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DNA Polyplexes as Combinatory Drug Carriers of Doxorubicin and Cisplatin: An In Vitro Study

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Abstract

Double helix nucleic acids were used as a combination drug carrier for doxorubicin (DOX), which physically intercalates with DNA double helices, and cisplatin (CDDP), which binds to DNA without an alkylation reaction. DNA interacting with DOX, CDDP, or both was complexed with positively charged, endosomolytic polymers. Compared with the free drug, the polyplexes (100 ~ 170 nm in size) delivered more drug into the cytosol and the nucleus and demonstrated similar or superior (up to a 7-fold increase) in vitro cell-killing activity. Additionally, the gene expression activities of most of the chemical drug-loaded plasmid DNA (pDNA) polyplexes were not impaired by the physical interactions between the nucleic acid and DOX/CDDP. When a model reporter pDNA (luciferase) was employed, it expressed luciferase protein at 0.7- ~ 1.4-fold the amount expressed by the polyplex with no bound drugs (a control), which indicated the fast translocation of the intercalated or bound drugs from the “carrier DNA” to the “nuclear DNA” of target cells. The proposed concept may offer the possibility of versatile combination therapies of genetic materials and small molecule drugs that bind to nucleic acids to treat various diseases.

Keywords

Combination therapy; DNA binding; DNA intercalation; Nanocarrier; pDNA; Polyplex

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Supporting information

The transfection efficiency (RLU/mg Protein) and dose-dependent cell viabilities of polymer/DNA and RPC-bPEI_{0.8}kDa⁴/chemical drug-DNA complexes in MCF7, MCF7/ADR-RES, A2780, A2780/ADR, and A2780/Cis cells and the size distribution of polymer/DOX-CDDP-DNA complexes are presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

For various diseases, combination therapy, which allows the use of lower doses of therapeutics, has greater therapeutic efficacy with fewer side effects than single drug therapy.^{1, 2} This approach has, in turn, promoted strong interest in the development of nano-sized carriers that can deliver combinatorial drugs with altered toxicity profiles.^{3–7} The therapy for a particular disease can be custom designed by selecting a suitable drug combination from a pool of various small molecule drugs and biological entities with diverse physico-chemical properties. Administering such a combination often requires a specific nanocarrier system with multiple compartments to load multiple drugs in a single carrier. For instance, liposomal and similar structures, such as polymersomes, can accommodate both a water-soluble drug in the aqueous core and a hydrophobic drug in the lipid bilayer.^{8–10} Charged biologics mostly require countercharged molecules/polymers to form electrostatic complexes.^{4, 11, 12} However, unlike hydrophobic agents, hydrophilic drugs often suffer from low loading efficiency and content due to the low volume ratio of the formed vesicles to the added drug solution volume when such vesicles are prepared by either a thin film hydration method or solvent displacement (e.g., nanoprecipitation).¹³ Although a remote loading technology using a pH or ammonium gradient allows certain hydrophilic drugs to cross the vesicle bilayer and thereby achieve high loading content in the vesicle core,¹³ this approach does not apply to the majority of water-soluble drugs. Thus, a small molecule drug is often chemically conjugated with various polymers or nanocarriers for co-delivery with hydrophobic therapeutics.^{14–16} However, the chemical modification is complicated, and therapeutic efficacy may be compromised as a result of this modification.

To co-deliver biologics (e.g., nucleic acids and proteins) and hydrophobic chemicals, polymeric micelles with hydrophobic cores and counter-charged shells have been employed.^{4, 17–20} However, constructing such nanocarriers for combination delivery requires time-consuming and laborious preparation steps such as micellization and separation.

Interactions between double helix nucleic acids and small molecules have been extensively investigated to identify drug candidates that intercalate with DNA or bind to DNA for various diseases.^{21, 22} Various forms of nucleic acids have been used to serve as drug carriers by taking advantage of such interactions.^{23–30} Examples include the use of DNA,²³ pDNA,²⁴ RNA aptamers,^{25–29} and polyGC³⁰ to deliver doxorubicin (DOX). However, before reaching target sites, DOX that is intercalated with DNA can dissociate because the physical intercalation between DOX and nucleic acids is reversible³¹ and because the nucleic acids can be degraded by DNases in serum. Additionally, caffeine in the blood modulates DOX intercalation with DNA, which reduces the cell-killing effects of DOX in vitro³² and causes DOX de-intercalation.^{33, 34} Thus, DOX-intercalated DNA should be protected to minimize the unwanted loss of DOX. It has been reported that DOX-intercalated polyGC or pDNA can be complexed and shielded with cationic gelatin or dendrimer and that such DOX-loaded nucleic acid nanoparticles showed effective gene expression and treated solid tumors in vivo.^{2, 30} However, it was not investigated how chemical drugs that interact with pDNA, affect nuclear DNA in the cells of interest or how pDNA, which interacts with chemical drugs, expresses its encoded protein. In addition,

although various candidate drugs for chemical drug-nucleic acid interaction exist, only DOX has been used.

When considering polycations for pDNA complexation, even though a branched polyethyleneimine (bPEI) with an average molecular weight of 25 kDa (bPEI_{25kDa}) is a gold-transfection polymer, its cytotoxicity is a critical concern for biomedical and pharmaceutical applications. Thus, many researchers have developed various polycations with stimulus (e.g., pH, enzymes, thiols)-triggered degradable properties. Of these, our group reported that bio-reducible bPEI derivatives interact electrostatically with pDNAs and protect them from nucleases in extracellular environments while releasing them in intracellular environments (e.g., the cytosol and the nucleus) after intracellular thiols (i.e., glutathione) trigger degradation of the polymers.³⁵ Additionally, these bio-reducible bPEI derivatives have been tested for intracellular protein delivery.³⁶ However, although reducible polymeric micelles have been used for the intracellular delivery of hydrophobic chemicals,³⁷ reducible polycations have not previously been used.

