Original Research Article

Effect of Cytotoxicity of Pegylated Liposomal Recombinant Human Erythropoietin-alfa on Neuroblastoma Cell Line SH-SY5Y

Samaneh Shahbaz Hoseineh[1], Azim Akbarzadeh[2]* and Hossein Attar[1]

[1]Department of Chemical Engineering, Science and Research Branch, Islamic Azad University, [2]Department of Pilot Nanobiotechnology, Pasteur Institute of Iran, Tehran, Iran

*For correspondence: **Email:** azimakbarzadeh1326@gmail.com; **Tel:** +989128387017, +982166465406; **Fax:** +982166465132

Abstract

Purpose: To evaluate the cytotoxic effect of pegylated liposomal Recombinant Human Erythropoietin-alfa (rHuEPO) nanoparticles synthesized by reverse phase evaporation technique on SH-SY5Y cell line.
Methods: To prepare the nanoparticles of the drug, rHuEPO, PEG3000, cholesterol and phosphatidylcholine were dissolved in buffer phosphate. The characteristics of the synthesized nanoparticles were determined by a zetasizer. Encapsulation efficiency, drug loading efficiency and drug release pattern were evaluated spectrophotometrically. The cytotoxicity effect of pegylated nanoliposomal rHuEPO was evaluated on SH-SY5Y cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
Results: Size and zeta potential of pegylated nanoliposomal drug and blank pegylated nanoliposomes were 571 ± 6.8 nm, -16.5 mV; 280 ± 4.71 nm and -27.1 mV, respectively. Encapsulation and drug loading efficiency were 89 ± 3.9 % and 0.8 ± 0.012 %, respectively. Drug release data indicate that 17 % of rHuEPO was released from pegylated liposomal nanoparticles over a period of 30 h. The difference in cytotoxicity between the free drug (IC_{50} = 110.1 ± 3.1 μg/ml) and nanodrug (IC_{50} = 87.2 ± 2.3 μg/ml) was statistically significant ($p < 0.05$).
Conclusion: This study shows that pegylated nanoliposomal rHuEPO has a powerful cytotoxic effect on SH-SY5Y cell line and is therefore a suitable alternative to the standard therapy, rHuEPO, for the chemotherapy of neuroblastoma.

Keywords: Recombinant Human Erythropoietin-alfa, Cytotoxicity, Drug delivery, Liposome, Reverse phase evaporation, SH-SY5Y cell line

INTRODUCTION

Recombinant human erythropoietin-alfa (rHuEPO) - a 165 amino acid glycoprotein is manufactured by recombinant DNA technology. rHuEPO is one of the most important drugs used in chemotherapy [1]. Additionally, non-targeted distribution of drug in living systems results in reducing efficiency and increasing side effects. Several side effects were reported to be associated with rHuEPO in consumers, such as skin rash or hives, edema (swelling of face, fingers, ankles, feet or lower legs), fever, hyperkalemia and increasing blood pressure. An effective way of overcoming the mentioned challenges is the encapsulation of drugs in nanocarriers, such as nanoliposomes.

Drug delivery is an effective approach of drug distribution to achieve therapeutic effect on

humans. Liposomes which are capable of encapsulating a broad range of drugs can be used as drug carriers in such systems.

Liposomes are defined as vesicles that result from phospholipids dispersed in aqueous medium in such a way that the aqueous filled center cavity is surrounded by phospholipid bilayer membranes. The carriers are small vesicles of spherical shape and are also one of the nano drug carriers that improve the delivery of therapeutic agents which are loaded with great variety of molecules including drugs [2-4]. Liposomes are biodegradable, biocompatible, biologically inert and non-toxic vesicles, and they can encapsulate both hydrophilic and hydrophobic drugs. They are also nonantigenic and nonpyrogenic [5].

In the current study, we synthesized liposomal rHuEPO nanoparticles by reverse phase evaporation technique to increase efficiency and decrease side effect of the drug. We have also used polyethylene glycol (PEG) because of its effect on improving stability [6,7].

EXPERIMENTAL

Materials

Phosphatidylcholine, cholesterol, polyethylene glycol 3000 (PEG3000) and MTT (0.5 mg/mL) were purchased from Sigma (USA). Isopropanol and ethanol were obtained from Merck Company (Germany). RPMI-1640 culture medium was purchased from Invitrogen (Invitrogen, USA). rHuEPO and neuroblastoma cell lines SH-SY5Y were supplied by Pasteur Institute of Iran. All other materials were analytical grade.

