a hydraulic pressure at 60 kN for 5 min.

Preparation of liposome

Here, 50 mg of phosphatidylcoline (PC), 10 mg of cholesterol and 80 mg of mPEG propionaldehyde (Pc/ Chol/ mPEG20000 propionaldehyde 65: 30: 5 mole ratio) were dissolved in 25 mL of chloroform in a round bottom flask. The obtained suspension was stirred for 1 h at room temperature and the solvent was removed in a rotary evaporator (Heidolph, Germany) (90 rpm, 45 °C) under vacuum for 60 min. A thin layer of lipid was formed at the wall of the round bottom flask. 25 mL of PBS (pH = 7.2) was added to the flask and was stirred for 1 h. The control solution that consists of empty liposomes was first prepared.

Preparation of nanodrug formulation

Nanoliposomal cisplatin was prepared by using a portion of the control solution of liposome. In this regard, 1 mL of cisplatin solution with concentration of 1000 µg/mL was added to 5 mL of the control solution at 25 °C and stirred vigorously and followed by sonicating (Elmasonic PH 350EL, USA) to obtain the nanodrug suspension with concentration of 200 µg/mL. The formulation was homogenized in order to reduce

the size of liposomes in a sonicating bath three times, each time for 60 s.

Measurement of encapsulated cisplatin

Encapsulation efficiency of the nanoliposomal formulation was determined by inductively coupled plasma spectroscopy (ICP-OES) (Varian, Vista-MPX, Japan). The unencapsulated cisplatin was separated from the suspension by ultracentrifugation (Beckman Coulter, Optima L-90K, USA) (45000 rpm, 4 °C, 60 min). One milliliter of the supernatant was dissolved in 5 mL of aqua rejia (3HCl: 1HNO_3) and was diluted to 10 mL. Thereafter, the absorbance of the supernatant was obtained spectrometrically at 214.42 nm. Encapsulation efficiency (EE) was calculated using Eq 1 [13]:

EE (%) = {(Cl – Cs)/Cl}100 …….. (1)

where, Cl and Cs are the concentrations of cisplatin in the nanoliposomal formulation before ultracentrifugation (in µg/mL) and in the supernatant (in µg/mL), respectively.

Characterization of nanoliposomal cisplatin

Morphological studies of nanoparticles were studied using scanning electron microscopy (SEM) (S-4160, Hitachi, Japan). The nanodrug samples were lyophilized at -70 °C for 18 h to obtain dried nanodrug. The samples were sprinkled on a double-sided adhesive tape and were sputter-coated with gold. The coated samples were investigated using SEM at 20 kV. Also the mean diameter, size distribution and zeta potential of particles were determined by dynamic light scattering (DLS) (Malvern, Zen 3600, UK).

Evaluation of *in vitro* cytotoxicity

MTT assay was used to assign cytotoxicity [1,18,22]. In this test, cells were cultured in colourless flat bottom 96-well-plate at density of 10^4 cells per well in 100 µL medium and incubated overnight. Cells were exposed to different concentrations of free cisplatin and nanoliposomal cisplatin (6.25, 12.5, 25, 50, 100, 200 µg/mL) for 48 h. The supernatant in each well was removed and 80 µL of MTT reagent (0.5 mg/mL) was added to each well and incubated at 37 °C for 2 h until development of formazan crystals. The MTT reagent was reduced by metabolically active cells to insoluble purple formazan dye crystals. The supernatant in each well was removed and 100 µL of isopropyl alcohol was added to each well to dissolve formazan crystals. Absorbance was measured at 570 nm using microplate reader (BioTek, VT, USA). IC_{50} of free cisplatin and nanodrug were calculated by the statistical package, Pharm-PCS software. Then cytotoxicity of free cisplatin and pegylated nanoliposomal cisplatin were evaluated after 48 h on human ovarian cancer cell line A2780CP with various concentrations of the nanodrug formulations (6.25, 12.5, 25, 50, 100, 200 µg/mL). Each experiment was carried out in triplicate on A2780CP cell line.

