

CD44 Receptor-Mediated Delivery of ROS/pH Dual-Sensitive Nanoparticles Based on Poly(Ethylene Glycol)-*b*-Poly(Doxorubicin)-*b*-Hyaluronic Acid Copolymer

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Purpose: This study aimed to develop and evaluate acidic pH- and reactive oxygen species (ROS)-sensitive triblock copolymer nanoparticles, composed of poly(ethylene glycol) (PEG), poly(doxorubicin) (DOX), and hyaluronic acid (HA), for targeted anticancer drug delivery.

Methods: PEG-PolyDOX (PPD) diblock copolymer and PEG-PolyDOX-HA (PPDHA) triblock copolymer nanoparticles were synthesized and characterized for their responsiveness to tumor microenvironmental conditions, including acidic pH and oxidative stress. In vitro assays were conducted using MDA-MB-231 breast cancer cells to assess cytotoxicity, apoptosis, necrosis, and CD44 receptor-mediated uptake. In vivo biodistribution and therapeutic efficacy were evaluated in an MDA-MB-231 xenograft mouse model compared with DOX and PPD nanoparticles.

Results: PPDHA nanoparticles exhibited morphological and size distribution changes, along with accelerated DOX release, under acidic pH and H₂O₂ conditions. They effectively induced apoptosis and necrosis in breast cancer cells and showed dose-dependent cytotoxicity. CD44 receptor-mediated uptake enabled preferential delivery of PPDHA nanoparticles to MDA-MB-231 cells. In vivo, PPDHA nanoparticles accumulated more efficiently in MDA-MB-231 tumors than in NIH3T3 tumors and significantly suppressed tumor growth compared with DOX or PPD nanoparticles.

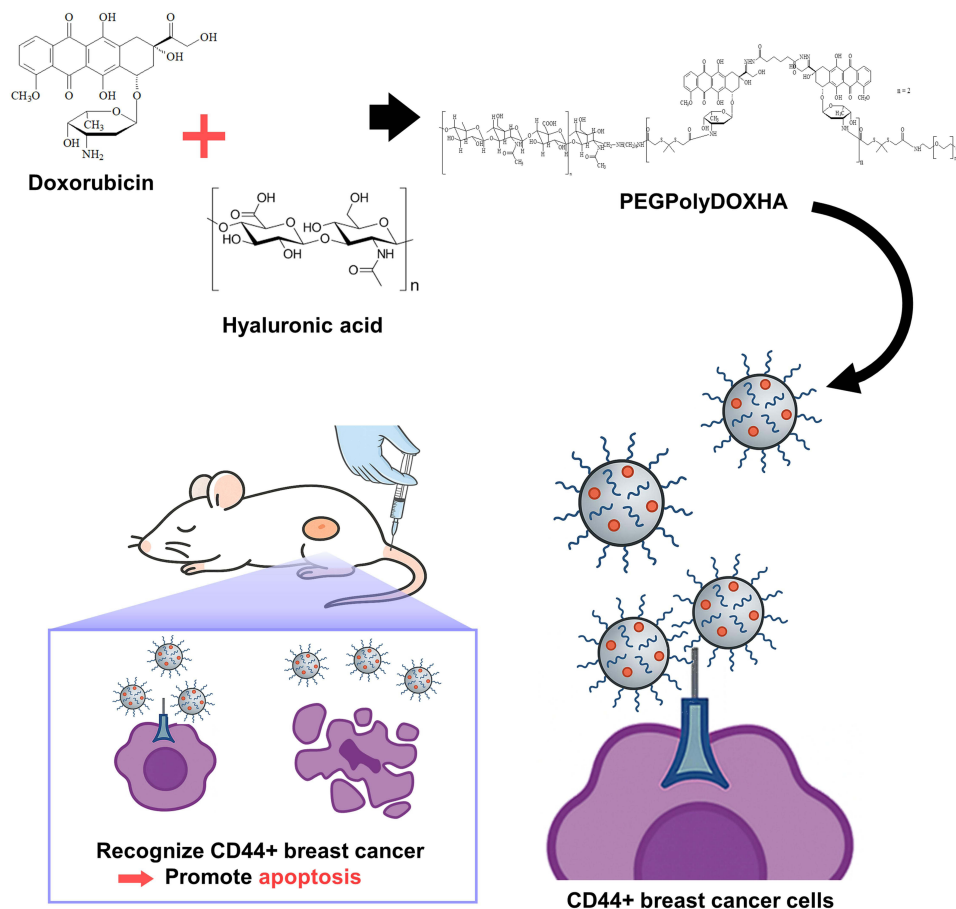
Conclusion: PPDHA nanoparticles demonstrated tumor-specific delivery and superior anticancer efficacy in vitro and in vivo. These findings suggest that PPDHA nanoparticles are promising candidates for targeted anticancer drug delivery.

Keywords: doxorubicin, block copolymer, acidic pH, oxidative stress, CD44 receptor

Introduction

Nano-dimensional vehicles were broadly studied for treatment of abnormal disorders.¹⁻³ Polymeric nanoparticles are regarded as ideal candidates for anticancer agent delivery against solid tumors with site-specificity because of their inherent characteristics such as various functionalities, ability to bypass biological barriers, tiny diameter, huge surface, and/or potential for targeting moiety decoration.^{3,4} In particular, polymeric nanoparticles can be designed to be degraded at specific target sites, such as solid tumors, to maximize cytotoxic anticancer agents in tumor tissues with minimal side effects on normal organs.³⁻⁵ For example, nanoparticles can be designed to be degraded at an acidic pH and facilitate the specific release of anticancer

Graphical Abstract



agents in the acidic environment of the tumor tissue.^{6,7} The acidic environment of tumor tissues is strongly associated with tumor progression/malignancy, making them difficult to treat using a chemotherapeutic approach.^{8,9} In the tumor microenvironment, the physiological status is frequently deteriorated due to the elevation of oxidative stress. That is, cancer cell proliferation, migration, and drug resistance are often facilitated by oxidative stress and acidic pH.^{8–11} Paradoxically, these malignant species in the tumor microenvironment have encouraged the development of tumor-specific vehicles using nanoparticles.^{10,12} Liu et al reported pH-sensitive nanosystems as an efficient cancer therapeutic strategy with tumor-specific delivery of anticancer agents in an acidic tumor microenvironment.¹⁰ Lee et al reported that oxidative stress and acidic pH-sensitive nanoparticles efficiently delivered anticancer drugs to metastatic tumors.¹³ They argued that the ROS-producing agent, piperlongumine, facilitated the oxidative stress-induced degradation of nanoparticles, accelerated tumor-specific nanoparticle delivery, and increased anticancer activity.

Doxorubicin (DOX) is widely used in chemotherapy to treat diverse types of cancer such as acute lymphocyte leukemia, Kaposi's sarcoma, breast cancer, lymphoma and bladder cancer.^{14–16} DOX is known to break DNA strands and inhibit the activity of topoisomerases, followed by the inhibition of cancer cell proliferation.¹⁴ Despite its significant anticancer activity, the severe side effects of DOX remain problematic, ie DOX has severe side effects against brain, liver and kidney.¹⁵ Multidrug resistant issues of DOX are frequently problematic in clinical application.¹⁶ The cardiotoxicity problem of DOX with cumulative and dose-dependent manner significantly reduces quality of patient's life and increases mortality of cancer patients.^{17,18} Especially, increased oxidative stress and generation of free-radicals by DOX are known to most probable mechanism for cardiotoxicity.¹⁸

To overcome these limitations, various delivery systems and polymeric conjugates have been developed. For example, poly(ethylene glycol) (PEG)-liposomes (PEGylated liposomes) have been used to treat HIV-associated Kaposi sarcoma.¹⁹ PEGylated liposome formulations for DOX are known to increase blood circulation time and preferentially accumulate in tumor tissues,¹⁹ modes of action that efficiently reduce the intrinsic side effects of DOX. Yokoyama et al reported that DOX-conjugated block copolymer micelles have excellent anticancer activity with decreased body-weight loss.²⁰ Polymeric micelles are ideal candidates for DOX delivery because they have a core-shell structure, that is, they consist of an outer shell of PEG and an inner core composed of a DOX-conjugated polypeptide block. These intrinsic features of polymeric micelles enable their accumulation in tumor tissues through enhanced permeation and retention and allow them to evade the reticuloendothelial system. In clinical trials, polymeric micelle formulations of DOX have shown favorable pharmacokinetic properties, maximum tolerated dose, and dose-limiting toxicities.²¹ Despite these advantages, however, some drawbacks of polymeric micelle systems, such as their lack of cancer cell specificity and inability to distinguish the abnormal tumor physiological conditions, remain problematic.