This study investigated nucleic acid interactions with DOX and/or *cis*-diamineplatinum (II) dichloride (cisplatin, CDDP) for the intracellular co-delivery of the two drugs and investigated the biological activity of the nucleic acids in the nanosized complexes. DOX and a hydrophilic CDDP were selected as model drugs for interaction with DNA. DOX reversibly intercalates with DNA, and the DOX-intercalated DNA forms a stable complex with DNA topoisomerase II to inhibit DNA replication.^{22, 38} Purine bases in DNA bind with CDDP, and the binding interaction matures to form CDDP-alkylated DNA.^{22, 39} The CDDP-DNA crosslink interferes with mitosis-driven cell division, leading to apoptotic cell death.²² A combinational therapy of DOX and CDDP has shown therapeutic synergism because CDDP-damaged DNA can be poorly repaired by the inhibition of topoisomerase II.¹⁴ Thus, in this study, we expected that the co-delivery of DOX and CDDP in a single carrier would result in synergistic therapeutic efficacy if these two drugs physically interact with nucleic acids. In addition, luciferase pDNA was used as a model pDNA carrier for DOX and CDDP; then, drug-loaded pDNA was complexed with bPEI_{25kDa} model polycations and their bio-reducible derivatives via electrostatic interactions. The drug-loaded polyplexes were investigated to determine the chemical drug-mediated cytotoxic effects and the pDNA (luciferase) transfection efficiency. The working mechanisms of the complexes in expressing luciferase and exerting the anticancer effects of the chemical drugs were investigated. In addition, we examined whether bio-reduction affects intracellular chemical delivery for enhanced cell-killing activity during the co-delivery of chemical drugs and nucleic acid drugs.

EXPERIMENTAL SECTION

Materials

Branched PEIs (bPEI_{0.8kDa} (M_w 0.8 kDa, M_n 0.6 kDa), bPEI_{25kDa} (M_w 25 kDa, M_n 10 kDa), 2-iminothiolane, L-cysteine hydrochloride monohydrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640 medium, Ca²⁺-free and Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS), triethylamine (TEA), dimethyl sulfoxide (DMSO), 4-(2-hydroxy-ethyl)-1-piperazine (HEPES), D-glucose, sodium bicarbonate,

recombinant human insulin, ethidium bromide (EtBr), dithiothreitol (DTT), Hoechst 33342 (HO), fetal bovine serum (FBS), penicillin-streptomycin antibiotics, trypsin-EDTA solution, paraformaldehyde (PFA), doxorubicin (DOX or adriamycin (ADR)), deoxyribonucleic acid sodium salt (from salmon testes; DNA or sDNA), *cis*-diamineplatinum (II) dichloride (cisplatin, CDDP), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and DNA ladders (IDNA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasmid DNA (pDNA) encoding firefly luciferase (gWiz-Luc or pLuc) was purchased from Aldevron, Inc. (Fargo, ND, USA). A luciferase assay kit and a BCATM protein assay kit were purchased from Promega Corporation (Madison, WI, USA) and Pierce Biotechnology, Inc. (Rockford, IL, USA), respectively.

In this study, the reducible polycations (RPCs) RPC-bPEI_{0.8kDa}1 and RPC-bPEI_{0.8kDa}4, which are 80.3 kDa and 5.7 kDa, respectively, were used after synthesis from low molecular weight bPEI_{0.8kDa} via thiolation and oxidation, as previously reported.^{40, 41} In brief, the primary amines of bPEI_{0.8kDa} (550 mg; 687.5 μ mol of bPEI_{0.8kDa} based on a M_w of 800; 3.2 mmol of amines based on the theoretical ratio (*i.e.*, 1°:2°:3°=25%:50%:25%) of amines in bPEI) were thiolated with 2-iminothiolane (2 or 1.2 equivalents of bPEI_{0.8kDa} for RPC-bPEI_{0.8kDa}1 or RPC-bPEI_{0.8kDa}4, respectively) in DPBS (55 mL; pH 7.0~7.4) for 12 hr at room temperature (RT). Upon completion, DMSO (18.33 mL; one-third DPBS by volume) and L-cysteine hydrochloride monohydrate (0.2 mmol or 0.24 mmol for RPC-bPEI_{0.8kDa}1 or RPC-bPEI_{0.8kDa}4, respectively) were added to the solution, and the DMSO-induced oxidative polymerization of thiolated bPEI_{0.8kDa} was conducted for 24 hr at RT. To remove the DMSO and excess reactants, the polymer solution was dialyzed for 24 hr in deionized water using a dialysis membrane (3.5 kDa of molecular weight cutoff). Finally, bPEI-based RPCs (RPC-bPEI_{0.8kDa}s) were lyophilized and stored at -20°C prior to use.

Cells and Cell Culture

MCF7 cells (a human breast adenocarcinoma cell line), MCF7/ADR-RES cells (a DOX-induced multidrug-resistant (MDR) subline of MCF7 cells), A2780 cells (a human ovarian carcinoma cell line), A2780/ADR cells (a DOX-induced MDR subline of A2780 cells), and A2780/Cis cells (a CDDP-induced MDR subline of A2780 cells) were used. The cells were cultured in culture medium (*i.e.*, RPMI 1640 medium) supplemented with D-glucose (2 g/L) and 10% heat-inactivated FBS under humidified air containing 5% CO₂ at 37°C. To maintain the MDR characteristics of the MCF7/ADR-RES cells and A2780/ADR cells, the cells were treated with DOX (400 ng/mL) weekly. Additionally, insulin (4 mg/L) was added to the medium for MCF7 cells and MCF7/ADR-RES cells.

Preparation and Physicochemical Characteristics of Polycation/Chemical Drug-Nucleic Acid Complexes

Using model polycations (*i.e.*, bPEI_{25kDa}, RPC-bPEI_{0.8kDa}1, and RPC-bPEI_{0.8kDa}4), model chemical drugs (*i.e.*, DOX and CDDP), and model nucleic acids (*i.e.*, DNA and pDNA), polycation/chemical drug-nucleic acid complexes were prepared. In brief, after nucleic acids had been incubated with chemical drugs for 30 min (for DOX) or various other time periods (2 ~ 30 min for CDDP) in HEPES buffer (20 mM, pH 7.4), chemical drug-bound nucleic acids (*i.e.*, DOX-nucleic acid, CDDP-nucleic acid, and DOX-CDDP-nucleic acid) were

mixed with polycations under predetermined complexation conditions. The solutions of polycation/chemical drug-nucleic acid complexes (20 μ L per 1 μ g nucleic acid) were incubated for 30 min at RT. To describe the complexation ratios of the resulting complexes, the N/P ratio and weight ratio (WR) were calculated based on the ratio of the amines (N) of polycations to the phosphate groups (P) of nucleic acids and the WR of polycations to nucleic acids, respectively.