Statistical analysis

The results are presented as mean ± standard deviation (SD, n = 3). Statistical analysis of data were performed by Student's t-test using SPSS software version 21.00. Differences were considered significant at $p < 0.05$.

RESULTS

Characteristic of mPEG propionaldehyde

The FTIR spectrum of synthesized mPEG propionaldehyde is shown in Fig. 1. The principal peak of the mPEG propionaldehyde was at 2886.8 cm^{-1} which can be assigned to the C-H stretching in aldehyde group.

^{1}H-NMR ($CDCl_3$, 500MHz, ppm): δ 9.77 (-OCH_2CH_2CHO, 1H, t), δ 2.66 (-CH_2CHO, 2H, q), δ 3.64 (PEG main chain, 1992H, m), δ 3.36 (-OCH_3, 3H, s).

Characteristics of nanoliposomal cisplatin

The mean diameter, size distribution and zeta potential of empty nanoliposomes and cisplatin-loaded nanoliposomes are outlined in Table 1. Incorporation of cisplatin into nanoparticles increased the size, size distribution and zeta potential of the nanoparticles. SEM image and particle size distribution of nanoliposomal cisplatin are shown in Figs 2 and 3, respectively. It can be seen the most of the particles had spherical shape. Encapsulation efficiency was obtained 98 ± 1 % based on Eq. 1.

Stability of nanodrug

The size and zeta potential of pegylated nanoliposomal cisplatin were stable significantly at the storage time. Stability of size and zeta potential of nanodrug formulations were recorded for 4 weeks. Therefore, the pegylated nanoliposomal cisplatin were considered stable at 4 °C over the observed time.

