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Paclitaxel-conjugated PEG and arginine-grafted bioreducible poly (disulfide amine) micelles for co-delivery of drug and gene

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ABSTRACT

We developed a paclitaxel-conjugated polymeric micelle, ABP-PEG$_{3.5k}$-Paclitaxel (APP) consisting of poly (ethylene glycol) (PEG) and arginine-grafted poly (cystaminebisacrylamide-diaminohexane) (ABP) for the co-delivery of gene and drug. The APP polymer self-assembled into cationic polymeric micelles with a critical micelle concentration (CMC) value of approximately 0.062 mg/mL, which was determined from measurements of the UV absorption of pyrene. The micelles have an average size of about 3 nm and a zeta potential of about +14 mV. Due to the positive surface charge, APP micelles formed polyplexes with plasmid DNA approximately 200 nm in diameter. The luciferase gene and mouse interleukin-12 (IL-12) gene was used to monitor gene delivery potency. APP polyplexes showed increased gene delivery efficiency and cellular uptake with higher anticancer potency than paclitaxel alone. These results demonstrate that an APP micelle-based delivery system is well suitable for the co-delivery of gene and drug.

1. Introduction

Cancer is a disease in which abnormal and uncontrolled cells divide in the body. Cancer cells generally grow very fast, spreading and destroying other parts of the body. Cancer has remained hard to cure despite recent technical advances in surgery and other therapies, including radiation, chemo, and hormone therapy. Based on the Cancer Facts & Figures 2012, about 1,596,670 cancer cases and 571,950 cancer deaths are estimated to occur yearly in the U.S. Breast cancer is the most common type of cancer among females (29% of the estimated new cases) while in the case of males, prostate cancer is the leading cancer (29% of the estimated new cases) [1]. Chemotherapy is the most common therapeutic approach and paclitaxel, a microtubule-stabilizing and cell division-blocking anti-cancer chemotherapeutic, is one of the most commonly used drugs [2]. Paclitaxel binds to tubulin and inhibits the breakdown of microtubules. This paclitaxel-induced microtubule stabilization blocks cell cycle in the late G2/M-phase, and leads to cell death. Based on the efficacy of that, Paclitaxel is widely used to treat a variety of patients with varying forms of cancer such as ovarian, breast, lung, head and neck cancers [3,4]. This drug is also used for the treatment of Kaposi's sarcoma and for the prevention of restenosis of coronary stents [5–7]. However, clinical applications of paclitaxel are limited by disadvantages such as poor water solubility (0.3 μg/mL) and instability in aqueous solution [8,9].

A co-solvent can increase the solubility of hydrophobic organic chemicals, such as paclitaxel. To solve solubility problem of paclitaxel, a co-solvent system, Cremophor® EL (a polyoxyethylated caster oil in 49.7% dehydrated alcohol), has been generally used in clinical applications. Still, this co-solvent system may cause other problems such as hypersensitivity reactions and neurotoxicity [10,11].

Paclitaxel has numerous hydrolytically sensitive ester groups. Degradation of paclitaxel mainly occurs by hydrolysis under basic and neutral conditions in aqueous solution [12]. In order to increase the therapeutic efficiency and overcome the disadvantages of the current delivery system, various methods including emulsification, micellization, liposome formation, microencapsulation and formation of polymeric micelles have been developed [13,14].

In cancer chemotherapy, long circulating carrier systems improve drug delivery potency. Polymeric micelles are one of the most promising candidates for cancer drug delivery [9,15]. Due to their unique structure, formed by amphiphilic copolymers, polymeric micelles can solubilize poorly water soluble drugs including paclitaxel and protect hydrolytically sensitive drugs from the aqueous environment. In addition, polymeric micelles can stay in the bloodstream long enough to provide accumulation at the target area, thereby increasing therapeutic efficacy [16].

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Previous work in our group demonstrated the effects of combination therapy of IL-12 and paclitaxel [17,18]. The combined approach showed significant inhibition of tumor growth. Then, arginine-grafted poly (cystaminebisacrylamide-diaminohexane) (ABP) has been showed high transfection efficiency with low cytotoxicity [19,20]. In this work, we designed a paclitaxel-conjugated polymeric micelle using the cationic bioreducible polymer ABP for the co-delivery of gene and drug. We evaluated physicochemical parameters of the micelle including particle size, surface charge, and functionality including cell transfection efficiency and anti-cancer potency. The luciferase gene and IL-12 gene were used to evaluate the efficacy for gene expression.