For this study, we synthesized a PEG-*b*-poly(DOX)-*b*-hyaluronic acid (HA) (PPDHA) copolymer with hydrazide and thioketal linkers conjugated to DOX. The hydrazide and thioketal linkers are responsible for the acidic pH and reactive oxygen species (ROS), respectively.^{22,23} The physicochemical properties and anticancer activity of PPDHA were evaluated in vitro and in vivo using breast carcinoma cells. The antitumor activities against MDA-MB-231 and MCF 7 cells were investigated with a tumor xenograft model.

Materials and Methods

Materials

Methoxy PEG-amine (MePEG-NH₂, 5,000 g/mol) was purchased from SunBio Co. (Incheon, Korea). HA (sodium hyaluronate, HA5K, Lot. No. 026570) with an average molecular weight (*M_n*) of 4,659 Da (size-exclusion chromatography coupled to a multi-angle laser light-scattering detector from the manufacturer's data) was purchased from Lifecore Biomedical Co., Ltd. (Chaska, MN, USA). Doxorubicin HCl (DOX) was purchased from LC Lab Co. (Woburn, MA, USA). Adipic acid dihydrazide (ADH), hexamethylenediamine (HMDA), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDAC), *N*-hydroxysuccinimide (NHS), triethylamine (TEA), and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemical Co. (St. Louis, Missouri, USA). Thioketal dicarboxylic acid (ThdCOOH) was purchased from Ruixi Biotech Co., Ltd. (Xi'an, China). Dialysis tubing having a molecular weight cutoff (MWCO) size of 1,000 g/mol, 2,000 g/mol, 3,500 g/mol, and 8,000 g/mol was purchased from Spectrum Lab, Inc. (Rancho Dominguez, CA, USA). Organic solvents, such as dimethyl sulfoxide (DMSO), ethyl alcohol (EtOH), and diethyl ether, were used as extra-pure grades.

Synthesis of PPDHA Triblock Copolymer

Step 1.

DOX dimer: DOX HCl (1 mM, 580 mg) was dissolved in 20 mL DMSO with excess TEA. ADH (0.5 mM, 87.1 mg) was added. The reaction was then incubated for 2 d at 40 °C. After reaction, residual solvents and byproducts were removed by dialysis procedure using a dialysis membrane (MWCO 1,000 g/mol).^{22,23} Reactants were introduced into dialysis membrane and then dialyzed against 1L of distilled water for 2 d. To avoid saturation of the solvent and byproducts, water was replaced every 3 h intervals. The yield of DOX dimer was estimated from the weight measurements as higher than 82% (w/w). [Yield (% w/w) = (weight of DOX dimer / (weight of DOX HCl + weight of ADH)) × 100]. DOX dimer was stored in the -20 °C until used for next step or analysis.

DOX tetramer: ThdCOOH (22.4 mg, 100 μM) dissolved in 10 mL DMSO was mixed with 2 equivalent of EDAC (200 μM, 38.4 mg) and NHS (200 μM, 23 mg). After 12 h at 30 °C, DOX dimer (245 mg, approximately 200 μM) was added. The mixture was allowed to react further for 2 d at 30 °C, after which the reactants were dialyzed against distilled water using a dialysis membrane (MWCO 2,000 g/mol) for 2 d. To avoid saturation of the solvent and byproducts, water was replaced every 3 h. The yield of DOX tetramer was greater than 86% (w/w). [Yield (% w/w) = (weight of DOX

tetramer/(weight of DOX dimer + weight of ThdCOOH)) $\times$ 100]. DOX tetramer was stored in the -20°C until used for next step or analysis.

Step 2.

DOX tetramer-ThdCOOH conjugates: ThdCOOH (224 mg, 1 mM) was dissolved in 10 mL DMSO. EDAC and NHS (1 equiv each) were added, followed by DOX tetramer (264 mg, approximately 100 μM). The mixture was allowed to react for 2 d at 30°C , and then the reactants were dialyzed against distilled water using a dialysis membrane (MWCO 2,000 g/mol) for 2 d. To avoid saturation of the solvent and byproducts, water was replaced every 3 h. DOX tetramer-ThdCOOH conjugates were stored in the -20°C until used for next step or analysis.

PEG-*b*-DOX tetramer (PPD): DOX tetramer-ThdCOOH conjugates (153 mg, approximately 50 μM) were dissolved in 20 mL DMSO with 1 equiv EDAC (9.6 mg) and NHS (5.8 mg). This reaction was incubated at 30°C for 12 h and, after that, MePEG-NH₂ (250 mg, 50 μM) was added. The mixture was allowed to react for 2 d and then dialyzed against distilled water for 2 d using a dialysis membrane (MWCO, 3,500 g/mol). The yield of PPD was higher than 93% (w/w). [Yield (% w/w) = (weight of PPD/(weight of DOX tetramer-ThdCOOH conjugates + weight of MePEG-NH₂)) $\times$ 100]. PPD conjugates were stored in the -20°C until used for next step or analysis.

Step 3.

HA-HMDA conjugates: HA-HMDA conjugates were synthesized as previously reported.²⁴ Briefly, HA (466 mg, approximately 0.1 mM) in 10 mL of H₂O/DMSO (3/7, v/v) was mixed with excess sodium cyanoborohydride and stirred for 12 h. Then 10 equiv of hexamethylene diamine (116.2 mg, 1 mM) was added and then further stirred for 24 h at room temperature. These reactants were dialyzed against water for 2 d using a dialysis membrane (MWCO, 3,500 g/mol), and the dialyzed solution was lyophilized for 3 d. HA-HMDA conjugates were stored in the 4°C until used for next step or analysis.

PPD-*b*-HA triblock copolymer (PPDHA): PPD diblock copolymer (161 mg, approximately 20 μM) dissolved in 10 mL DMSO was mixed with EDAC (3.84 mg, 20 μM) and NHS (2.3 mg, 20 μM), followed by incubation for 12 h at 30°C . Following this, HA-HMDA conjugates (100 mg, approximately 20 μM) were added. The mixture was allowed to react for 2 d and then dialyzed against distilled water for 2 d using a dialysis membrane (MWCO, 8,000 g/mol). The yield of PPDHA was higher than 92.5% (w/w). [Yield (% w/w) = (weight of PPDHA / (weight of PPD + weight of HA-HMDA conjugates)) $\times$ 100]. PPDHA conjugates were stored in the -20°C until used for fabrication of nanoparticles or analysis.

Chemical Structure of Copolymer

The chemical structures of the conjugates and copolymers were evaluated by ¹H nuclear magnetic resonance (NMR) spectroscopy (Varian Unity Inova 500 MHz NB High-Resolution Fourier transform (FT)-NMR spectrometer, Varian Tech. Inc., Santa Clara, CA, USA). The synthesized conjugates and copolymers were dissolved in DMSO, D₂O, or D₂O/DMSO mixtures for the analysis. A high-resolution time-of-flight (HR-TOF) mass spectrometer (AccuTOF GC-X, JEOL, Ltd., Tokyo, Japan) was used to estimate the molecular weights of the DOX dimer and tetramer.

Preparation and Characterization of Nanoparticles

To prepare the nanoparticles, the PPDHA copolymer (20 mg) was reconstituted in 5 mL of deionized water.

To measure DOX content in the nanoparticles, nanoparticles (2 mg) were reconstituted into 0.5 mL deionized water and then mixed with 4.5 mL of phosphate-buffered saline (PBS, 0.01 M, pH 6.0) with 20 mM H₂O₂. This solution was incubated in a shaker incubator (100 rpm, 37°C) for 12 h and then diluted with DMSO 10 times for measurement of the DOX concentration using an ultraviolet-visible spectrophotometer (UV-1601 UV-VIS spectrophotometer, Shimadzu, Kyoto, Japan) at 479 nm.²³ This procedure was repeated three times. The DOX content was calculated as follows: experimental DOX content (w/w) = (DOX weight in nanoparticles/total nanoparticle weight) $\times$ 100.

A DOX release study was performed as follows: DOX (5 mg) reconstituted into 5 mL of deionized water was introduced into a dialysis tube (MWCO, 3,500 g/mol) and then this was introduced into a 50 mL conical tube with 45 mL acetate buffer (pH 6.8 and 6.0) or PBS (0.01 M, pH 7.4) with or without H₂O₂. Drug release studies were performed in a shaker incubator (100 rpm, 37°C). At predetermined intervals, the entire buffer solution was collected to measure the

concentration of DOX released at 479 nm using a UV-Vis spectrophotometer. Fresh medium was then added to the conical tube, and the drug release assay was continued. This process was repeated thrice.

Physicochemical Properties of PPDHA Nanoparticles

The particle size was analyzed using a Zetasizer (Nano-ZS, Malvern, Worcestershire, UK). Nanoparticles were reconstituted in deionized water (0.1% w/v) based on polymer weight) or buffer solution (PBS, pH 7.4; acetate buffer, pH 6.0 and 6.8) in the presence of H₂O₂ and then incubated for 3 h at 37 °C.