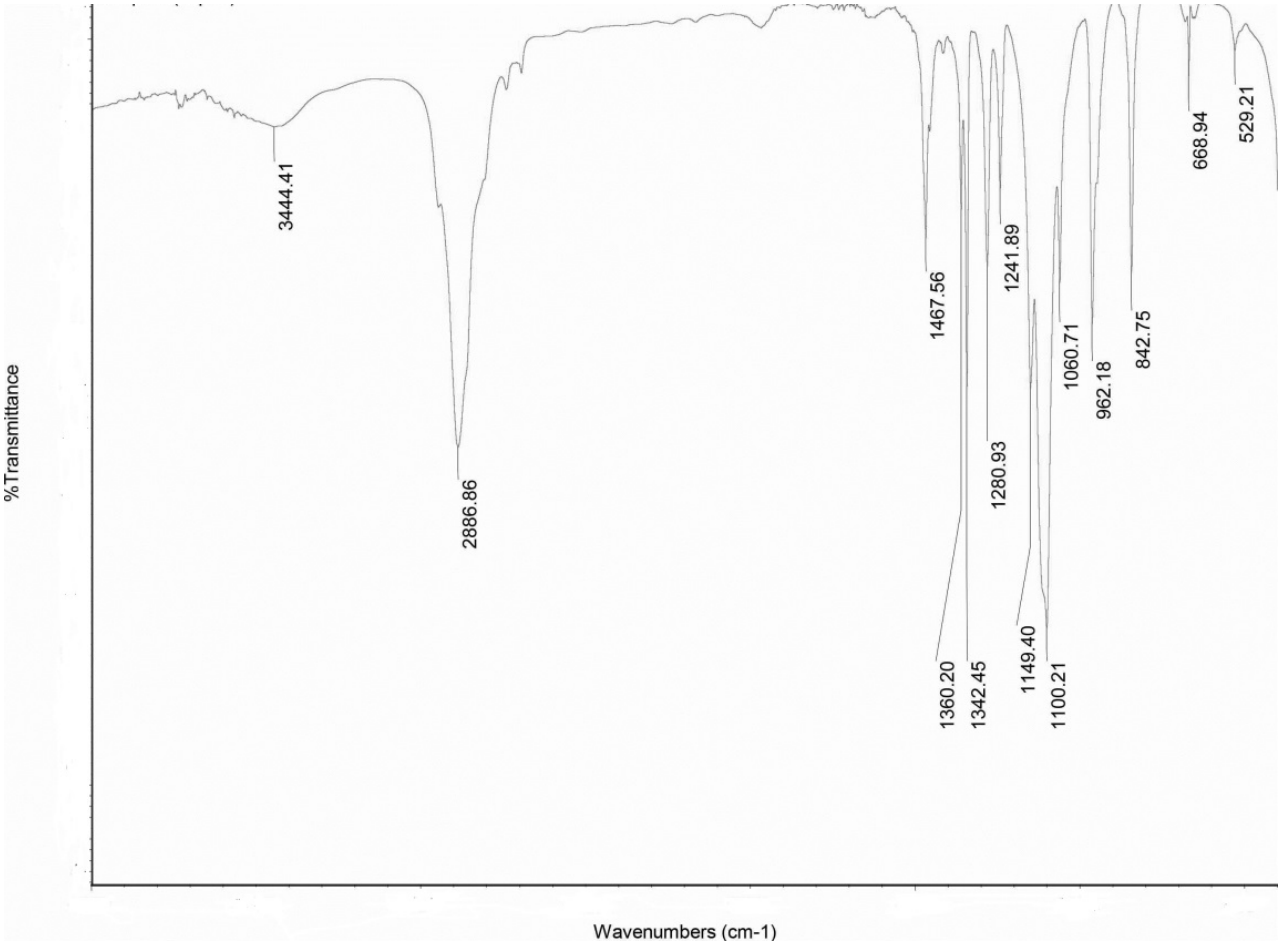

Figure 1: FTIR spectrum of synthesized mPEG propionaldehyde

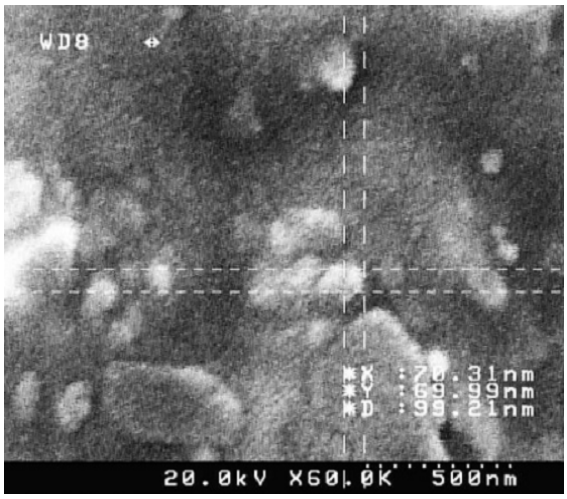

Figure 2: SEM image of nanoliposomal cisplatin

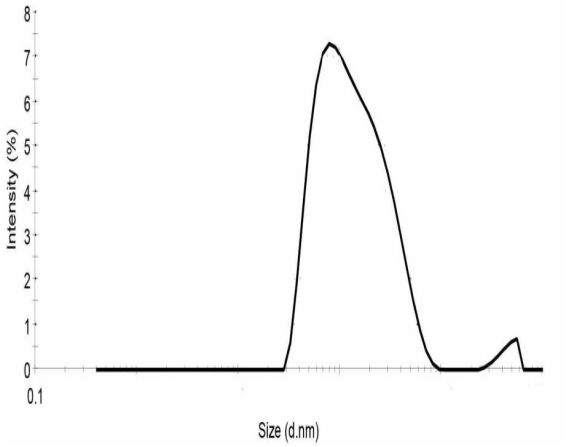

Figure 3: Particle size distribution of nanoliposomal cisplatin

Table 1: Physicochemical properties of pegylated liposomal nanoparticles

Formulation	Mean diameter (nm)	Zeta potential (mV)	Polydispersity index (PdI)
Empty nanoliposomes	108 ± 2.2	-27.2	0.34
Cisplatin-loaded nanoliposomes	125 ± 2.9	-16.6	0.55

All data are presented as mean ± SD (n=3)

Cytotoxicity of nanoliposomal cisplatin

IC_{50} values obtained for free cisplatin and pegylated nanoliposomal cisplatin were 93.6 ± 3.1 µg/mL and 67.8 ± 2.3 µg/mL, respectively. The cytotoxicity of pegylated nanoliposomal cisplatin is higher than that of free cisplatin on human ovarian cancer cell line A2780CP.

