International Journal of Pharmaceutics 382 (2009) 254–261

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Polyethylene glycol-complexed cationic liposome for enhanced cellular uptake and anticancer activity

Suk Hyun Jung[a,b], Soon Hwa Jung[a], Hasoo Seong[a], Sun Hang Cho[a], Kyu-Sung Jeong[b], Byung Cheol Shin[a,*]

[a] Biomaterials Research Center, Korea Research Institute of Chemical Technology, Yuseong, Deajeon, Republic of Korea
[b] Center for Bioactive Molecular Hybrids and Department of Chemistry, Yonsei University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 2 April 2009
Received in revised form 3 July 2009
Accepted 1 August 2009
Available online 8 August 2009

Keywords:
Liposome
Cationic lipid
Polyethylene glycol
Intracellular uptake

ABSTRACT

Liposomes as one of the efficient drug carriers have some shortcomings such as their relatively short blood circulation time, fast clearance from human body by reticuloendothelial system (RES) and limited intracellular uptake to target cells. In this study, polyethylene glycol (PEG)-complexed cationic liposomes (PCL) were prepared by ionic complex of cationically charged liposomes with carboxylated polyethylene glycol (mPEG-COOH). The cationic liposomes had approximately 98.6 ± 1.0 nm of mean particle diameter and 45.5 ± 1.1 mV of zeta potential value. While, the PCL had 110.1 ± 1.2 nm of mean particle diameter and 18.4 ± 0.8 mV of zeta potential value as a result of the ionic complex of mPEG-COOH with cationic liposomes. Loading efficiency of model drug, doxorubicin, into cationic liposomes or PCL was about 96.0 ± 0.7%. Results of intracellular uptake evaluated by flow cytometry and fluorescence microscopy studies showed higher intracellular uptake of PCL than that of Doxil®. In addition, *in vitro* cytotoxicity of PCL was comparable to cationic liposomes. In pharmacokinetic study in rats, PCL showed slightly lower plasma level of DOX than that of Doxil®. *In vivo* antitumor activity of DOX-loaded PCL was comparable to that of Doxil® against human SKOV-3 ovarian adenocarcinoma xenograft rat model. Consequently, the PCL, of which surface was complexed with PEG by ionic complex may be applicable as drug delivery carriers for increasing therapeutic efficacy of anticancer drugs.

1. Introduction

Liposomes are spherical vesicle composed of phospholipid bilayer membranes. Liposomes have been extensively studied in an attempt to enhance the therapeutic efficacy of various drugs in the field of drug delivery system (Drummond et al., 1999; Sharma and Sharma, 1997; Bajoria and Sooranna, 1998). However, liposomal drugs for intravenous injection have been found to be plagued by their rapid opsonization and by being taken up by the reticuloendothelial system (RES) located mainly in the liver and spleen (Andresen et al., 2005; Moghimi and Patel, 1998). In general, this rapid uptake of drug carriers leads to a short blood circulation time of the incorporated drug. Notably, this problem has been resolved by modification of the surface of liposomes with polyethylene glycol (PEG) or covalent conjugation of PEG to drug carrying molecule (Moghimi and Szebeni, 2003; Torchilin and Trubetskoy, 1995). PEG possesses ideal properties for modulating pharmacokinetic behavior of drug; non-toxicity, good solubility in aqueous solution, low immunogenicity and antigenicity (Gabizon and Papahadjopoulos, 1988; Klibanov et al., 1990). Also, PEG conjugation to bioactive molecules has no deleterious effect on conformation or activity of them. These properties of PEG have been explained by its high mobility associated with conformational flexibility and water-binding ability (Ceh et al., 1997; Shimada et al., 2000). Despite a remarkable prolongation of circulation half-life of the incorporated drug by the PEG-modified liposomes, intracellular uptake of the PEG-liposomes which were delivered to the target cells could be lowered by electrostatic repulsion between negatively charged surface of PEG-liposomes and cell membranes (Chandaroy et al., 2002).