To check stability of nanoparticles, PPD or PPDHA nanoparticles in deionized water (4 mg/mL) were stored in refrigerator at 4°C and, after 1 day, 3 days, 5 days and 7 days, particle size was measured.

The morphology of the PPDHA nanoparticles was observed by transmission electron microscopy (H-7600, Hitachi Instruments Ltd., Tokyo, Japan). One drop of the aqueous nanoparticle solution was placed on a carbon film-coated copper grid, dried at room temperature, and stained with phosphotungstic acid (0.1% w/w in water). The observations were performed at 80 kV.

In Vitro Cell Culture Study

Cell culture: NIH3T3 mouse fibroblasts and MDA-MB-231 and MCF7 human breast cancer cells were purchased from the Korean Cell Line Bank (Seoul, Korea). MDA-MB-231 and MCF7 human breast cancer cells were incubated with RPMI 1640 medium (supplemented with 1% antibiotics and 10% fetal bovine serum) under 5% CO₂ and 37 °C. NIH3T3 mouse fibroblast cells were incubated with DMEM medium (supplemented with 1% antibiotics and 10% fetal bovine serum) under 5% CO₂ and 37 °C.

Anticancer activity: MDA-MB-231 and MCF7 cells were seeded in 96 wells (1×10⁴ cells/well) and then incubated at 5% CO₂ (37 °C) overnight. PPDHA nanoparticles in PBS were sterilized with a syringe filter (0.8 µm) and then diluted with cell culture media. DOX was dissolved in DMSO and then diluted with cell culture media at least 100 times. The cells were then treated with DOX or PPDHA nanoparticles in vitro. Cancer cells were incubated for 1 or 2 d in 5% CO₂ (37 °C). To evaluate cell viability, MTT (30 µL, 5 mg/mL in PBS) solution was added to the cell culture, followed by 4 h incubation at 5% CO₂ (37 °C). After that, the supernatants were discarded, and then 100 µL DMSO was added to solubilize formazan. A microplate reader (Infinite M200 pro multimode microplate reader, Tecan Trading AG Inc., Männedorf, Switzerland) was used to measure cell viability at 570 nm. All results of the cell cytotoxicity study were expressed as average ±SD from 8 wells.

Apoptosis/necrosis analysis of cancer cells: For apoptosis/necrosis analysis, MDA-MB-231 cells (3×10⁵ cells) were treated with DOX or PPDHA nanoparticles for 1 d in the 5% CO₂ incubator at 37 °C. The cells were harvested and washed with PBS, then resuspended in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) containing FITC-annexin V (1 g/mL, sc-4252 FITC, Santa Cruz Biotech., Inc., Dallas, Texas, USA) to stain apoptotic cells and propidium iodide (PI, 10 g/mL) to stain necrotic cells for 20 min. Apoptosis/necrosis of cancer cells was analyzed using a flow cytometer (FACScan flow cytometer, Becton Dickinson Biosciences, San Jose, CA, USA) at 488 nm (FITC-annexin) and 575 nm (PI). All procedures were performed in the dark.

Western blotting: Cell lysates were prepared using RIPA buffer supplemented with a protease inhibitor cocktail. Protein concentrations were determined using a BCA protein assay kit (Takara Bio, Shiga, Japan). Equal amounts of protein were separated by SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were blocked with 5% skim milk in PBS-T (1× PBS containing 0.05% Tween-20) and incubated overnight at 4 °C with the indicated primary antibodies (1:2000 dilution in 5% BSA in PBS-T). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bethyl Laboratories, TX, USA) for 2 h at room temperature. The following primary antibodies were used: anti-PARP and anti-cleaved PARP (GeneTex, CA, USA); anti-cleaved caspase-3 (Cell Signaling Technology, MA, USA); anti-Bax (Proteintech, IL, USA); and anti-caspase-3, anti-Bcl-2, and anti-β-actin (Santa Cruz Biotechnology, CA, USA). Antibody-bound proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Takara Bio, Shiga, Japan).

Delivery test: The intracellular delivery of PPDHA nanoparticles was observed using a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan). DOX or PPDHA nanoparticles were added to cancer cells seeded onto cover glasses

in 6 wells (1×10^5 cells/well) for 2 h. Following this, the cells were washed with PBS and fixed with 10% paraformaldehyde solution (10 min). They were then immobilized using an immobilization solution (Immunomount, Thermo Electron Co., Pittsburgh, PA, USA) and observed under a fluorescence microscope in the dark.

In vivo Antitumor Activity of Nanoparticles Using Tumor Xenograft Model

Tumor xenograft model: To determine the antitumor activity of PPDHA nanoparticles, male BALB/c nude mice (20–25 g, 4–5 weeks old) were used (Orient, Seongnam, Korea). All mice were freely fed with supported water until use. MDA-MB-231 cells (1×10^7 cells) were subcutaneously administered to the backs of the mice. Drug treatment was initiated when the largest solid tumor diameter was 4–5 mm. For DOX injection, DOX was dissolved in ethanol/Cremophor EL and diluted at least 10 times with PBS (pH 7.4, 0.01 M). PPDHA nanoparticles were reconstituted in deionized water and then sterilized with a 1.2 μ m syringe filter. These were administered intravenously through the tail veins. The treatment groups were as follows: control (PBS), DOX, and PPDHA nanoparticles. Five male mice were used for each treatment group, with an injection volume of 100 ~ 200 μ L. The treatment dose was 10 mg/kg as DOX. The time of the first injection was determined as Day 0. After 3 d, each treatment was administered intravenously once more. The tumor volume growth was assessed every 5 d and calculated using the following formula: $V = [a \times (b)^2]/2$. a = largest diameter; b = smallest diameter.

Fluorescence optical imaging of the whole body and each organ of mice: NIH3T3 cells (left side) and MDA-MB-231 cells (right side) (1×10^7 cells) were subcutaneously administered to the backs of the mice. When the largest diameter of the solid tumor reached >6 mm, PPDHA nanoparticles were intravenously administered into the mouse tail vein (DOX dose: 10 mg/kg). The injection volume was 200 μ L. The mice were anesthetized with avertin after 24 h, and the fluorescence intensity of the whole body was observed. For fluorescence observation of each organ, the mice were sacrificed via CO₂ inhalation, and the fluorescence intensity of each organ was observed. The fluorescence images were obtained using a MaestroTM small animal imaging instrument (Cambridge Research and Instruments, Inc., Woburn, MA, USA) in the dark.

Statistical Analysis

The statistical significance of results was analyzed using Student's *t*-test (SigmaPlot® v.11.0, Systat Software Inc., San Jose, CA, USA). The minimum level of statistical significance was set at $p < 0.05$.