The physicochemical characteristics (*e.g.*, particle size and surface charge) of the polyplexes were measured using a Zetasizer 3000HS (Malvern Instrument, Inc., Worcestershire, UK) at a wavelength of 677 nm with a constant angle of 90° at RT. For surface charge and particle size measurements, the concentrations of nucleic acid in the polyplex solutions were 2.5 μ g/mL and 5 μ g/mL, respectively.

To monitor the loading efficiency of the chemical drugs into the polymer/chemical drug-nucleic acid complexes, the polyplex solution was centrifuged at 4000 rpm in an ultrafiltration unit (Millipore centrifugal filter units with molecular weight cut-off (MWCO) 3.5 kDa) at 4°C for 10 min to remove the unloaded chemical drugs. The amounts of DOX and CDDP in the filtrate were evaluated using a DOX absorbance standard curve at 485 nm and inductively coupled plasma mass spectrometry (ICP-MS), respectively. The loading efficiency (LE) of the chemical drugs into the polyplexes was calculated using the following equation.

$$\text{Loading efficiency (LE) of chemical drugs (\%)} = \frac{\text{Weight of chemical drugs in polyplex}}{\text{Weight of chemical drugs in feed}} \times 100 (\%)$$

To monitor the release of DOX from the polymer/chemical drug-nucleic acid complexes, the polyplexes (1 mL) were incubated in the presence of heparin (50 μ g/mL) at 37°C for 1 hr and then transferred into a dialysis unit (MWCO 3.5 kDa). The polyplex-containing dialysis unit was dialyzed against DIW (4 mL) at 80 rpm and 37°C. At predetermined time-points, the solution outside the dialysis unit was replaced with fresh DIW (4 mL), and the dialysate was lyophilized. After the lyophilized powder was dissolved in DMSO, the DOX concentration was calculated by observing its fluorescence at 479 nm (excitation) and 593 nm (emission).

In Vitro Cytotoxicity of Drug-Free Polyplexes and In Vitro Cell-killing Effects of Chemical Drug-Loaded Polyplexes

To examine the in vitro cytotoxicity of chemical drug-free polycation/nucleic acid complexes and of polycation/chemical drug-nucleic acid complexes, an MTT-based assay was selected. The cells were seeded at a density of 5×10^3 cells/well in 96-well plates. The seeded cells were cultured for 24 hr before adding various concentrations of chemical drug-free polyplexes, chemical drug-loaded polyplexes, or free chemical drugs. With polyplexes or free chemical drugs, MCF7, A2780, and A2780/Cis cells were cultured for 2 d, whereas DOX-resistant cell lines (*i.e.*, MCF7/ADR-RES and A2780/ADR cells) were incubated for 5 d. In the case of the 5 d experiments, the culture medium was replaced with fresh medium at 3 d. After 2 d or 5 d of treatment with polyplexes or free chemical drugs, MTT solution (10

μL; 5 mg/mL) was added to the cells (0.1 mL of culture medium), and an additional 4-hr incubation was conducted. On discarding the MTT-containing medium, the formazan crystals produced by living cells were dissolved in DMSO, and their absorbance was measured at 570 nm using a microplate reader.

In Vitro Transfection of Chemical Drug-Free Polyplexes and Chemical Drug-Loaded Polyplexes

As previously reported,^{42–44} an in vitro transfection study of polyplexes was performed in 6-well plates, and the cells were seeded at a density of 5×10^5 cells/well. The seeded cells were cultured for 24 hr prior to adding polyplexes, and the culture medium containing 10% FBS was replaced with a serum-free and insulin-free medium 1 hr before polymeric transfection. The cells were exposed to the polyplexes (20 μL per 1 μg pDNA) for 4 hr and then incubated for an additional 44 hr with a serum-containing medium. For MCF7 and MCF7/ADR-RES cells, insulin was additionally added to the serum-containing medium. After finishing the 48-hr transfection experiments, the cells were thoroughly rinsed with $\text{Ca}^{2+}(-)\text{Mg}^{2+}(-)\text{DPBS}$ and then lysed using a reporter lysis buffer. The relative luminescence units (RLU) and protein content in the cell lysates were evaluated using the manufacturer's protocols for the luciferase assay and the BCATM protein assay, respectively. To compare the transfection efficiencies of the polymer/chemical drug-pDNA complexes and the polymer/pDNA complexes, the normalized transfection efficiency was used, as expressed by the following equation:

$$\text{Normalized Transfection Efficiency (Fold)} = \frac{\text{Transfection Efficiency (RLU/mg Protein) of polymer/chemical drug-pDNA complexes}}{\text{Transfection Efficiency (RLU/mg Protein) of polymer/pDNA complexes}}$$

Cellular Uptake, Nuclear Uptake, and Intracellular Distribution of Chemical Drugs Delivered with Chemical Drug-Loaded Polyplexes

To assess the cellular uptake and nuclear uptake of free chemical drugs and polymer/chemical drug-DNA complexes, cells were seeded in 6-well plates at a density of 5×10^5 cells/well in culture medium (2 mL) and incubated for 24 hr. After a 4-hr treatment with free chemical drugs (i.e., DOX + CDDP) or polymer/DOX-CDDP-DNA complexes ([DOX] = [CDDP] = 5 μM), the cells were rinsed twice with DPBS and detached. To determine the cellular uptake of free chemical drugs or polymer/DOX-CDDP-DNA complexes, the intracellular fluorescence of DOX was measured using a flow cytometer (FACScanto II, Becton–Dickinson, Franklin Lakes, NJ, USA) with a primary argon laser (532 nm) and a fluorescence detector (578±15 nm). The DOX uptake by the cells was analyzed from a viable population, as determined by gating, of at least 1×10^4 cells.