DISCUSSION

The chemical structure of the synthesized mPEG20000 propionaldehyde, as a coating agent in drug delivery, were confirmed by FTIR and ^{1}H-NMR. In the FTIR spectrum of the synthesized $mPEG_{20000}$ propionaldehyde there is a peak at 2886.8 cm^{-1} which can be assigned to the C-H stretching in an aldehyde group. In other words, this band confirms the presence of the functional aldehyde group in the samples. The ^{1}H-NMR spectrum of the synthesized $mPEG_{20000}$ propionaldehyde indicate the protons of the aldehyde group and methylene adjacent to the aldehyde group (CH_2CHO). These proton appear as a triplet peak at 9.77 ppm and a quintet peak at 2.66 ppm. So, ^{1}H-NMR results confirmed the presence of the functional aldehyde group in the synthesized $mPEG_{20000}$ propionaldehyde.

The physicochemical properties of nanoparticles are presented in Table 2. It can be observed that the size of the drug-loaded nanoparticles is higher than that of the empty nanoparticles. In addition, the zeta potential of the drug-loaded nanoparticles was more positive than the empty nanoparticles. These results are consistent with the results obtained by Hoseineh *et al* and Eskolaky *et al* [18,22]. The results of the mean diameter of the nanoparticles using DLS after their fabrication confirmed the size of nanoparticles in the nano scale. In the current study, the mean diameters of the prepared nanoparticles were approximately 100 nm. This size is less than the size of nanoparticles prepared by Krieger *et al* which is approximately 110 nm [1]. This phenomenon can be due to the applied preparation method. In this study, the nanoliposomes were prepared using reverse phase evaporation (REV) method and the lipid composition was Pc/Chol/($mPEG_{20000}$ propionaldehyde) in 65/30/5 mole ratio, while Krieger *et al* applied film method (FM) and the lipid composition was Pc/Chol/$mPEG_{2000}$-PE in 65/30/5 mole ratio [1]. The physicochemical properties of nanoparticles can be influenced by experimental conditions of manufacturing such as concentration, temperature and sonication time [15,21]. The nanoparticles prepared under different conditions showed smaller sizes and a narrower size distribution profile [8,15,22].

The selection of solvent was important as it exerts a critical influence over the reaction time required in the REV method. Therefore, chloroform and ethyl alcohol were tested as solvents in this study, being chloroform the most appropriate one, since it significantly decreased the reaction time. These results suggested that solubility in the different solvents had effect on nanoparticles size and the shorter solubility time, the lower nanoparticles size.

MTT assay is a valid test to evaluate the efficacy of the nanodrug formulation *in vitro* environment, and hence it was used in this study to obtain the cytotoxicity of pegylated nanoliposomal cisplatin. IC_{50} values of pegylated nanoliposomal cisplatin and free drug were 67.8 µg/mL and 93.6 µg/mL, respectively. The results showed the IC_{50} of nanoformulation on human ovarian cancer cell line A2780CP were less than that of the free drug. The cytotoxicity of the nanoformulation was approximately 1.4 times that of the free drug. These results are consistent with the results obtained by Hoseineh *et al* and Eskolaky *et al* [18,22]. The results showed decreasing of IC_{50} values of nanoformulations lead to enhance the cytotoxicity of nanoformulations.

CONCLUSION

Pegylated nanoliposomal cisplatin have been successfully synthesized by reverse phase evaporation method in this study. Pegylated nanoliposomal cisplatin shows higher cytotoxicity than the free drug on human ovarian cancer cell line A2780 CP after 48 h of incubation. These findings provide a new approach to the application of liposomal drug carriers in cancer chemotherapy. *In vivo* studies are, however, required to further develop the formulation.

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