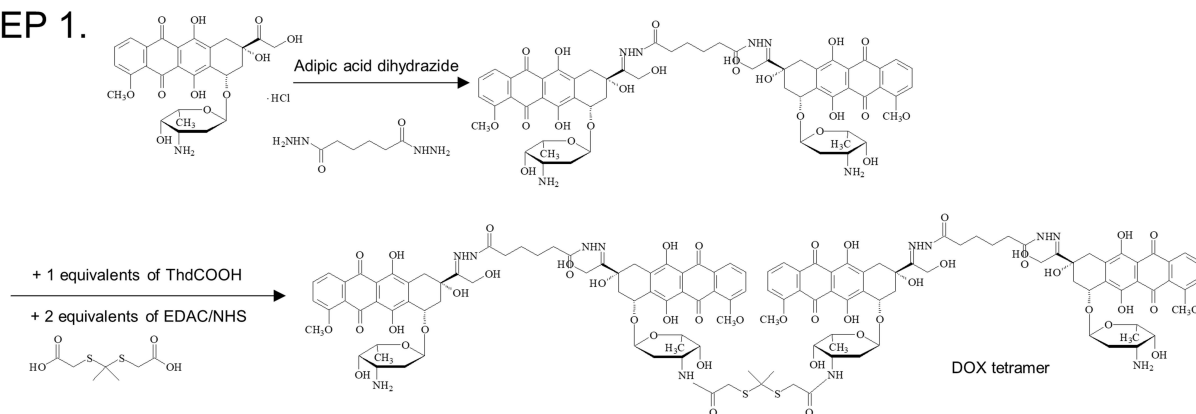
Results

Synthesis of PPDHA Block Copolymer

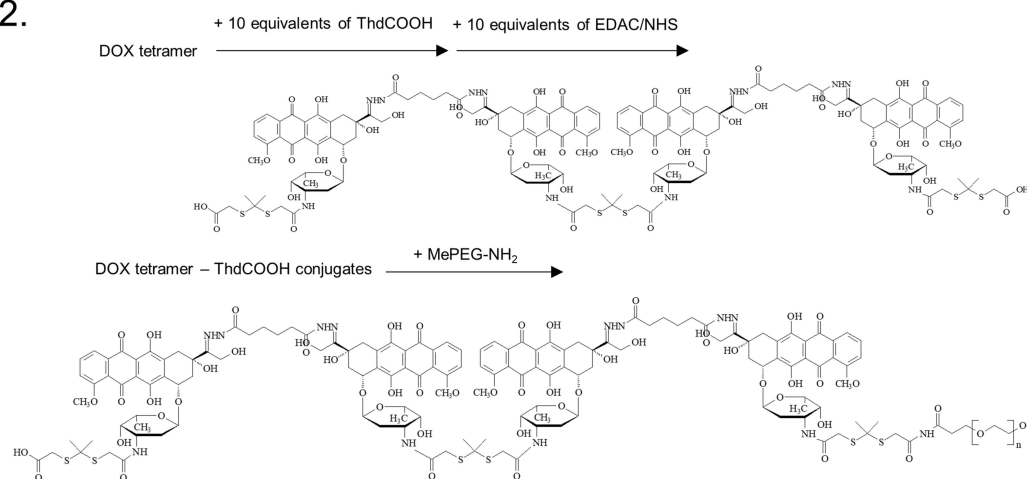
To synthesize the PPDHA block copolymer, a DOX tetramer was synthesized and conjugated to PEG and HA, as shown in Figure 1 and Figure S1–S2. First, the DOX dimer (Figure S1C) was synthesized using ADH, as shown in Figure 1 and Figure S1C. The molecular structures and their specific peaks from ¹H NMR spectra are shown in Figure S1A–C. Subsequently, the carboxylic acids on both sides of ThdCOOH were activated with the carbodiimide (EDAC/NHS) and, after that, the amine group of the DOX dimer was attached to produce the DOX tetramer. The specific peaks of the ThdCOOH and DOX tetramers are shown in Figure S1D and E. As shown in Figure S1C and D, specific peaks of DOX, ADH, and ThdCOOH in DOX dimer or DOX tetramer were characterized between 1.5 ~ 8 ppm, 1.5 ~ 2.5 ppm, and 1.5 ~ 3.0 ppm, respectively. To produce DOX tetramer-ThdCOOH conjugates, the amine end group on both sides of the DOX tetramer was conjugated with ThdCOOH, and peaks of DOX, ADH, and ThdCOOH confirmed at 0.5 ~ 8.0 ppm as shown in Figure S1E. Subsequently, one end of the carboxylic acid of the DOX tetramer-ThdCOOH conjugate activated using carbodiimide (EDAC/NHS) and then amine-terminated PEG was connected to produce the PPD diblock copolymer (Figure 1 and Figure S2A). The protons of the PEG ethylene group were confirmed at 3.7 ppm (Figure S2A).

HA reacts with sodium cyanoborohydride to form a reductive end group, as shown in Figure S2B. HA with an aminated end was then prepared using HMDA (HA-HMDA conjugates), and the peaks of HA and HMDA was verified between 1.5 ~ 5.5 ppm. The carboxylic acid end of the PPD diblock copolymer was activated using the EDAC/NHS

STEP 1.



STEP 2.



STEP 3.

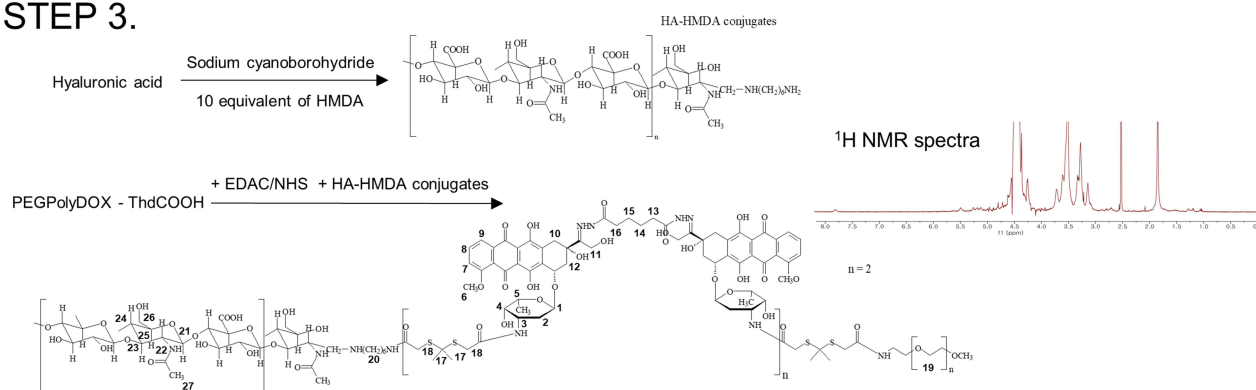


Figure 1 Synthesis scheme of PEGPolyDOXHA (PPDHA) triblock copolymer: Step 1. Synthesis of DOX dimer and DOX tetramer. Two molecules of DOX were attached to the amine groups of ADH (DOX dimer). After that, carboxylic acid of ThdCOOH was activated with EDAC/NHS system and then DOX dimer conjugated to the both sides of COOH (DOX tetramer). Step 2. Synthesis of PEGPolyDOX (PPD) diblock copolymer. Ten equivalents of ThdCOOH were added to DOX tetramer to conjugate ThdCOOH to the end amine group of DOX tetramer and then MePEG-NH₂ was conjugated to the one end of DOX tetramer-ThdCOOH conjugates. Step 3. Synthesis of PPDHA triblock copolymer. HA-HMDA conjugates were conjugated to the another end of DOX tetramer-ThdCOOH conjugates. ¹H NMR spectra of PPDHA triblock copolymer in DMSO-D₂O.

system and then reacted with HA-HMDA conjugates to produce the PPDHA triblock copolymer (Figure 1). The peaks of HA, EPG, and DOX tetramer were confirmed at 0.5 ~ 8.0 ppm.

As shown in Figure S3 and Table 1, HR-TOF was employed to estimate the *M_r* of the DOX dimer (Figure S3A) and tetramer (Figure S3B). The experimental *M_r* of the DOX dimer and DOX tetramer were estimated as 1,225 g/mol and 2,638 g/mol, respectively. The experimental *M_r* of the DOX dimer and DOX tetramer were 1,221 g/mol and 2,621 g/mol,

Table 1 Characterization of HA/Poly (Doxorubicin)/PEG Triblock Copolymer

	Molecular Weight (g/mol)	
	Theoretical ^a	Experimental ^b
DOX dimer	1,225	1,221
DOX tetramer	2,638	2,621
PPD diblock copolymer	8,032	–
PPDHA triblock copolymer	12,773	–

Notes: ^aTheoretical molecular weight (M.W.) of DOX dimer and DOX tetramer was calculated from M.W. of DOX, ADH and ThdCOOH. Theoretical molecular weight (M.W.) of PPD diblock copolymer and PPDHA triblock copolymer was calculated from DOX tetramer, PEG (5,000 g/mol) and HA (4,659 g/mol). ^bM.W. Experimental M.W. was derived from High resolution Time-of-flight (TOF) mass spectra (Figure S3).

respectively, indicating that these results were not significantly different from the theoretical M_r and that the DOX dimer or DOX tetramer was successfully synthesized by repeated conjugation of DOX to poly(doxorubicin) (PolyDOX).

Characterization of PPDHA Nanoparticles

To prepare the nanoparticles, the PPD and PPDHA block copolymers were simply reconstituted into aqueous solutions such as PBS, and their morphologies and/or particle size distributions were investigated, as shown in Figure 2. As shown in Figure 2 and Table 2, PPD block copolymer and PPDHA block copolymer successfully formed nanoscale particles in aqueous solution of 102.6 nm and 82.3 nm. The nanoparticles of the PPDHA block copolymer were spherical with a diameter smaller than 200 nm (Figure 2A). PPDHA nanoparticles have narrow and monomodal size distribution patterns, indicating that they have the potential to form nanoscale particles, as shown in Figure 2B. To check stability of nanoparticles, PPD or PPDHA block copolymer, they were reconstituted into deionized water and then stored in the refrigerator at 4 °C as shown in Figure S4, both of PPD or PPDHA nanoparticles maintained their average particle sizes and showed no precipitants or aggregates were observed, ie average particle sizes both of PPD or PPDHA nanoparticles were almost similar compared to initial values even though their average particle sizes were slightly increased. They were stable at least for 7 days as shown in Figure S4.

Furthermore, the PPDHA nanoparticles were smaller than the PPD nanoparticles (Table 2). The DOX content was estimated from the degradation of nanoparticles in aqueous solution, as shown in Table 2. The experimental DOX content of the DOX dimer, DOX tetramer, PPD nanoparticles, and PPDHA nanoparticles did not differ significantly from the theoretical values. Small changes in DOX content between the theoretical and experimental values are ascribed to variation between the theoretical and experimental M_r , with the actual DOX content slightly lower than the theoretical value.