2. Materials and methods

2.1. Materials

Branched polyethylenimine (25 kDa, bPEI), *N*-Boc-1,6-hexanediamine (N-Boc-DAH), trifluoroacetic acid (TFA), triisobutylsilane (TIS), piperidine, *N,N′*-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino) pyridine (DMAP), ethylenediaminetetraacetic acid (EDTA), dimethyl thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF) and DL-buthionine-sulfoxamine (BSO) were purchased from Sigma–Aldrich (St. Louis, MO). *N,N′*cystaminebisacrylamide (CBA) was purchased from Polysciences, Inc. (Warrington, PA). Luciferase assay kit was purchased from Promega (Madison, WI). Succinimidyl 3-(2-pyridyldithio) propionate (SPDP), mouse IL-12 p40 enzyme-linked immunosorbent assay (ELISA) kit and BCA protein assay kit were purchased from Pierce (Rocford, IL). Thiol PEG carboxyl (HS-$PEG_{3.5k}$-COOH, Mw = 3.5 kDa) was purchased from JenKem Technology USA Inc. (Allen, TX). The reporter plasmids encoded luciferase (gWiz-Luci) was purchased from Aldevron (Fargo, ND). IL-12 plasmid used in this study previously was constructed and named p2CMVmIL-12 [21]. Dulbecco's modified eagle medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), SYBR® safe DNA gel stain and YOYO®-1 iodide were purchased from Invitrogen (Carlsbad, CA). Paclitaxel (Genexol®) was kindly provided by Samyang Genex Co. (Seoul, Korea).

2.2. Synthesis of APP

The polymer, arginine-grafted bioreducible poly (disulfide amine), ABP, was synthesized as previously reported [19]. ABP was dissolved in 0.1 M phosphate-buffered saline (PBS, pH 7.2, 0.15 M NaCl). 1.2 equiv. of SPDP dissolved in DMF was added to the ABP solution and stirred for 1 h at room temperature and then the mixture was dialyzed and lyophilized. For the PEGylation of paclitaxel, HS-$PEG_{3.5k}$-COOH was dissolved in anhydrous dichloromethane, and 2.6 equiv. of paclitaxel, 2.0 equiv. of DCC and 2.0 equiv. of DMAP were added into the paclitaxel solution at 0 °C. The mixture was further reacted for 24 h at 4 °C. The precipitate was filtered out and filtrate was extracted with methylene chloride/H_2O. The organic phase was condensed and poured into ice-cold diethyl ether to precipitate the product. Then, ABP-SPDP (1.0 equiv.) and $PEG_{3.5k}$-paclitaxel (2.5 equiv.) were dissolved in 50 mM PBS with 10 mM EDTA. The mixture was further reacted for 4 h at 4 °C. The reaction was monitored by Thin-Layer Chromatography (TLC) with ninhydrin staining and UV spectroscopy for the released pyridine-2-thione. And then, the mixture was extracted, dialyzed and lyophilized. The structure of APP was verified by ^{1}H NMR spectra.

2.3. Physical properties

The molecular weight of ABP and PEGylated paclitaxel was estimated by size-exclusion chromatography (SEC) (Superdex 75 column, calibrated with standard poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA)) using AKTA FPLC system. ABP and PEGylated paclitaxel were dissolved at a concentration of 3 mg/mL. Acetate buffer (0.1 M ammonium acetate, 30% acetonitrile, pH 5.5) was used as eluent. Flow rate was 0.4 mL/min.

The critical micelle concentration (CMC) value of the APP was determined from the measurements of UV absorption (242, 262 and 272 nm) of pyrene [22]. An acetone solution of pyrene and APP was prepared and added to water. The acetone in the solution was then evaporated. The final concentration of pyrene in each sample solution was 1.2 μM and the final concentration of the APP micelles solutions was 0.01–0.30 mg/mL.

The average size and zeta potential of the APP micelle was examined by using the Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) with a He–Ne laser (633 nm) at 25 °C. The concentration of the APP solution was 0.20 mg/mL.

2.4. Particle size and surface charge measurement of polyplexes

The average sizes and zeta potentials of ABP and APP polyplexes at 25 °C were examined using the Nano ZS. 100 μL of polyplex solutions (4 μg of plasmid DNA) were prepared in HEPES buffered saline (10 mM HEPES, 1 mM NaCl, pH 7.4) at various weight ratios ranging from 1 to 20. After 30 min of incubation, polyplex solutions were diluted to a final volume of 600 μL before measurement.

2.5. Gel retardation assay

10 μL of polyplexes were prepared in Hepes buffered saline (10 mM HEPES, 1 mM NaCl, pH 7.4) at various weight ratios ranging from 1 to 20, followed by incubation for 30 min. 0.7% agarose gel containing SYBR® safe DNA gel staining solution was prepared in TAE (10 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA) buffer. 2 μL of loading dye was added to each polyplex sample, and analyzed by electrophoresis in TAE buffer at 100 V for 35 min. The migration of DNA bands was visualized by a UV illuminator using a Gel Documentation System (Bio-Rad, Hercules, CA).