For nuclear uptake, after the cells were prepared and exposed to free chemical drugs or polymer/DOX-CDDP-DNA complexes for 4 hr, as in the experimental procedure for cellular uptake, the cells were rinsed twice with DPBS and detached. The samples for nuclear uptake of free chemical drugs or polymer/DOX-CDDP-DNA complexes were prepared using the Nuclei PURE Prep nuclei isolation protocol. Nuclear DOX fluorescence

was measured using a flow cytometer (FACScanto II, Becton–Dickinson, Franklin Lakes, NJ, USA) with a primary argon laser (532 nm) and a fluorescence detector (578±15 nm).

To investigate the intracellular locations of DOX in polyplex-treated cells, the cells (1×10^5 cells) were seeded on coverslips, and the study was performed as previously described in the in vitro transfection study. At 2 hr post-transfection (30 min prior to sampling), HO was added to stain the nucleus. The cells were rinsed with Ca^{2+} (-) Mg^{2+} (-)DPBS and fixed with 4% PFA. The cells were evaluated using a laser scanning confocal microscope (FV1000, Olympus, Center Valley, PA, USA) with excitation lasers (diode for 408 nm and HeNe for 543 nm) and variable band-pass emission filters. Confocal images were collected in 500 nm sections and used to construct images of whole cells.

Translocation of Chemical Drugs from One DNA to Another DNA

To understand how chemical drugs bound to “carrier DNA” translocate to “target DNA” (i.e., nuclear DNA) to express the in vitro cell-killing effects of chemical drugs and how “carrier DNA” (i.e., pDNA) bound to chemical drugs expresses its corresponding protein, fluorescent EtBr and DAPI were used as model molecules for intercalating chemical drugs and minor-groove binding chemical drugs, respectively; sDNA (salmon testes DNA) and IDNA (ladder DNA) were selected as “carrier DNA” and “nuclear DNA”, respectively. After mixing either EtBr or DAPI with sDNA at 10 moles of EtBr or DAPI per nucleotide mole of sDNA, respectively, the EtBr-intercalated sDNA (EtBr-sDNA) or DAPI-minor groove-bound sDNA (DAPI-sDNA) was incubated for 30 min. Then, either EtBr-sDNA or DAPI-sDNA was mixed and incubated with IDNA at different molar ratios of sDNA and IDNA for predetermined times. The DNA mixture was transferred into 1% agarose gel, and the DNA-loaded gel in 0.5x TBE buffer was electrophoresed at 50 mV for 120 min. EtBr-intercalated DNA and DAPI-minor groove bound DNA were detected using a UV illuminator.

RESULTS AND DISCUSSION

Optimal Conditions of Chemical Drug-Nucleic Acid Interaction

To prepare efficient polymer/chemical drug-nucleic acid complexes, we first determined the optimal molar ratios between DOX and DNA. As previously reported,⁴⁵ when DOX was mixed with DNA, the emission fluorescence of DOX at approximately 595 nm was dramatically decreased upon increasing the amount of DNA (Figure 2(a)). Mixing DOX with DNA at 1:1 and 1:2 molar ratios of [DOX]:[nucleotide] reduced the fluorescence of DOX to approximately 33% and 9%, respectively, compared to free DOX, indicating that DOX was intercalated in the double-stranded DNA. Above a 1:3 molar ratio, the fluorescence of the intercalated DOX reached approximately 5% of the intrinsic fluorescence of free DOX.

Then, MCF7 cells were treated with the DOX-intercalated DNA prepared at different mixing ratios of DOX and nucleotides to find the optimal ratio for the highest cell-killing effect. The IC_{50} (the drug concentration that causes 50% growth inhibition) values of DOX-DNA (1:1), DOX-DNA (1:2), and DOX-DNA (1:3) were close to the IC_{50} (0.96 μM) of free DOX, whereas DOX-DNA (1:4) and DOX-DNA (1:5) had slightly lower IC_{50} values (~ 0.8

μM) (Figure 2(b)). Although DOX-DNA complexes at a 1:4 molar ratio and DOX-DNA complexes at a 1:5 molar ratio had similar IC₅₀ values, the DNA in DOX-DNA (1:4) was able to deliver 1.25-fold more moles of DOX than DNA in DOX-DNA (1:5) for the same amount of DNA because the DOX content in DOX-DNA (1:4) and DOX-DNA (1:5) was approximately 41.7 wt% and 33.3 wt%, respectively. However, this study used DOX-DNA prepared at a 1:5 molar ratio for further experiments because a higher DOX content in DOX-DNA could increase the hydrophobicity, resulting in insoluble DOX-DNA at high concentrations. Additionally, DOX-DNA (1:5) was preferred over DOX-DNA (1:4) because a higher DNA content in DOX-DNA could be beneficial when the DNA is replaced with therapeutic nucleic acids for the co-delivery of DOX and nucleic acids. Specifically, DOX-DNA (1:5) completely intercalated the added DOX, as the filtrate did not contain any DOX after centrifuging the DOX-DNA (1:5).

Unlike DOX, CDDP is a DNA alkylating agent that eventually forms a chemical bond with the purine base of the nucleotide (i.e., N7 of guanine). The extent of DNA alkylation by CDDP is time-dependent and gradually increased after incubation for approximately 30 min.³⁹ Once DNA is alkylated by CDDP, both DNA and CDDP become inactive; therefore, we must find an optimal incubation time for CDDP-DNA to keep both CDDP and DNA active. Assuming an equal molar content of the four bases (i.e., A, T, C, G) in DNA, CDDP-DNA (1:4) could be an optimal binding molar ratio. However, as mentioned, CDDP-DNA (1:5) is preferable to CDDP-DNA (1:4) because a higher DNA content in CDDP-DNA could be beneficial for the co-delivery of CDDP and therapeutic nucleic acids. Thus, although the chemical and physical properties of CDDP are quite different from the properties of DOX, the molar mixing ratio of CDDP-DNA was set to 1:5, as in the DOX-DNA experiments. After CDDP was added to the DNA solution, the CDDP-DNA was incubated for 2 to 30 min. Then, the CDDP-bound DNA was complexed with a model polycation (e.g., RPC-bPEI_{0.8kDa}4) to form RPC-bPEI_{0.8kDa}4/CDDP-DNA complexes (WR 5) to avoid premature dissociation of CDDP from the DNA. To monitor the cell-killing effects of the CDDP-bound DNA, the resulting complexes were added to A2780 cells instead of MCF7 cells because A2780 cells were slightly more sensitive to CDDP than MCF7 cells in our experiments. As shown in Figure 3, all RPC-bPEI_{0.8kDa}4/CDDP-DNA complexes (WR 5), constructed using CDDP-DNA (1:5) prepared with different incubation times, presented greater cytotoxicity than free CDDP. However, the complexes prepared at different incubation times did not present significantly different cell-killing effects. This result implies that the duration of 30 min for incubation is not long enough for a DNA alkylation reaction, but 2 min for incubation followed by complexation with polycations was sufficient for CDDP delivery and was selected for use in further experiments for simplicity.

