Potential of pH-sensitive polymer-anchored cationic liposomes for combinatorial anticancer therapy with doxorubicin and siRNA

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To explore a potential of pH-sensitive polymer-liposome complexes for the tumor-specific combinatorial delivery of anticancer agents and siRNA, conventional liposomes (ConL), polymer-liposome complexes (PLC) and polymer-cationic liposome complexes (PCLC) were prepared. Pluronic P104-based multiblock copolymer (MBCP-2) was included as pH-sensitive polymer. Physicochemical properties, release under different pH, cytotoxicity and in vitro cellular uptake of DOX-loaded liposomes were investigated. From the release test, an acidic pH was determined to be an important factor for release from the PLC vehicles. The novel PLC vehicle itself showed low cytotoxicity demonstrating suitable viability. Observing cellular uptake of DOX by confocal microscopy imaging, a greater amount of DOX was delivered to cells with the pH-sensitive polymer-anchored vehicles than that with free DOX and ConL. It was verified that the novel vehicles could effectively deliver both DOX and GFP-siRNA. Novel pH-sensitive PCLC have a potential for targeted therapy of anticancer agents and gene therapy under acidic tumor microenvironment.

Key words: pH-sensitive polymer – Cationic liposomes – Complexes – siRNA – Doxorubicin.

Doxorubicin (DOX), a DNA-intercalating anthracycline antibiotic, has been used widely as a chemotherapy drug for various cancers including leukemias, breast, liver, and lung. However, chemotherapy has many obstacles such as toxicity to normal cells. To overcome this difficulty, the accurate targeting to cancer cells and the modified release of drugs have been investigated. Accurate cancer cell targeting demands specific requirements, such as acidic conditions [1]. Therefore, acidic-sensitive vehicles are suitable for this approach [2, 3].

Another alternative approach for cancer therapy is gene therapy using small interfering RNA (siRNA), small hairpin RNA (shRNA), or plasmid DNA. In particular, siRNA can initiate RNA interference (RNAi), which produces a gene silencing effect by causing downregulation of the targeted mRNA [4]. Usually associated with multidrug resistance or apoptosis of cancer cells, RNAi, mediated by siRNA, has emerged as one of the most advanced and versatile tools for biological research, as well as one of the most promising therapeutic strategies for various human diseases such as viral infections, genetic diseases, cardiovascular disorders, and cancers. In terms of therapeutics, combinational therapies for cancer have been more effective than single therapies.

Co-delivery systems, that could simultaneously deliver drugs and siRNA to the same cells *in vitro* and *in vivo* have been proposed to inhibit gene expression or to achieve the synergistic/combined effect of drug and gene therapies [5-9]. Various delivery systems, either viral or nonviral, have been investigated for siRNA delivery *in vitro* and *in vivo*. Recently, nonviral vectors such as cationic polymers have received growing interest due to their many advantages over their viral counterparts, including ease of production, improved safety, and low immune responses that enable repeated use.

Among various delivery systems, liposomal formulations have been developed for modified release of drugs. In the case of DOX, several products including Myocet, Caelyx (EU), and Doxil (USA) are commercially available. Recent advances in liposome chemistry have resulted in various types of stimulus-responsive polymer-containing liposomes. These liposomes are prepared by simply adding a stimulus-responsive polymer to the liposome dispersion or by mixing lipids and polymers during the preparation of vesicles [10]. Stimuli-responsive liposomes, especially those sensitive to changes in pH, are attractive for the cytoplasmic delivery of polar drugs because they can be readily internalized by cells. Such liposomes are stably internalized by cells mainly via an endocytic pathway, and they are destabilized at low pH (~5) in the endosome; hence, the drugs can be easily released into the cytoplasm [1, 11]. Stimulus-induced conformational changes are thought to allow the release of drugs at low pH (endosomes) and thus increase the cellular uptake of water-soluble compounds.

For this purpose, novel polymer, Pluronic P104-based multiblock copolymer (MBCP-2), was applied as a pH-sensitive polymer that would selectively degrade under locally acidic physiological conditions [2]. A class of thermosensitive biodegradable multiblock copolymers with acid-labile acetal linkages was synthesized from Pluronic triblock copolymers (Pluronic P85 and P104) and di-(ethylene glycol) divinyl ether [2].