Preparation of nanoparticles

Nanoparticles were synthesized by reverse phase evaporation technique. rHuEPO, PEG3000, cholesterol and phosphatidylcholine (1:2.8:23:97 weight ratio) were dissolved in 50 mL of 98 % ethanol using magnetic stirrer (150 rpm, 30 min, room temperature) to obtain a transparent yellow suspension. Then, the solvent phase was evaporated by rotary evaporator (90 rpm, 1 h, 50 ^{o}C) (Heidolph Co., Germany). Afterwards, the resultant film was dissolved in 10 mL buffer phosphate (pH = 7.4). The control solution was also prepared without drug. The formulations were stirred for 24 h (150 rpm, room temperature) and sonicated (Bandelin Sonopuls HD 2070, Bandelin Elec., Germany) for 5 min at 60 Hz. In addition, the counterpart concentrations of standard drug were prepared in phosphate buffer (pH = 7.4).

Characterization of nanoparticles

The mean diameter, size distribution and Zeta potential of particles were determined by Zetasizer (Zen 3600; Malvern Instruments Ltd, Malvern Worcestershire, UK). Morphology of nanoparticles was studied by scanning electron microscope (AIS2300; Seron Technologies Inc.; Korea).

Encapsulation and drug loading efficiency were computed as in Eqs 1 and 2. For this purpose, 2 mL of each suspension were centrifuged (15000 rpm, 4 °C, 90 min). Thereafter, the absorbance of the supernatant of both formulations was obtained spectrophotometrically (UV-Vis 1601PC, Shimadzu Co., Japan) at 280 nm.

Encapsulation efficiency (%) = $\{(C_i - C_s)/C_i\}100$ .....(1)

where C_i is the initial concentration of the drug in mg/ml and C_s is the concentration of the drug in supernatant in mg/ml.

Loading efficiency (%) = $(N_a/N_w)100$ .... (2)

where N_a is the amount of drug in the nanoparticle and N_w is the weight of the nanoparticle all in mg.

In vitro drug release studies

Drug release was determined by membrane diffusion technique. For this purpose, 1 mL of each formulation (pegylated nanoliposomal rHuEPO and the standard form) was poured in dialysis bag (cut off 50000 Dalton, Sigma). The dialysis bag was submerged in phosphate buffer (pH = 7.4) and stirred (100 rpm, 37 °C). At predetermined time intervals, 1 mL of phosphate buffer was taken and then substituted with fresh phosphate buffer. As a result, the amounts of released drug in phosphate buffer were measured using spectrophotometric method at definite time intervals.

Evaluation of cytotoxicity

The cytotoxicity of pegylated nanoliposomal rHuEPO on neuroblastoma cell line SH-SY5Y was investigated by MTT assay in 96-well plate reader. The cells were cultured using 1×10^4 cells in each well in RPMI-1640 containing 10 % fetal bovine serum and 1 % penicillin/streptomycin (37 °C, 5 % CO_2). Twenty four hours later -when cells adhered to the floor of the wells, cells were treated with standard

rHuEPO and rHuEPO loaded nanoparticles in concentrations of 0.008, 0.016, 0.0323, 0.0646, 0.129, 0.258, 0.516, 1.033 and 2.066 µM. After incubating for 48 h, supernatant was removed, and 100 µM MTT solution (0.5 mg/mL, pH = 7.4) was added to each well and incubated for 3 h. Later, MTT solution was removed, and 200 µM of isopropanol 100 % (v/v) was added to dissolve formazan crystals. Absorbance was determined at 570 nm (BioTek Instruments, VT, USA). Experiments were repeated three times. IC_{50} was calculated by the statistical package, Pharm-PCS software.

Statistical analysis

SPSS version 21.00 software was used, and student t-test was applied to comparing statistical differences and *p* < 0.05 was considered significant.

RESULTS

Characteristics of nanoparticles

The mean diameter, size distribution and zeta potential of blank pegylated liposome nanoparticles and pegylated liposomal rHuEPO are outlined in Table 1. As the values show, incorporation of drug into nanoparticles increased the size, size distribution and zeta potential of the nanoparticles.

The encapsulation and drug loading efficiency were 89 ± 3.9 and 0.8 ± 0.012 %, respectively.

***In vitro* drug release**

The drug release data for rHuEPO from pegylated nanoliposomal and standard drug are shown in Figure 2.

Figure 2: The release pattern of rHuEPO from pegylated liposomal drug nanoparticles synthesized by reverse phase evaporation technique (▲) and standard drug (□). Pattern is presented as a percentage of release at different time intervals. Results are presented as mean ± 5 % error from three independent tests

Table 1: Physicochemical properties of pegylated nanoliposomal rHuEPO

Formulation	Mean diameter (nm)*	Zeta potential (mV)*	Polydispersity Index (PDI)*
Pegylated liposomal nanoparticles without drug	280±4.71	-27.1	0.431
Pegylated liposomal nanoparticles with drug	571±6.8	-16.5	0.505