Cationic liposomes have been studied as drug carriers because of their selective accumulation in tumor endothelial cells (Wu et al., 2007). Enhancement of cellular uptake as well as *in vitro* cytotoxicity has been demonstrated and those results were attributed to the ability of cationic liposomes to interact with cells via electrostatic interaction, which could induce endocytosis of the liposomes and also facilitate drug release to the cytosol by endosomal escape (Dass, 2003). These properties of cationic liposomes have been applied to the delivery of nucleic acid, in spite of cytotoxicity of cationic lipids themselves at high concentration and rapid clearance of cationic liposomes from circulation. Thus, we hypothesized that liposomes modified with PEG and cationic lipid could prolong

* Corresponding author. Tel.: +82 42 860 7226; fax: +82 42 860 7229.
E-mail address: bcshin@krict.re.kr (B.C. Shin).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.08.002

the circulation time in bloodstream and enhance cellular uptake of the liposomes to target cells. In this study, PEG-complexed cationic liposomes (PCL) were prepared by ionic complexation of cationically charged liposomes with anionic PEG derivative, mPEG-COOH. The complexed amount of PEG in PCL was determined by ^{1}H NMR analysis. Intracellular uptake of doxorubicin (DOX)-loaded PCL was investigated by using flow cytometry assay and fluorescence microscopic observation. *In vitro* cytotoxicity of PCL was evaluated by MTT assay. Dissociation of PEG from PCL was evaluated by ^{1}H NMR analysis. Pharmacokinetic behavior of various liposomal formulation of DOX was investigated after intravenous (i.v.) injection of each formulation into SD rats. *In vivo* antitumor activity was evaluated after i.v. injection of each formulation into human ovarian adenocarcinoma xenograft tumor-bearing mice.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine (soy hydrogenated, HSPC), cholesterol (CHOL) and 1,2-distearoyl-*sn*-glycero-3-trimethylammonium-propane (DSTAP) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Doxil® was purchased from ALZA Corporation (Mountain View, CA, USA). Doxorubicin hydrochloride (DOX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and daunorubicin hydrochloride were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO, USA); fetal bovine serum (FBS), penicillin–streptomycin, paraformaldehyde and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco BRL/Life Technologies (New York, NY, USA). Poly(ethylene glycol) methylether (mPEG2000) was purchased from Sigma Chemical Company (St. Louis, MO, USA). All other materials were of analytical grade and used without further purification.

2.2. Preparation of DOX-loaded cationic liposomes

Cationic liposomes were prepared by thin film-hydration method and their lipid composition was as follows: HSPC:CHOL:DSTAP = 12.6:8.3:4.5 (mass ratio, total 15.95 mg/ml). DOX loading into inner core of the cationic liposomes was carried out by using the remote loading method (Han et al., 2006, 2007). Briefly, the lipids with the above composition were dissolved in 5 ml of chloroform, dried to a thin film in a rotary evaporator (Buchi Rotavapor R-200, Switzerland) and then suspended in 10 ml of 250 mM ammonium sulfate solution. The liposomal solution was extruded five times through a polycarbonate filter (pore size; 200, 100 and 80 nm, Whatman, USA) with a high pressure extruder (Northern Lipids Inc., USA). Unloaded ammonium sulfate was removed through dialysis in distilled water for 48 h at 4 °C by using cellulose dialysis tubing (MWCO 12000–14000, Viskase Co., IL, USA). Liposomal solution and DOX solution (5 mg/ml) (1:1, v/v) were mixed and then incubated for 2 h at 60 °C. The mixture was dialyzed to remove the unloaded DOX for 48 h at 4 °C. The DOX-loaded liposomes were stored at 4 °C until use. The concentration of DOX in the liposomes was measured by using UV–vis spectrophotometry at 497 nm (UV-mini, Shimadzu, Japan) and the loading efficiency was calculated according to the following equation:

$$\text{loading efficiency}\,(\%) = \frac{F_t}{F_i} \times 100 \quad (1)$$

where F_t is the concentration of DOX in the liposomes after their dissolution in organic solvent mixture consisting of chloroform:methanol:distilled water (2:1:0.05, v/v) and F_i is the initial concentration of DOX. The particle size and zeta potential of the liposomes were measured by using light scattering with a particle diameter analyzer (ELS-Z, Otuska, Japan).