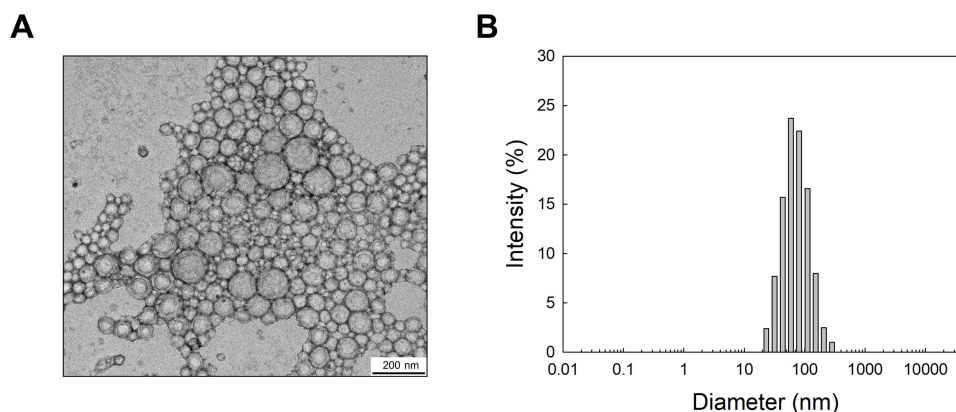


Figure 2 Characterization of PPDHA nanoparticle morphology and size distribution. (A) TEM photograph of PPDHA nanoparticles. Phosphotungstic acid (0.1%, w/w in water) was used for negative staining of nanoparticles. (B) Typical particle size distribution of PPDHA nanoparticles. Nanoparticles reconstituted in deionized water (0.1%, w/v based on polymer weight).

Table 2 Physicochemical Properties of PPD Nanoparticles and PPDHA Nanoparticles

	Drug Contents (% w/w) ^a		Average Particle size (nm) ^b
	Theoretical	Experimental	
DOX dimer	88.7	88.1	–
DOX tetramer	82.4	81.6	–
PPD NP	27.1	26.7	102.6±4.9
PPDHA NP	17.0	16.2	82.3±3.8

Notes: ^aTheoretical drug contents of DOX was calculated from M.V. of each chemical. Experimental drug contents were estimated from measurements of UV spectrophotometer.

^bAverage particle size: Average ± standard deviation (S.D.) from three different measurements.

To investigate the role of PPDHA nanoparticles under acidic pH and oxidative stress, they were reconstituted in aqueous solutions of various pH levels with or without hydrogen peroxide (H₂O₂) (Figure 3). The nanoparticles were spherical at alkaline pH (pH 7.4). However, in acidic solution they began to aggregate and disintegrate (Figure 3A(i-iii)). Furthermore, the nanoparticles seriously disintegrated in the presence of H₂O₂ (Figure 3A(iv-vi)); in particular, their aggregation and/or disintegration further progressed under acidic pH and oxidative stress, indicating that PPDHA nanoparticles are sensitive to acidic environments and/or oxidative stress in the tumor microenvironment. Figure 3B(i-vi) and C supported these results through the changes of size distributions and drug release behavior. The particle size distribution changed from a monomodal pattern at alkaline pH to bi- or multimodal patterns, and their distribution patterns became more complex under oxidative stress (H₂O₂), ie the aggregation and/or disintegration of PPDHA nanoparticles occurred under acidic pH and oxidative stress. As expected, DOX release from nanoparticles was accelerated in the acidic pH, whereas DOX release from nanoparticles at alkali pH (pH 7.4) was minimized. Furthermore, the drug release rate was higher in the presence than in the absence of H₂O₂.

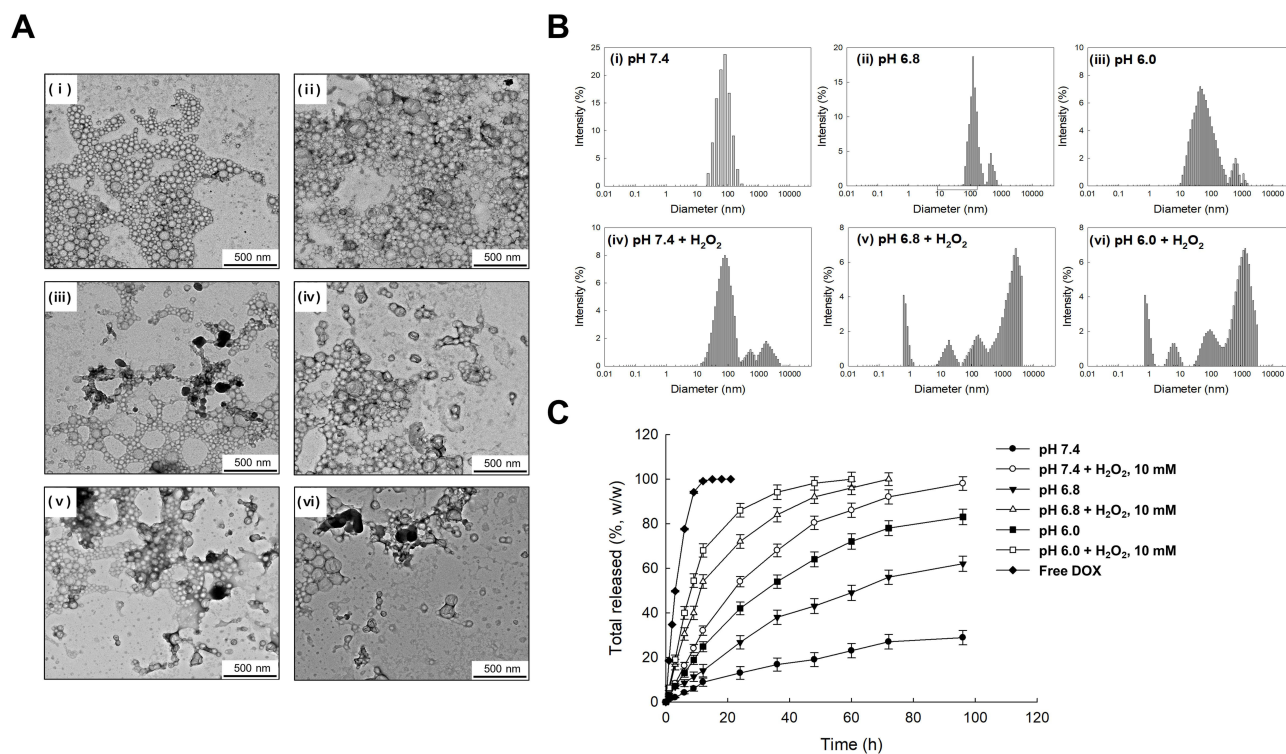


Figure 3 Effect of H₂O₂ on physicochemical property changes of PPDHA nanoparticles. To study the effect of oxidative stress, PPDHA nanoparticles were incubated with buffer solution (+ H₂O₂) for 3 h at 37°C followed by particle size measurement or morphological observation. **(A)** TEM photograph of PPDHA nanoparticles. (i) pH 7.4; (ii) pH 6.8; (iii) pH 6.0; (iv) pH 7.4 + H₂O₂, 10 mM; (v) pH 6.8 + H₂O₂, 10 mM; (vi) pH 6.0 + H₂O₂, 10 mM. **(B)** Particle size distribution of PPDHA nanoparticles. **(C)** The effect of oxidative stress on the drug release behavior of PPDHA nanoparticles.

(Figure 3C), indicating that the acidity and/or oxidative stress accelerated the release rate of DOX from the nanoparticles, which is the abnormalities of tumor microenvironment.

In vitro Cell Culture Study

Figure 4 shows the anticancer activity of DOX, PPD, and PPDHA nanoparticles against MDA-MB-231 and MCF7. As shown in Figure 4A, both PPD and PPDHA nanoparticles showed dose-dependent cytotoxicity against breast cancer cells and DOX, although the cytotoxicity of PPD and PPDHA nanoparticles was slightly lower than that of DOX. These results might be due to the sustained release behavior of the nanoparticles, which was slightly lower than that of DOX. In particular, the cytotoxicities of PPD and PPDHA nanoparticles were similar to that of DOX after 2 d (Figure 4A). Normal-cell viability assays performed using HaCaT keratinocytes and L929 fibroblasts showed that PPDHA nanoparticles maintain high cell viability across a wide concentration range, demonstrating minimal toxicity toward non-tumorigenic cells (Figure S5). DOX dimer and tetramer showed similar cytotoxicity against breast cancer cells, as shown in Figure S6. Furthermore, IC_{50} value between DOX, DOX dimer and DOX tetramer, PPD nanoparticles and PPDHA nanoparticles was not significantly changed even though PPDHA nanoparticles was slightly lower than that of other treatment as shown in Table 3.