Preparation and Physicochemical and Drug Release Properties of Polymer/Chemical Drug-Nucleic Acid Complexes

To complex the chemical drugs with nucleic acids, we selected non-reducible bPEI_{25kDa} and its reducible derivatives (i.e., RPC-bPEI_{0.8kDa}1 and RPC-bPEI_{0.8kDa}4) with endosomal escaping activity.³⁵ Based on our previous experience,³⁵ the complexation ratios of N/P 5 between polycations and pDNA for bPEI_{25kDa}/chemical drug-DNA complexes, WR 2 for RPC-bPEI_{0.8kDa}1/chemical drug-DNA complexes, and WR 5 for RPC-bPEI_{0.8kDa}4/

chemical drug-DNA complexes were used. The resulting polymer/chemical drug-DNA complexes had positive surface charges (~ 20 mV). When DOX and CDDP were loaded into nanoparticles, the bPEI_{25kDa}/chemical drug-DNA complexes were 140~170 nm in diameter (Figure 4). The RPC-bPEI_{0.8kDa}¹/chemical drug-DNA complexes and the RPC-bPEI_{0.8kDa}⁴/chemical drug-DNA complexes were 100 ~ 150 nm in diameter. As shown in Figure S1, the size distribution of the polyplexes was unimodal with approximately 0.2~0.3 polydispersity. However, when loading DOX into the polymer/chemical drug-DNA complexes, the maximum concentration of DOX was limited to 10 μ M and 5 μ M in the RPC-bPEI_{0.8kDa}¹/chemical drug-DNA complexes and RPC-bPEI_{0.8kDa}⁴/chemical drug-DNA complexes, respectively, because the DOX-loaded polyplexes precipitated at higher DOX concentrations.

When using model polyplexes such as bPEI_{25kDa}/DOX-CDDP-DNA, RPC-bPEI_{0.8kDa}¹/DOX-CDDP-DNA, and RPC-bPEI_{0.8kDa}⁴/DOX-CDDP-DNA complexes, the polyplexes encapsulated almost all of the DOX in the initial mixture. The complete loading of DOX into the polyplexes may have resulted from the almost complete intercalation of DOX into the DNA at the 1:5 molar ratio. However, the CDDP loading efficiencies were $66.5 \pm 2.6\%$ for bPEI_{5kDa}/DOX-CDDP-DNA complexes, $69.6 \pm 0.3\%$ for RPC-bPEI_{0.8kDa}¹/DOX-CDDP-DNA complexes, and $71.6 \pm 2.6\%$ for RPC-bPEI_{0.8kDa}⁴/DOX-CDDP-DNA complexes expressed as the mean $\pm$ standard deviation ($n=3$). The lower CDDP loading compared with DOX loading may have resulted from the different interaction strengths of CDDP and DOX with nucleic acids (i.e., the differences between the weak binding of CDDP to nucleic acids and the relatively strong intercalation of DOX with nucleic acids).

The release of chemical drugs from the polymer/chemical drug-DNA complexes was tested using a model polyplex, namely bPEI_{25kDa}/DOX-DNA complexes. The polycation was dissociated from DOX-DNA because of the difficult DOX release from the polyplexes. In this study, heparin was used as a model anion because intracellular polyanions such as nucleic acids could competitively decomplex polymer/chemical drug-DNA complexes. Compared with the release rate of free DOX from a dialysis tube (MWCO 3.5 kDa), the released amount of DOX indicates that the DOX release rate from DOX-DNA is approximately 3-fold slower than for free DOX under the experimental conditions (Figure 5).

Cell-killing Characteristics and Intracellular Distribution of Polymer/Chemical Drug-Nucleic Acid Complexes

To evaluate the therapeutic efficacy of DOX and CDDP with different nucleic acids, DOX-DNA (1:5), CDDP-DNA (1:5), or DOX-CDDP-DNA (0.5:0.5:5) was complexed with bPEI-based non-degradable (bPEI_{25kDa}) or degradable polymers (RPC-bPEI_{0.8kDa}¹ and RPC-bPEI_{0.8kDa}⁴). Then, the polymer/chemical drug-DNA complexes were used to treat breast cancer cells (wild-type MCF7 cells and DOX-resistant MCF7/ADR-RES cells) and ovarian cancer cells (wild-type A2780 cells, DOX-resistant A2780/ADR cells, and CDDP-resistant A2780/Cis cells). The cell-killing effect of polymer/chemical drug-DNA complexes in DOX-resistant cell lines was evaluated after treatment for 5 d, whereas the other three cell lines were exposed to the complexes for 2 d.

The cytotoxicity of polymer/DNA complexes was evaluated before the polymer/chemical drug-DNA complexes to exclude cationic polyplex-mediated killing activity against tumor cell lines (Figure S2). When preparing polymer/DNA complexes at [DNA] 10 µg/mL and [DNA] 3–20 µg/mL in MCF7 cells and MCF7/ADR-RES cells, respectively, their cell viabilities were above 80%. This result suggests that polymer-mediated cytotoxicity could be negligible at [Drug] 6 µM in MCF7 cells for 2 d and [Drug] 12 µM in MCF7/ADR-RES cells for 5 d because the mixing molar ratio between chemical drugs and DNA was 1:5. Similarly, in A2780 cells, A2780/ADR cells, and A2780/Cis cells, [DNA] 3.5 µg/mL, [DNA] 7 µg/mL, and [DNA] 7 µg/mL, respectively, caused negligible cytotoxicity of polymer/DNA complexes, indicating that the cytotoxicity of polymer/chemical drug-DNA complexes at [Drug] 2 µM for 2 d, [Drug] 4 µM for 5 d, and [Drug] 4 µM for 2 d, respectively, might be influenced by the chemical drugs but not the cationic polymers.