In this study, we combined two approaches: the pH-sensitive delivery of DOX and the cationic liposomal delivery of siRNA. We investigated the pH-sensitive properties of polymer-liposome complexes (PLC) attributable to the pH-sensitive polymer and the cationic lipids that allow the delivery of siRNA. The potential for cancer therapy using polymer-cationic liposome complexes (PCLC) as novel therapy-delivery vehicles was investigated.

I. MATERIALS AND METHODS

1. Materials

Egg yolk phosphatidylcholine (EPC), cholesterol (CHOL), and 1,2-dioleoyl-3- trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Multi-block copolymer (MBCP-2) from triblock copolymer PEO-PPO-PEO (Pluronic P104) was supplied by the School of Pharmacy, the University of Mississippi (Oxford, MS, USA) [3]. DOX-HCl, ammonium phosphate dibasic [$(NH_4)_2HPO_4$], chloroform (CH_3Cl), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). GFP siRNA (sense, GCA UCA AGG UGA ACU UCA A(dTdT); antisense, UUG AAG UUC

ACC UUG AUG C(dTdT)) and Bcl-2 siRNA (sense, CUC UGU GGA UGA CUG AGU A(dTdT); antisense, UAC UCA GUC AUC CAC AGA G(dTdT)) were obtained from Bioneer Co. (Daejeon, Korea). Sephadex G-50 spin-columns were purchased from Geneaid Biotech Ltd. (Taipei, Taiwan). All reagents and chemicals used were reagent grade.

2. Cell culture

The rat hepatoma cells (H4II-E), stabilized to express GFP, were a kind gift from Dr. S.K. Kim (Chungnam National University, Daejeon, Korea). Human hepatocellular carcinoma (HepG2), human cervix adenocarcinoma (HeLa), and human breast adenocarcinoma (MDA-MB-231, MCF-7) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) in a humidified incubator supplied with 5 % CO_2 and maintained at 37 °C for 24 h. All media were supplemented with 10 % heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco).

3. Preparation of conventional liposomes and polymer-liposome complexes

Conventional liposomes (ConL), polymer-liposome complexes (PLC), and polymer-cationic liposome complexes (PCLC) were prepared by the lipid film method [12]. All lipids and MBCP were dissolved in chloroform at the ratio of lipid as shown in *Table I*. The organic mixture was removed using a rotary evaporation under reduced pressure with the temperature of the water bath adjusted to 40 °C. Prior to hydration, the morphology of lipid film was observed. One milliliter of 300 mM $(NH_4)_2HPO_4$ (pH 7.4) was added to this lipid film and hydrated by vigorous vortexing. The resulting suspension was sonicated for 60 min at 37 °C and was passed 10 times through an extruder (Northern Lipids Inc., Vancouver, BC, Canada) equipped with double-layered 0.2-μm Nucleopore polycarbonate membrane filters (Whatman, Clifton, NJ, USA).

4. Loading of doxorubicin by ion gradient

To evaluate the possibility of using the polymer-coated liposomes to co-deliver a drug and siRNA, DOX was encapsulated as a model drug in the pH-sensitive polymer-coated liposomes by transmembrane ammonium-phosphate ion gradient [13, 14]. A Sephadex G-50 spin-column was used to exchange the outer $(NH_4)_2HPO_4$ for 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS, pH 7.4). DOX dissolved in HBS (8.5 mg/mL) was added to empty vehicles at a drug-to-lipid ratio of 1:4 (w/w) and incubated at 4 °C for 12 h with occasional mixing. Size-exclusion chromatography was performed using a Sephadex G-50 spin-column again to purify the liposomes and remove the free DOX, according to manufacturer's purification protocol.

Table I - Composition of conventional liposomes and polymer-liposome complexes (per mL).