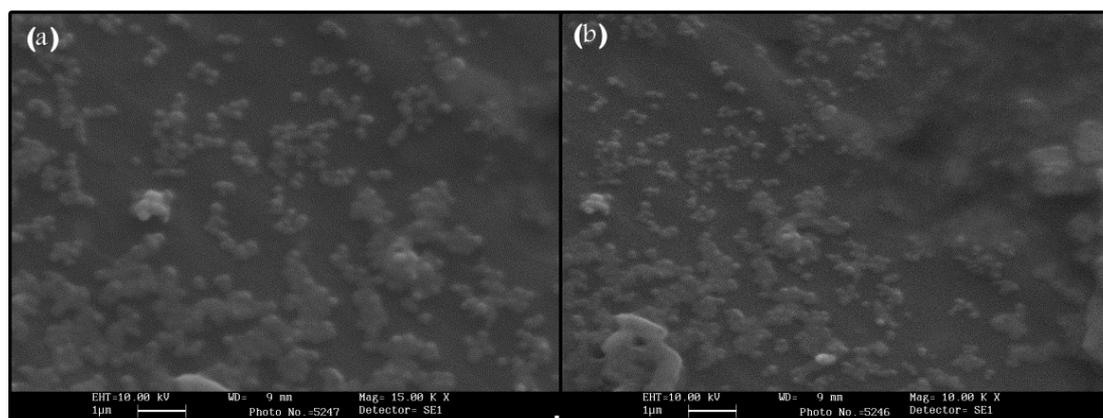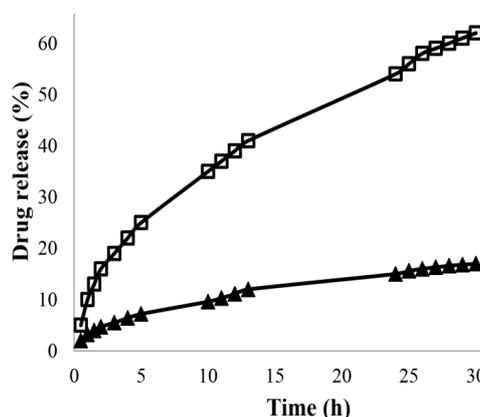*All data are presented as mean ± SD (n = 3)*

Figure 1: SEM image of pegylated liposomal rHuEPO nanoparticles (a) and blank pegylated liposomal nanoparticles (b) synthesized by reverse phase evaporation technique

Evaluation of cell cytotoxicity

The IC_{50} for pegylated nanoliposomal rHuEPO and the standard form was 87.2 ± 2.3 µg/ml and 110.1 ± 3.1 µg/ml, respectively.

DISCUSSION

Liposome drug delivery systems play an important role in the formulation of potent drugs to improve therapeutics. Currently, drug-loaded liposome nanoparticles are designed to reduce the side effects of therapeutic agents and also increase the drug accumulation in the target sites in some clinical applications [8]. In addition, liposomes could bypass the biological barriers whilst protecting entrapped drugs and deliver them to target organs, and it helps release of the drug at the therapeutic level [9].

Successful preparation of liposomal nanoparticles through reverse phase evaporation technique was achieved. The experiment was performed three times and results showed that the preparation technique was sufficiently valid and reliable.

Also, PEG was used in the synthesis of nanoparticles, because it is stable in the blood circulatory system and is not easily removed by the reticulo endothelial system (RES). In addition, PEG helps to increase pharmacokinetic properties and change insoluble drugs to soluble ones [10-13].

The results of the particles diameter measurement using Zetasizer after their fabrication confirmed the particles size in the nano scale [14]. Increasing the mean diameter of nanoparticles from 280 to 571 nm led to reduced stability due to the loaded drug in the nanoparticles.

The release process consisted of two phases, quick and slow release. In both formulations, a burst release of drug occurred in the first 3 h. Release rate decreased with time which is probably because of presence of PEG. The results indicated 62 ± 3.1 % and 17 ± 0.72 % of the drug were released into phosphate buffer for the standard drug and pegylated liposomal drug, respectively. Previous studies showed PEG also brings about increased stability and probability of drug delivery to tumor with high efficiency [15].

The cytotoxicity of pegylated liposomal rHuEPO nanoparticles on neuroblastoma cell line SH-SY5Y was evaluated by the MTT assay. Firstly, it was determined that nanoparticles containing no drug had no cytotoxic effect on the cells. The results showed that pegylated liposomal formulation had more toxic effect compared with the standard drug. Regarding definition of IC_{50}, the cytotoxicity of the new formulation was estimated to be 1.3 times more than the standard drug for SH-SY5Y cell line.

CONCLUSION

Nanotechnology-based drug delivery systems, especially liposomes, are currently a rapidly advancing field of science. Pegylated nanoliposome particles have been successfully synthesized by reverse phase evaporation technique in this study. Significant improvement in the efficiency of pegylated liposome nanoparticles rHuEPO compared with the standard form of the drug has been achieved. Further studies on the formulation including in vivo experiments are recommended.

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