Free DOX is internalized by cells via direct membrane penetration or hydrophobicity-induced adsorptive endocytosis, whereas CDDP is internalized via fluid-phase endocytosis or specific transport-mediated internalization. This difference indicates that the internalization rate of DOX could be faster than the internalization of CDDP. Positively charged polymer/chemical drug-DNA complexes could be difficult to internalize via direct membrane penetration but might internalize via ionic attraction-mediated adsorptive endocytosis and then escape from endolysosomal compartments through bPEI-induced proton buffering. When evaluating the cellular uptake of polymer/chemical drug-DNA complexes by model cells such as MCF7 cells, which are drug sensitive, and MCF7/ADR-RES cells, which are drug resistant, the cellular uptake of chemical drugs with or without drug carriers showed different trends depending on the drug sensitivity/resistance of the cells. As expected, the intracellular DOX fluorescence in bPEI_{25kDa}/DOX-CDDP-DNA, RPC-bPEI_{0.8kDa}1/DOX-CDDP-DNA, or RPC-bPEI_{0.8kDa}4/DOX-CDDP-DNA complex-treated MCF7 cells was 1.1-fold, 1.3-fold, or 1.2-fold lower than in the free chemical drug-treated cells, respectively, indicating that less drugs were delivered to the cells by the nanocarriers than by carrier-free delivery (Figure 6(a)). However, the intracellular DOX fluorescence in the polymer/DOX-CDDP-DNA complex-treated MCF7/ADR-RES cells was 1.5–2.0-fold higher than in the free chemical drug-treated cells, indicating more drugs were delivered to the cells by the nanocarriers than by carrier-free delivery (Figure 6(b)). These different cell-dependent DOX delivery activities of the polyplexes might have resulted from the different cellular efflux abilities of drug-sensitive and drug-resistant cells in response to the cellular uptake of chemical drugs.

Although the polymer/chemical drug-DNA complexes escaped from the endolysosomes, the polymer must dissociate from the polyplexes to release the chemical drugs. Thus, it was expected that the polymer/chemical drug-DNA complexes would have somewhat lower cell-killing effects than DOX alone but somewhat higher cell-killing effects than CDDP alone. However, the *in vitro* cell-killing effects of the polymer/DOX-DNA, polymer/CDDP-DNA, and polymer/DOX-CDDP-DNA complexes were similar or superior to (up to 7-fold higher than) the effects of the free chemical drugs regardless of the polymers and chemical drugs used (Figure 7). Specifically, in MCF7 cells, the IC₅₀ value (0.8 µM) of DOX-DNA (1:5) (Figure 2(b)) was similar to the IC₅₀ value of the RPC-bPEI_{0.8kDa}1/DOX-DNA complexes. Although additional steps are required for drug release from polyplexes, unlike DOX-DNA,

bPEI_{25kDa}/DOX-DNA and RPC- bPEI_{0.8kDa}4/DOX-DNA complexes showed an approximately 1.2-fold greater cell-killing effect than did DOX-DNA (1:5) (Figure 7 and Figure S3). Thus, we expect that the polymer/chemical drug-DNA complexes are more capable of inducing cell-killing effects than are the chemical drug-DNA complexes.

In addition, it was expected that RPC-bPEI_{0.8kDa}1- and RPC-bPEI_{0.8kDa}4-based complexes would show better cell-killing effects than bPEI_{25kDa}-based complexes because the thiol-triggered biodegradation of RPC-bPEI_{0.8kDa}1 and RPC-bPEI_{0.8kDa}4 in the cytosol or the nucleus could cause the faster release of chemical drugs compared with non-degradable bPEI_{25kDa}. Furthermore, it was thought that RPC-bPEI_{0.8kDa}4 would exhibit better cell-killing effects than RPC-bPEI_{0.8kDa}1 because RPC-bPEI_{0.8kDa}4 has a lower molecular weight than RPC-bPEI_{0.8kDa}1.35 However, the cell-killing effects of polycation/chemical drug-DNA complexes did not show remarkable differences based on the polycation used.

The superior cell-killing effects of polycation/chemical drug-DNA complexes compared with free chemical drugs were further supported by the intracellular fluorescence intensity of DOX delivered with or without polymeric nanocarriers. To clearly elucidate the delivery effects of chemical drugs according to the cationic polyplex used, DOX-resistant MCF7/ADR-RES cells were used because DOX-resistant cells can prevent the cellular internalization of free DOX. In addition, although DOX and CDDP were co-delivered by a single nanocarrier, the intracellular fluorescence of only DOX was monitored because CDDP itself was not detected by fluorescence or absorbance. The intracellular DOX fluorescence evaluated by confocal microscopy showed that DOX delivered by bPEI_{25kDa}/DOX-CDDP-DNA or RPC-bPEI_{0.8kDa}1/DOX-CDDP-DNA complexes resulted in stronger fluorescence in the cytoplasm and the nucleus than did free DOX (Figure 8(a)). In addition, as determined by nuclear isolation, polymer/DOX-CDDP-DNA complexes were taken up by the nuclei 2.0~2.6-fold more than the free chemical drugs were (Figure 8(b)). These results demonstrate why the chemical drug-loaded polyplexes exhibited much more potent cell-killing effects than the free chemical drugs.