Component	Formulation				
	ConL[a]	PLC[b]-5*	PLC[b]-10*	PLC[c]-5*	PLC[c]-10*
EPC[d]	14	14	14	14	14
CHOL[d]	6	6	6	6	6
DOTAP[d]	-	-	-	4	4
MBCP[e]	-	5	10	5	10
Total[d]	20	20	20	24	24

[a]Conventional liposome consists of EPC:CHOL = 7:3. [b]Polymer-liposome complexes (PLC) were prepared using the molar ratio EPC:CHOL = 7:3 with small amounts of MBCP added. [c]Polymer-cationic liposome complexes (PCLC) were prepared using the molar ratio EPC:CHOL:DOTAP = 7:3:2 with small amounts of MBCP added. [d]μmole. [e]w/w %. *The numbers 5 and 10 indicate the percentage of MBCP added in formulation.

The loading efficiency was determined by UV spectroscopy. The DOX concentration was measured at 495 nm by UV spectrophotometer (Mini 1240, Shimadzu, Kyoto, Japan) after lysis with Triton X-100 (final concentration 1 % v/v).

5. Particle size and zeta potential of vehicles

The particle sizes of the empty vehicles and DOX-loaded vehicles were determined by light scattering spectrophotometry (ELS-8000, Photal, Tokyo, Japan). The samples were diluted with deionized water, and then transferred into a quartz cuvette in an ELS-8000 dynamic light scattering instrument. The zeta pontential of the vehicles was measured with an electrophoretic light-scattering spectrophotometer. Data were analyzed using a software package (ELS-8000 software) supplied by the manufacturer.

6. *In vitro* release test

To investigate pH-dependent release patterns of DOX from vehicles, *in vitro* release study was performed under two pH conditions. After 100 μL of DOX-loaded vehicles (100 nmole DOX) was added to 96-well plates containing 100 μL of two types of buffers: acetate buffer (pH 5.0) and phosphate-buffered saline (PBS, pH 7.4), the plate was stored at 37 °C, with shaking at 100 rpm (n = 3). At the designated time, the samples were withdrawn from the well; the sampled volume was replaced with acetate buffer (pH 5.0) or PBS (pH 7.4). The released DOX was separated from DOX-loaded liposome formulations by ultrafiltration method using Amicon Ultra (3 kDa). To break down the ConL, PLC, and PCLC, a Triton X-100 solution (5 %, v/v) was added to each well. The released DOX was determined with a fluorometer (LS 55, PerkinElmer Inc., San Jose, CA, USA) with excitation at 488 nm and emission at 580 nm [15].

7. Gel retardation assay

To compare the complex formation between vehicles and siRNA, different amounts of ConL and PCLC were added to 10 pmole of GFP-siRNA. The mixture was incubated for 30 min at room temperature with occasional pipetting. After incubation, the complexes were electrophoresed on agarose gel (2 %) and visualized by ethidium bromide staining.

8. Confocal laser scanning microscopy

To visualize the co-delivery of DOX and GFP-siRNA, confocal laser-scanning microscopy was applied. H4ll-E cells were seeded and grown on 22 × 22 mm coverslips placed in 6-well plates at a density of 5×10^5 cells/well. The cells were transfected with siRNA in naked form, complexed with Lipofectamine or DOX-loaded PCLC in basal DMEM. Simultaneously, DOX loaded in ConL or PCLC was delivered. Control cells were untreated and maintained in basal DMEM. At 3 and 24 h post-transfection, the cells were rinsed with PBS and fixed using 4 % paraformaldyde in PBS for 10 min. Following a second rinsing procedure, two drops of mounting solution (Fluoromount aqueous mounting medium, Sigma-Aldrich) were added between the coverslips and the slide glass. Then, the cells were observed under a confocal microscope (Leica TCS NT, Leica Microsystems, Wetzlar, Germany) equipped with a argon laser and associated filters for simultaneous 488 nm excitation.

9. Cytotoxicity

The cytotoxicity of empty vehicles and various DOX-loaded vehicles was determined in HepG2, H4II-E, HeLa, MDA-MB-231, and MCF-7 cells. The cell lines were transferred from a 100-mm cell culture plate into a 96-well plate at a density of 1×10^4 cells per well, except HeLa cells, which were plated at 5×10^3 cells per well. After overnight incubation at 37 °C, the cells were exposed to 200 μL of Bcl-2 siRNA, DOX-loaded vehicles, or empty vehicles containing media for 24 h. Quantitatively, 100 pmole or 200 pmole of DOX was treated