2.6. Transfection experiments and luciferase assay

MCF-7 (human breast adenocarcinoma cell line) and A549 (human lung adenocarcinoma epithelial cell line) cells were seeded at 3.0×10^4 cells/well in 24-well plates in 500 μL of DMEM containing 10% FBS and incubated at 37 °C for one day before transfection. Polyplexes were prepared with 1 μg of plasmid DNA at different weight ratios in 50 μL of FBS-free medium, and the mixtures were incubated for 30 min at room temperature. 25 kDa bPEI and ABP were used as controls. The medium was replaced by 450 μL FBS-free medium before transfection. Following 4 h treatment with polyplexes, the medium was replaced by 500 μL medium containing 10% FBS. Cells were incubated further for 2 days before measurement of luciferase activity.

To perform the luciferase assay, the cells were washed with DPBS and lysed for 30 min at room temperature with 150 μL of reporter lysis buffer (Promega, Madison, WI). The luciferase activity of 15 μL cell lysate was measured by using 100 μL of luciferase assay reagent on a Tecan Infinite M200 Pro (Tecan Group Ltd., Männedorf, Switzerland) and the protein content was measured by using a Micro BCA assay reagent kit (Pierce, Rockford, IL). All experiments were performed in triplicate.

2.7. ELISA for IL-12

MCF-7 cells were seeded at 3.0×10^4 cells/well in 24-well plates in 500 μL of DMEM containing 10% FBS and incubated at 37 °C for one day before transfection. Polyplexes were prepared with 1 μg of plasmid DNA (p2CMVmIL-12) at weight ratio of 10:1 in 50 μL of FBS-free medium, and the mixtures were incubated for 30 min at room temperature. 25 kDa bPEI and ABP were used as controls. The medium was replaced by 450 μL FBS-free medium before transfection. Following 4 h treatment with polyplexes, the medium was replaced by 500 μL medium containing 10% FBS. Cells were incubated further for 1 day before measurement. The levels of IL-12 secreted in culture supernatants were determined by using mouse IL-12 p40 ELISA kit (Pierce, Rockford, IL).

To perform the ELISA, 50 μL sample diluent and 50 μL culture supernatants were added to each well of anti-mouse IL-12 p40 pre-coated 96-well strip plate and incubated for 1 h at room temperature. The wells were washed three times with wash buffer and incubated for 1 h at room temperature with 100 μL/well of biotinylated antibody reagent. The microplate wells were washed three times, 100 μL of streptavidin-HRP solution were added to each well and incubated for 30 min at room temperature. After being washed, ELISA plate was developed with 100 μL/well of substrate solution for 30 min at room temperature in the dark. Then, the reaction was stopped by adding 100 μL of stop solution to each well and the absorbance was measured at 450 nm with a reference of 550 nm using the Tecan Infinite M200 Pro.

2.8. Cellular uptake assay

MCF-7 and A549 cells were seeded at 1.0×10^5 cells/well in 12-well plates in 2 mL of DMEM containing 10% FBS and grown at 37 °C for one day. Plasmid DNA was labeled with YOYO®-1 iodine (1 molecule of the YOYO®-1 per 50 base pair of nucleotide) for 30 min before use. Polyplexes were prepared with 0.5 μg of YOYO®-1 labeled plasmid DNA at weight ratio of 10:1 in 40 μL of FBS-free medium, and the mixtures were incubated for 30 min at room temperature. The polyplexes were added to the cells and incubated for 4 h at 37 °C in serum-free medium. Then, medium was removed carefully from the wells. The cells were washed two times with ice-cold DPBS. After trypsinization, the cells were collected by centrifuge at 1500 rpm and suspended in 500 μL of 1% FBS in DPBS. The degree of cellular uptake was examined by using the BD FACScan analyzer (Becton Dickinson, San Jose, CA). And, the acquired data was analyzed using the CellQuest software. A minimum of 10,000 cells was assessed for each sample and all samples were evaluated in triplicate.

2.9. Cytotoxicity of polymers

To measure the cytotoxicity of the polymers, ABP, ABP-$PEG_{3.5k}$ and APP, MTT assays were performed. MCF-7 and A549 cells were seeded at 5000 cells/well in 96-well plates in 90 μL of DMEM containing 10% FBS and incubated at 37 °C for one day. Then 10 μL of polymer solution at various concentrations was added and cells were incubated for 2 days before assay.