These results indicate that DOX derivatives such as DOX dimers, DOX tetramers, PPD nanoparticles, and PPDHA nanoparticles maintain their anticancer activity during the polymerization process. The IC_{50} values of PPD and PPDHA nanoparticles were not significantly changed compared to DOX in either MDA-MB-231 cells or MCF7 cells, indicating that the nanoparticles have promising anticancer activity similar to that of DOX itself. As shown in Figure 4B and C, MDA-MB-231 cell apoptosis/necrosis was induced by treatment with PPD and PPDHA nanoparticles; that is, PPD or PPDHA nanoparticles induced early/late apoptosis and necrosis of MDA-MB-231 cells as effectively as DOX alone (Figures 4B and Figure S7). In addition, both nanoparticles induced apoptotic proteins as effectively as DOX (Figure 4C). PARP expression decreased, whereas cleaved PARP increased dose-dependently in both DOX and nanoparticles. Furthermore, caspase-3 expression decreased,

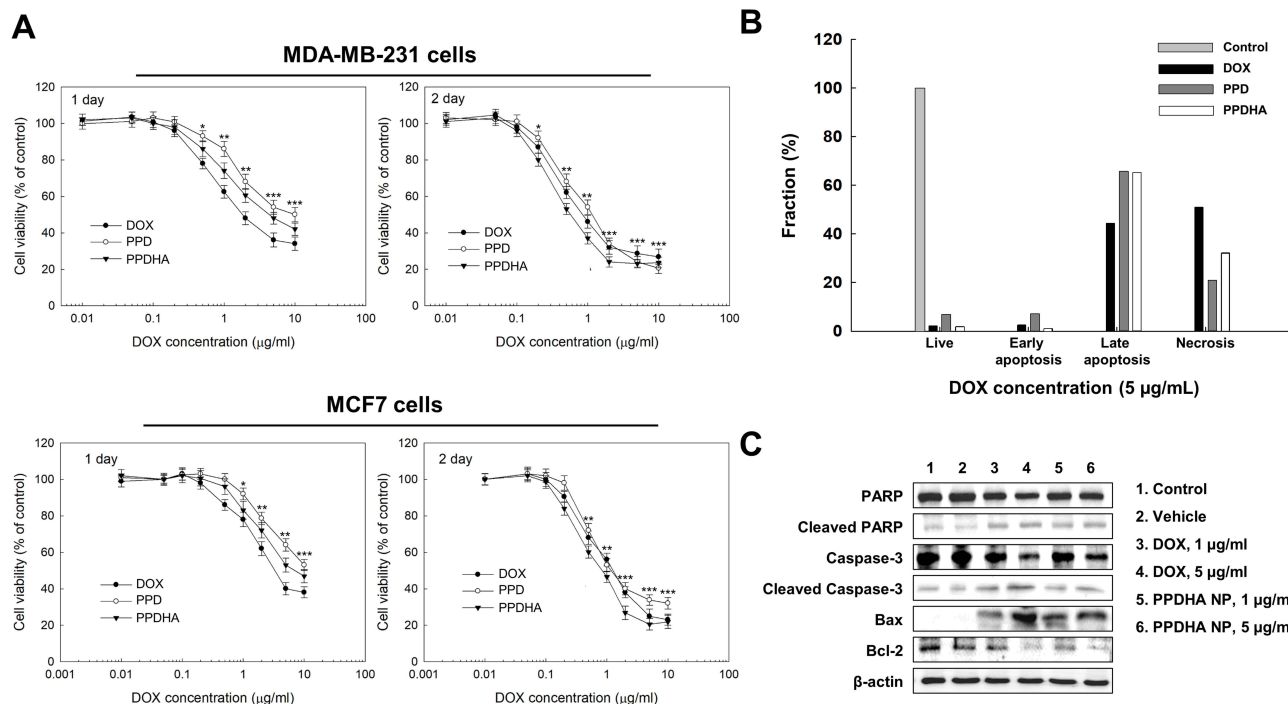


Figure 4 Cytotoxicity and apoptosis-inducing effects of PPD and PPDHA Nanoparticles. **(A)** Cytotoxicity of DOX, PPD and PPDHA nanoparticles (0.01–10 μg/mL) (MDA-MB-231 cells (top); MCF7 cells (bottom)) evaluated by the MTT assay. **(B)** FITC-annexin V and PI was used for staining cancer cells (MDA-MB-231 cells) after 1 d of treatment with DOX, PPD and PPDHA nanoparticles (5 μg/mL). Flow cytometry was employed to assess the apoptotic rate, and the bar chart depicts the relative percentages of live, necrotic, early apoptotic, and late apoptotic cells. **(C)** The expression of apoptosis-related proteins analyzed using Western blotting after 1 d of treatment with DOX or PPDHA nanoparticles (1 and 5 μg/mL). The significance is as follows * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group.

Table 3 IC₅₀ of DOX or HA/Poly(Doxorubicin)/PEG Nanoparticles

	IC ₅₀ (g/l) ^a	
	MDA-MB-231 cells	MCF 7 cells
DOX	0.86	1.27
DOX dimer	0.82	
DOX tetramer	0.91	
PPD NP	1.21	1.22
PPDHA NP	0.6	0.83

Notes: ^aIC₅₀ was estimated from Figure 4A and Figure S6(b) at 2 d.

whereas cleaved caspase-3 increased dose-dependently both of DOX and nanoparticles. Figure 4C showed the increase of BAX expression and the decrease of Bcl-2 expression, indicating that the cancer cell apoptosis/necrosis was induced efficiently.

PPD and PPDHA nanoparticle intracellular delivery was investigated using breast cancer cells in vitro (Figures 5 and Figure S8). PPD and PPDHA nanoparticles were delivered to the intracellular region of cancer cells (MDA-MB-231 cells, Figure S8A; MCF7 cells, Figure S8B) as successfully as DOX itself. Although PPD and PPDHA nanoparticles have similar