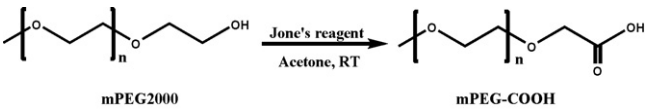

Fig. 1. Synthesis of mPEG-COOH.

2.3. Synthesis of mPEG2000-COOH

mPEG2000-COOH was synthesized as shown in Fig. 1. Poly(ethylene glycol) methylether (10 g, 5 mmol) was dissolved in 100 ml of acetone at room temperature. The solution was cooled to 0 °C by placing the flask in ice bath. Later, 5 ml of Jones reagent (containing 0.02 M of CrO_3) was added to the flask drop by drop over a 15 min period. The flask was removed from ice bath and then the reaction mixture was stirred for 20 h at room temperature. The reaction was quenched by adding 3 ml of isopropyl alcohol. Subsequently, 1 g of finely activated carbon powder was added and stirred for 2 h. The reaction products were filtered with wet celite, and the filtrate was evaporated in a rotary evaporator. The viscous liquid was dissolved in 50 ml of water and extracted with chloroform. The extracts were evaporated to obtain the product and dried under vacuum for 48 h. Yield: 98%; ^{1}H NMR (δ, ppm, $CDCl_3$): 3.38 (s, 3H, CH_3), 3.66 (m, ~44H, OCH_2CH_2O), 4.16 (s, 2H, CH_2) (Dhanikula and Hildgen, 2006; Nakazono et al., 2002).

2.4. Preparation of PEG-complexed cationic liposomes (PCL)

To complex the mPEG2000-COOH on the surface of cationic liposomes, pH of the mPEG2000-COOH solution was adjusted up to 10 by using 1 M of sodium hydroxide solution for 30 min at room temperature. The alkaline mPEG2000-COOH solution was added to cationic liposome solution (1:1, v/v) and the mixture was incubated for 2 h. The mixture was dialyzed by using dialysis tubing (MWCO 100000, Spectrum Laboratories, Inc., CA, USA) to remove the uncomplexed mPEG2000-COOH. Amount of the complexed PEG on the liposomal surface was measured by using ^{1}H NMR analysis (Automated NMR System, Buruker, Germany) of PCL.

2.5. Cell line and animals

B16F10, a murine melanoma cell line, and SKOV-3, human ovarian adenocarcinoma were cultured in DMEM added with 10% (v/v) heat-inactivated FBS and 10 μl/ml penicillin–streptomycin. The cultures were sustained at 37 °C in a humidified incubator containing 5% CO_2. The cells were maintained within their exponential growth phase.

Female BALB/c mice (5–6 weeks old, 18–22 g) and SD rats (7 weeks old, 200–220 g) were purchased from Harlan Int. (IN, USA). All of the procedures involved in the animal experiments were performed according to approved protocols and in accordance with the recommendations of the NIH guideline for the proper use and care of laboratory animals.

2.6. Cytotoxicity study

Cytotoxicity of liposomes was determined by MTT assay. Murine melanoma cells, B16F10 cells, were transferred to 96-well tissue culture plates at 1×10^4 cells per each well and incubated for 24 h at 37 °C prior to drug treatment. The culture medium was replaced with medium containing serial dilutions of various liposomes. Then, 20 μl of MTT stock solutions (5 mg/ml) were added to each well and the plates were incubated for 4 h at 37 °C. The