Transfection Characteristics of Polymer/Chemical Drug-Nucleic Acid Complexes

When plasmid DNA (pDNA) was applied instead of DNA, we investigated whether pDNA could express its encoded protein even with DOX and CDDP interaction. For this study, cytotoxic chemical drugs and pDNA in polymer/chemical drug-pDNA complexes, at approximately 300 nM and 1.52 μ M (1 μ g/well), respectively, were added to 5×10^5 cells/well cultured in 2 mL of culture medium, and pDNA expression was monitored at 2 d post-transfection. The experimental conditions of the in vitro transfection studies (Figure 9) included 100-fold more cells and a 20-fold larger medium volume than for the in vitro cell-killing studies (Figure 7). That is, if the same concentration of chemical drugs was used, the cells in the former experiments were exposed to a 5-fold smaller amount of chemical drugs than in the latter experiments. Additionally, the transfection efficiencies of the bPEI_{25kDa}/pDNA, RPC-bPEI_{0.8kDa}1/pDNA, and RPC-bPEI_{0.8kDa}4/pDNA complexes were $(4.72 \pm 0.76) \times 10^9$ RLU/mg Protein, $(1.15 \pm 0.34) \times 10^8$ RLU/mg Protein, and $(4.10 \pm 0.85) \times 10^7$ RLU/mg Protein, respectively, in MCF7 cells and were set as efficiencies of 1.0 to easily compare the transfection efficiencies of the chemical drug-incorporated pDNA complexes.

In other cells, the transfection efficiencies (RLU/mg) of the chemical drug-free polyplexes used to normalize the transfection efficiencies of the polymer/chemical drug-pDNA complexes are summarized in Table S1.

When applying polymer/CDDP-pDNA (2 min) complexes prepared using a 2-min interaction between CDDP and pDNA, the normalized transfection efficiencies of most polymer/CDDP-pDNA complexes were similar (0.6~1.2-fold) to the normalized transfection efficiencies of the polymer/pDNA complexes in all five cell lines and were not influenced by the type of polymer used in the complexes (Figure 9), which indicated very weak chemical drug-induced changes in the transfection efficiencies of the polymer/chemical drug-pDNA complexes. However, interestingly, when applying a polymer/CDDP-pDNA (30 min) complex prepared by interacting pDNA with CDDP for 30 min, most polymer/CDDP-pDNA (30 min) complexes exhibited 4~50-fold lower transfection efficiencies than the polymer/pDNA complexes in all five cell lines, regardless of the polymer type (Figure 9). In contrast to the transfection efficiencies of the polymer/CDDP-pDNA (2 min) complexes, the markedly reduced transfection efficiencies of the polymer/CDDP-pDNA (30 min) complexes indicate that 2 min of interaction between CDDP and pDNA may form mostly CDDP-bound pDNA, whereas 30 min of interaction between CDDP and pDNA may form primarily CDDP-alkylated pDNA, as the given CDDP concentration (300 nM in the transfection studies, approximately 60 nM of the estimated concentration in the cell-killing studies) did not induce significant cytotoxicity in any of the five cell lines (Figure 7). This result could further suggest that CDDP-bound pDNA produces pDNA protein without the influence of CDDP but that CDDP-alkylated pDNA limits the protein expression of pDNA, as in the well-known action mechanism of CDDP.²²

In contrast to the CDDP effects on the transfection efficiency of polymer/CDDP-pDNA complexes, the presence of DOX had different impacts on the transfection efficiency of polymer/DOX-pDNA and polymer/DOX-CDDP-pDNA (2 min) complexes, depending on the cell lines. Indeed, most polymer/DOX-pDNA and polymer/DOX-CDDP-pDNA (2 min) complexes showed similar normalized transfection efficiencies (0.7~1.4-fold) to the polymer/pDNA complexes in MCF7, MCF7/ADR-RES, and A2780/ADR cells, whereas DOX-loaded polyplexes interestingly showed 1.6~8.3-fold higher normalized transfection efficiencies compared with chemical drug-free polyplexes in A2780 and A2780/Cis cells (Figure 9). These cell line-dependent transfection effects of DOX-incorporated polyplexes might be influenced by the chemical drug (i.e., DOX only or both DOX and CDDP)-induced cell death because either DOX or DOX-CDDP at the estimated concentration (approximately 60 nM) in cell-killing studies caused 50–70% cell viability 2 d post-treatment in A2780 and A2780/Cis cells. Although it is unclear how DOX enhanced the co-delivered pDNA expression, a few reports have shown similar DOX-induced transfection enhancement.²

Translocation of Chemical Drugs from One DNA to Another DNA

The transfection efficiencies of polymer/CDDP-pDNA (2 min) and polymer/CDDP-pDNA (30 min) complexes could explain whether the interaction between CDDP and pDNA is mediated through binding or alkylation. However, although many studies using the co-

delivery of DOX and therapeutic genes have reported that the drugs synergistically kill cells in vitro and solid tumors in vivo, they have not shown how DOX-intercalated pDNA induces pDNA expression.¹ As shown in Figure 9, the polyplexes made with DOX-intercalated pDNA also sometimes showed weak or strong transfection enhancement depending on the cells. Thus, we attempted to determine whether chemical drugs that interacted with “carrier DNA” are transferred to “nuclear DNA” in the nuclei of cells. As shown in Figure 2(a), DOX-intercalated DNA had an approximately 10-fold lower fluorescence intensity than free DOX, showing that free DOX is not distinguished from DNA intercalated with 10-fold more DOX. Therefore, instead of DOX, EtBr was used as a model drug for intercalation and DAPI for minor groove binding because the fluorescence intensities of these model drugs when interacting with DNA are much higher than for free model drugs. In particular, the fact that the model drugs exhibit reversible binding/dissociating characteristics with DNA, as does DOX, and that the chemical drugs were released from the chemical drug-DNA complex (Figure 5) could provide a possible estimation of the DOX translocation patterns from one DNA to another DNA, although the release rates of the model drugs from model drug-DNA complexes (i.e., EtBr-DNA or DAPI-DNA) are not identical to the release rate of DOX from DOX-DNA. In addition, salmon DNA (sDNA) was used as a model carrier of chemical drugs and ladder DNA (IDNA) as the drug target in the nucleus, and sDNA and IDNA have different sizes.