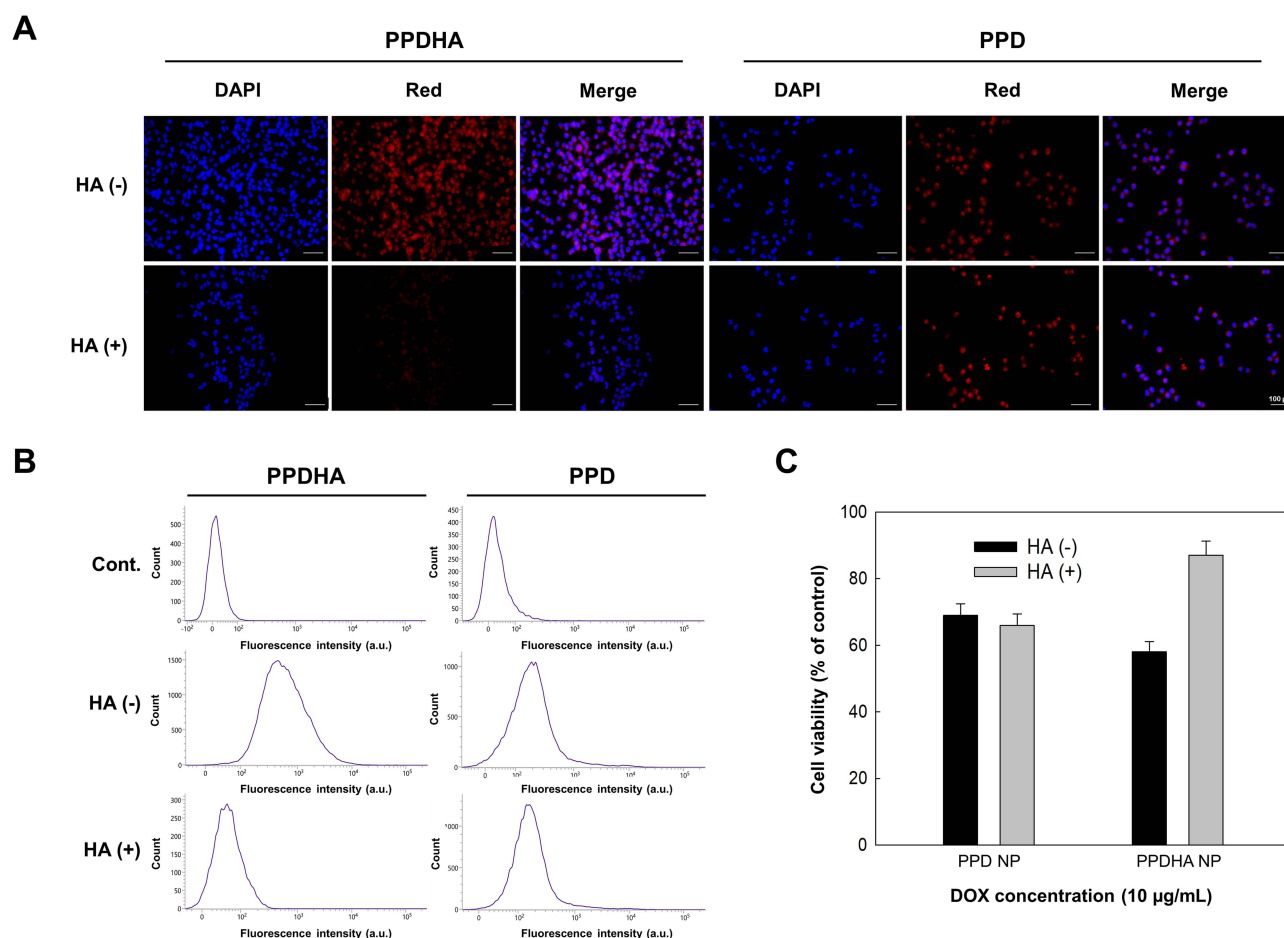


Figure 5 The effect of CD44 receptor blocking against MDA MB 231 cells. Free HA (100 µg/mL) was pre-treated to cancer cells for 1 h to block CD44 receptors, after which PPDHA or PPD nanoparticles (as a DOX concentration, 1.0 µg/mL) were added to cancer cells for 2 h (MDA-MB-231 cells). Intracellular uptake of nanoparticles against MDA-MB-231 cells: (A) Fluorescence microscopic observation. Image magnification, × 400; scale bar, 100 µm. (B) Flow cytometer analysis. (C) The effect of CD44 receptor blocking on cytotoxicity of PPDHA or PPD nanoparticles (based on DOX concentration, 10 µg/mL). Before nanoparticle addition, free HA (100 µg/mL) was pre-treated to cells for 1 h to block CD44 receptors. Then, the cells were exposed to nanoparticles for 6 h. Media were then replaced with fresh media followed by further incubation for 18 h. The significance is as follows ****p* < 0.001 compared with the control group. ns, not significant.

anticancer activities against breast cancer cells, PPDHA nanoparticles only revealed receptor sensitive manner against CD44 receptor of MDA-MB-231 cells (Figures 5A and B). The intracellular delivery of PPDHA nanoparticles significantly decreased when the pretreatment of free-HA was used to block the CD44 receptor of MDA-MB-231 cells (Figure 5A(Left) and B(Left)), whereas that of PPD did not change (Figure 5A(Right) and B(Right)). Furthermore, the cellular cytotoxicity of the PPDHA nanoparticles was significantly altered by blocking of CD44 receptor (Figure 5C), whereas the PPDHA nanoparticle cytotoxicity did not change. These results confirmed the CD44-sensitive intracellular delivery capacity and cytotoxicity of PPDHA nanoparticles. Furthermore, neither PPD nor PPDHA nanoparticles responded to pretreatment with the free CD44 receptor in NIH3T3 cells, as shown in Figure S9A and B; that is, the intracellular delivery of PPDHA nanoparticles did not significantly change between HA (-) and HA (+). Furthermore, the cellular cytotoxicity of both PPD and PPDHA nanoparticles did not change before and after blocking the CD44 receptor (Figure S9C), indicating that CD44 receptor-mediated delivery capacity of PPDHA nanoparticles was attained.

In vivo Antitumor Activity of Nanoparticles

Figure 6A(i,ii) show the effect of acidic pH and oxidative stress on the changes of fluorescence intensity of the PPDHA nanoparticles. The fluorescence intensity of the PPDHA nanoparticles increased in acidic solutions and under oxidative stress (in the presence of H_2O_2) (Figure 6A(i)). Furthermore, the fluorescence intensity of PPDHA nanoparticles was

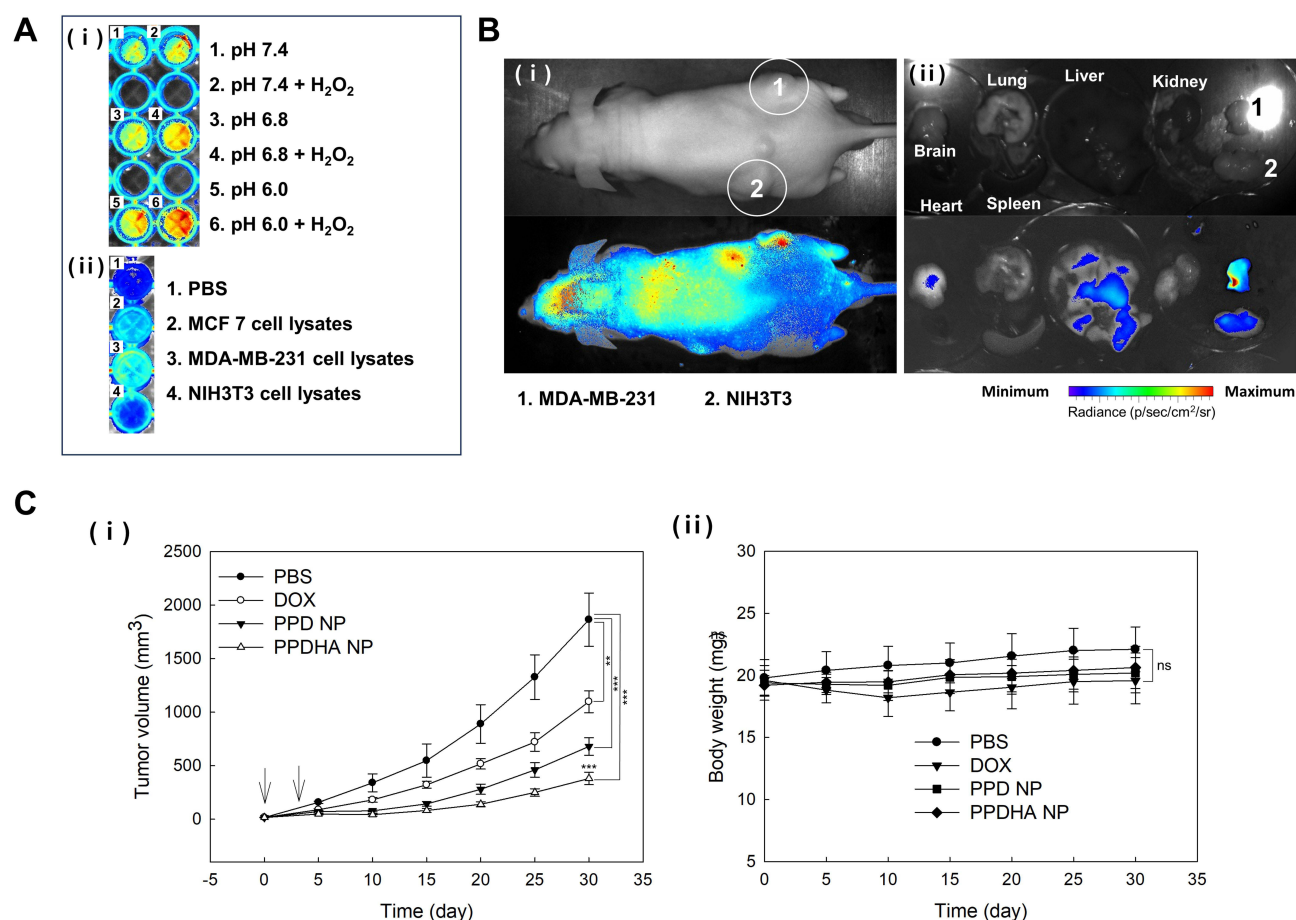


Figure 6 Antitumor activity of DOX, PPD nanoparticles, and PPDHA nanoparticles against MDA-MB-231 tumor xenograft model. **(A)** (i) Effect of acidic pH and oxidative stress on the changes of fluorescence image of PPDHA nanoparticle. (ii) Effect of cell lysates on PPDHA nanoparticle fluorescence images. PPDHA nanoparticles were incubated with each solution or cell lysates at 37 °C for 6 h. **(B)** Fluorescence images of whole body (i) and organs (ii). PPDHA nanoparticles (Based on DOX, 10 mg/kg) were administered intravenously via tail vein and, 24 h later, the mouse was anesthetized to observe whole body fluorescence. Mice were sacrificed to observe each organ. The radiant efficiency of color bar was calculated by $[p/sec/cm^2/sr]/[\mu W/cm^2]$. **(C)** Effect of DOX, PPD nanoparticles, or PPDHA nanoparticles on tumor volume changes (i) and body weight changes (ii). DOX, PPD nanoparticles or PPDHA nanoparticles (Based on DOX, 10 mg/kg) were i.v. injected (Based on DOX, 10 mg/kg). Five mice were used, and tumor volumes or body weights were checked at 5 d intervals. Results are calculated and the average $\pm$ S.D. from 5 mice were expressed. The significance is as follows ** $p < 0.01$, *** $p < 0.001$ compared with the control group. ns, not significant.

higher in MDA-MB-231 cell lysates than in NIH3T3 cells (Figure 6A(ii)). The PPDHA nanoparticles have sensitivity against acidic pH and oxidative stress in the tumor region better than their normal counterparts.