First, we sought to determine how quickly chemical drugs can be transferred to “nuclear DNA” in the nucleus. Thus, after EtBr was mixed with sDNA for 30 min, the resulting EtBr-intercalated sDNA (EtBr-sDNA) was further mixed and incubated with IDNA for approximately 0, 5, 10, and 15 min. As shown in Figure 10(a), upon adding EtBr-sDNA to IDNA, the EtBr in the EtBr-sDNA was immediately transferred to the IDNA, resulting in the formation of EtBr-intercalated IDNA (EtBr-IDNA). Similarly, when using DAPI instead of EtBr, the DAPI in the DAPI-minor groove bound sDNA (DAPI-sDNA) was also immediately transferred to the IDNA, forming DAPI-minor groove bound IDNA (DAPI-IDNA) (Figure 10(b)). Second, we wanted to determine whether all drugs that interacted with “carrier DNA” could be delivered to “nuclear DNA” in the nucleus. To pursue this aim, EtBr-sDNA was incubated with IDNA at different weight ratios of sDNA to IDNA for 15 min because a much higher amount of “nuclear DNA” could exist than the amount of “carrier DNA.” When applying a low weight ratio of sDNA to IDNA (e.g., 1:1), a small portion of the EtBr in the EtBr-sDNA was transferred to the IDNA, whereas most of the EtBr remained in the EtBr-sDNA. However, with increasing amounts of IDNA, the EtBr in the EtBr-sDNA moved into the IDNA. At a weight ratio of sDNA to IDNA of 1:20, EtBr-sDNA released almost all the intercalated EtBr, and the released EtBr intercalated with IDNA (Figure 11(a)). When applying DAPI instead of EtBr, similar results were observed, as shown in Figure 11(b). These results strongly indicate that chemical drugs interacting with nucleic acids as drug carriers can be very quickly moved into the nuclear DNA of cells and that almost all of the chemical drugs carried by nucleic acids can be transferred into the nuclear DNA.

CONCLUSIONS

To simultaneously deliver two or more drugs with different physical, chemical, and biological characteristics, physical loading methods have generally involved laborious preparation steps and, sometimes, chemical conjugation. With these nanosized drug carriers, there is difficulty in controlling the drug loading. However, using both chemical drug-nucleic interactions and ionic attraction allows for the adjustment of the loading content and combinations of various chemical drugs and biologics. Although three model drugs, including a reporter pDNA, a hydrophilic anticancer drug, and a hydrophobic anticancer drug, were used in this study, the findings can be extended to other chemical drugs that interact with nucleic acids via binding, intercalation, minor groove binding, and major groove binding. In addition to expanding the types of chemical drug candidates used, their counterparts can include various types of therapeutic or non-functional nucleic acids, such as pDNAs, short interfering RNAs (siRNA), microRNAs (miRNA), oligonucleotides, nucleic acid aptamers, peptide nucleic acids (PNA), and locked nucleic acids (LNA). Chemical drug-nucleic acid complexes are further shielded by positively charged polymers via an electrostatic attraction. The resultant nanosized polymer/chemical drug-nucleic acid complexes effectively deliver both biological therapeutics and hydrophilic/hydrophobic chemical therapeutics into cells. Thus, the nanosized drug carriers that we have designed are potential nanodrugs because various chemical and biological drug candidates could be applied to various disease models (e.g., tumors, microbial diseases, hypertension, depression, allergy, neurodegenerative diseases, and infectious diseases), and their loading content and composition can be customized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic preparation of polymer/chemical drug-nucleic acid complexes and the proposed concept of their intracellular delivery.

Figure 2(a)

Figure 2(b)

Figure 2. (a) DOX/DNA molar ratio-dependent DOX-DNA intercalation ($n=1$) and (b) DOX/DNA molar ratio-dependent cell-killing effects of DOX-intercalated DNA in MCF7 cells after 2 d of treatment (mean $\pm$ standard error; $n=6$).

Figure 3. Cell-killing effects of RPC-bPEI_{0.8kDa}⁴/CDDP-DNA complexes (WR 5) prepared with different interaction times between DNA and CDDP in A2780 cells after 2 d of treatment (mean $\pm$ standard error; n36).

Figure 4.
Size of polymer/chemical drug-DNA complexes (mean $\pm$ standard deviation; n=3).

Figure 5. Time-dependent release of DOX from bPEI_{25kDa}/DOX-DNA complexes in dialysis tube (MWCO 3.5 kDa) (mean $\pm$ standard deviation; n=3).

Figure 6. Cellular uptake of free chemical drugs and chemical drug-loaded polyplexes in (a) MCF7 cells and (b) MCF7/ADR-RES cells after 4 hr of incubation. (DOX = 5 μ M)

Figure 7(a)

Figure 7(b)

Figure 7(c)

Figure 7(d)

Figure 7(e)

Figure 7.

Cell-killing effects of free chemical drugs and polymer/chemical drug-DNA complexes in (a) MCF7, (b) MCF7/ADR-RES, (c) A2780, (d) A2780/ADR, and (e) A2780/Cis cells. DOX-resistant cells and other cells were treated with free chemical drugs and chemical drug-loaded polyplexes for 5 d and 2 d, respectively (mean $\pm$ standard error; n36).

Figure 8(a)

Figure 8(b)

Figure 8.

(a) Intracellular DOX distribution and (b) nuclear uptake of free chemical drugs and polymer/chemical drug-DNA complexes in MCF7/ADR-RES cells after 2 hr and 4 hr of incubation, respectively. (DOX = 5 μ M)

Figure 9. Transfection efficiency of polymer/chemical drug-pDNA complexes in (a) MCF7, (b) MCF7/ADR-RES, (c) A2780, (d) A2780/ADR, and (e) A2780/Cis cells at 2 d post-transfection (mean $\pm$ standard error; n38).

Figure 10.

Time-dependent translocation of model chemical drug loaded in model “carrier DNA” to model “nuclear DNA”: (a) translocation of EtBr as a model intercalating drug and (b) translocation of DAPI as model minor groove binding drug. Salmon DNA (sDNA) and ladder DNA (IDNA) were used as model “carrier DNA” and model “nuclear DNA”, respectively.

Figure 11.

“Nuclear DNA” amount-dependent translocation of model chemical drug loaded in model “carrier DNA” to model “nuclear DNA”: (a) translocation of EtBr as model intercalating drug and (b) translocation of DAPI as model minor groove binding drug. Salmon DNA (sDNA) and ladder DNA (IDNA) were used as model “carrier DNA” and model “nuclear DNA”, respectively.