To confirm the CD44 receptor sensitive manner, MDA-MB-231 and NIH3T3 normal cells were implanted in the backs of the mice and then PPDHA nanoparticles were i.v. administered, as shown in Figure 6B. On whole-body imaging (Figure 6B(i)), the MDA-MB-231 tumor fluorescence intensity was significantly higher than that in NIH3T3 tumors. Furthermore, MDA-MB-231 tumors revealed higher fluorescence intensity than that of NIH3T3, indicating that PPDHA nanoparticles have MDA-MB-231 tumor specificity compared to normal tissues and NIH3T3 tumors (Figure 6B(ii)). As expected, the antitumor activities of the PPD and PPDHA nanoparticles were superior to those of DOX or PBS, as shown in Figure 6C. PPDHA nanoparticles showed superior antitumor activity against MDA-MB-231 cells and efficiently suppressed tumor volume (Figure 6C(i)). Body weight did not significantly change in either the PPD or PPDHA nanoparticle treatments, whereas body weight decreased slightly under DOX treatment (Figure 6C(ii)). PPDHA nanoparticles have superior antitumor activity and CD44 receptor sensitivity.

Discussion

The TME physiology is significantly distinguished from that of its normal counterpart. In particular, an acidic environment and elevated oxidative stress are regarded as intrinsic TME properties.^{9,11,25} Both acidic pH and elevated oxidative stress are associated with tumor malignancy and aggravate the status of patient tumors.^{11,26} These tumor microenvironment abnormalities have been considered in nanoscale vehicle design. In particular, nanoparticles can be designed to respond specifically to the tumor microenvironment physiological status and liberate anticancer drugs from the tumor tissue with minimal drug release in their normal counterparts.^{6,10,13,22,24} For example, hybrid HA nanoparticles, which are sensitive to acidic pH of cancer cells, efficiently delivered anticancer drugs to breast carcinoma cells through the disintegrating the inner core of nanoparticle under acidic environment.²⁴ Liu et al reported that nanoparticles composed of PEG/PHS/poly(L-lactide) showed faster DOX release at acidic pH than at pH 7.4 and were specifically cytotoxic to cancer cells.¹⁰ Chitosan nanoparticles having selenocysteine-histidine moieties delivered anticancer drugs in an acidic pH- and ROS-sensitive manner and efficiently suppressed the pulmonary metastasis of colon cancer cells.²⁴ Yoon et al also reported that photosensitizer-conjugated hyaluronic acid (HA) using thioketal linker has targetability against CD44-receptor of HeLa human cervical cancer cells and sensitivity against oxidative stress.²⁷ They argued that nanophotosensitizers can be delivered to the intracellular region of cancer cells via CD44 receptor-mediated mechanism and then they sensitively degrade by hydrogen peroxide with dose-dependent manner.

Napoli et al (2004) reported that unilamellar vesicles containing poly(propylene sulfide) (PPS) could be used for oxidative stress-sensitive drug delivery and disease biodetection.²⁸ They reported that under oxidative stress, hydrophobic PPS blocks could be converted to hydrophilic blocks and modify the physicochemical properties of the unilamellar vesicles. However, most nanoscale vehicles require stimuli-sensitive polymer backbones, such as PHS or PPS, for the targeted delivery of anticancer agents, which are physically entrapped in the nanoscale vehicle inner cores. Despite its significant anticancer activity, micelles are based on the synthetic copolymers composed of hydrophilic and hydrophobic domain to payload or conjugate DOX.^{28–31} The strength of our study is to synthesize polyDOX as a hydrophobic domain and PEG or HA as a hydrophilic domain, ie the anticancer agent, DOX itself, polymerized as a hydrophobic domain to be polyDOX and then PEG or HA as a hydrophilic domain was attached to the end of polyDOX to be block copolymer. Practically, DOX molecules were connected by hydrazide linkers (ADH) for acidic pH sensitivity and a thioketal group for ROS-sensitivity, then polymerized as a DOX tetramer (PolyDOX). PolyDOX tetramers can act as a hydrophobic block, as shown in Figure 1. They formed spherical nanoparticles in aqueous solution and liberated DOX in an acidic pH- and ROS-sensitive manner, as shown in Figure 3. They disintegrated in an acidic environment, and their disintegration was accelerated in the presence of H₂O₂ (Figure 3). This physicochemical behavior may accelerate intratumoral delivery of anticancer drugs. The acidic pH and/or oxidative stress stimulated the DOX liberation from the nanoparticles (Figure 3). The limitation of PPDHA nanoparticles in this study is that DOX was only connected each other and has no functional group in their chemical structure. Even though DOX itself has a strong fluorescence intensity and showed fluorescence detection in vivo animal tumor xenograft model, polyDOX can be modified with near-infrared (NIR) fluorescence dye for detection of tumor model in deep tissues.

Tumor malignancies are frequently associated with the expression of various receptors and proteins.^{32,33} These receptors and/or proteins are typically expressed on the cell surface and control cell adhesion, migration, proliferation, invasion, and metastasis. Furthermore, these receptors bind to specific molecules and control the intracellular delivery of foreign materials via receptor-mediated pathways.^{34,35} For example, CD44 receptors are typically expressed on the cell surface and are critical for cancer progression and metastasis.^{36,37} They play a critical role in the malignancy of tumors, and in particular, variant isoforms are strongly associated with poor prognoses of tumor patients.^{38,39} Since CD44 receptors on the surface of cancer cells specifically recognize HA, HA-decorated nanoparticles are regarded as an ideal candidate for the targeted delivery of bioactive agents against CD44-positive cancer.^{24,40–42} HA-decorated poly(DL-lactide-co-glycolide) nanoparticles were selectively delivered to CD44-positive cells and showed higher antitumor activity against a patient-derived xenograft model than paclitaxel alone.⁴² PPDHA nanoparticles also showed CD44 receptor specificity; that is, the intracellular delivery capacity of PPDHA nanoparticles can be blocked by blocking of the CD44 receptor (Figure 5). Furthermore, PPDHA nanoparticles were preferentially delivered to CD44 receptor-positive cells (MDA-MB-231) rather than to NIH3T3 tumors. Furthermore, these behaviors of PPDHA nanoparticles efficiently suppressed the growth of MDA-MB-231 tumors (Figure 6). They can respond to acidic pH and oxidative stress and specifically disintegrate (Figure 6A). These results indicate that PPDHA nanoparticles can deliver MDA-MB-231 cells in a CD44-specific manner. Subsequently, they efficiently degrade, and DOX is released into the tumor microenvironment with minimal side effects against normal tissues/organs. PPDHA nanoparticles are promising candidates for anticancer drug targeting.

Conclusion

PPD diblock and PPDHA triblock copolymers were synthesized for the targeted delivery of DOX against physiology intrinsic to the tumor microenvironment, such as acidic pH and oxidative stress. Nanoparticles were prepared by simple reconstitution in an aqueous solution and had tiny particle sizes (< 200 nm) with spherical morphologies. The nanoparticle morphology significantly distorted under acidic pH and oxidative stress conditions, and the monomodal distribution of nanoparticle properties also changed multimodal pattern. The DOX release rate also accelerated, indicating that the PPDHA nanoparticles has sensitivity against acidic pH and oxidative stress. Breast cancer cell viability (MDA-MB-231 cells and MCF 7 cells) was suppressed by treatment of nanoparticles with dose-dependent manner. In vitro cell culture study using MDA-MB-231 cells, they induced apoptotic proteins such as caspase-3, cleaved caspase-3, PARP, cleaved PARP, BAX, and Bcl-2. Nanoparticles were intracellularly delivered both of MDA-MB-231 cells and MCF7 cells. In particular, PPDHA nanoparticles were delivered through CD44 receptor of MDA-MB-231 cells; that is, their delivery capacity and cytotoxicity were inhibited by blocking of CD44 receptor. In contrast, PPD nanoparticles have no sensitivity against CD44 receptor of breast cancer cells. In vivo MDA-MB-231 tumor xenograft model, the PPDHA nanoparticles preferentially delivered to MDA-MB-231 tumors rather than that of NIH3T3 tumors. They efficiently suppressed the increase of tumor volume of MDA-MB-231 cells compared to DOX itself. These results indicate that PPDHA nanoparticles can be specifically delivered to tumors rather than to their normal counterparts and can efficiently suppress tumor growth. We suggest that PPDHA nanoparticles are promising candidates for anticancer drug delivery.

Ethical Statement

The study was carried out in compliance with the “Guide for the Care and Use of Laboratory Animals”. The study adhered to the institutional regulations on animal use at Chosun University and conformed to the criteria established by the Institutional Animal Care and Use Committee (IACUC) of Korea. The Research Chosun University Institutional Animal Care and Use Committee (Approval No. CIACUC2025-S0022) approved all the procedures of those experiments, ensuring animal rights, welfare, minimal stress, and no harm or suffering.

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Disclosure

The authors report no conflicts of interest in this work.

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