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Dextran/Poly(*L*-histidine) Graft Copolymer for pH-Responsive Drug Delivery

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***Abstract*—**pH-sensitive drug targeting using nanoparticles for cancer chemotherapy have been spotlighted in recent decades. Graft copolymer composed of poly (*L*-histidine) (PHS) and dextran (DexPHS) was synthesized and pH-sensitive nanoparticles were fabricated for pH-responsive drug delivery of doxorubicin (DOX). Nanoparticles of DexPHS showed pH-sensitive changes in particle sizes and drug release behavior, i.e. particle sizes and drug release rate were increased at acidic pH, indicating that DexPHS nanoparticles have pH-sensitive drug delivery potentials. Antitumor activity of DOX-incorporated DexPHS nanoparticles were studied using CT26 colorectal carcinoma cells. Results indicated that fluorescence intensity was higher at acidic pH than basic pH. These results indicated that DexPHS nanoparticles have pH-responsive drug targeting.

***Keywords*—**pH-sensitive polymer, nanoparticles, block copolymer, poly (*L*-histidine).

I. Introduction

TARGETED drug delivery based on pH-responsive nanoparticles has spotlighted in recent decades because solid tumor tissues have acidic environment compared to normal tissues [1]-[3]. Especially, since the imidazole group of histidine is known to ionize at acidic pH and express cationic properties, PHS is an ideal candidate for pH-sensitive drug delivery against solid tumors [4], [5]. Practically, PHS-derivatives or copolymers have extensively investigated for intracellular delivery of gene medicine and anticancer drugs [4]-[6].

Recently, we synthesized novel block or graft copolymers composed of PHS and poly(2-hydroxyethyl methacrylate) or dextran, and investigated physicochemical properties of pH-responsive nanoparticles [6], [7]. Furthermore, pH-sensitive nanoparticles also enhanced photosensitizing efficacy for the HuCC-T1 cholangiocarcinoma cells [8].

In this study, we fabricated pH-responsive nanoparticles using dextran/PHS graft copolymer and investigated antitumor activity against CT26 colorectal carcinoma cells. Since PHS is a polypeptide and has biocompatibility, DexPHS graft copolymer may have fully biocompatible properties. pH-sensitive drug delivery against CT26 cells and pH-sensitive cellular cytotoxicities were investigated *in vitro*.

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II. Experimental

A. Materials

Dextran (M.W.=6,000g/mol), PHS (M.W.=approximately, 5,000g/mol), triethylamine (TEA), N-hydroxysuccinimide (NHS), succinic anhydride, dimethylaminopyridine (DMAP), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide HCl (EDC), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St Louis, MO). The dialysis membranes with molecular weight cutoffs (MWCO) of 8,000 g/mol were purchased from Spectra/PorTM dialysis membrane (Spectrum Laboratories Inc, Rancho Dominguez, CA). DOX (doxorubicin HCl) was purchased from LC labs (Woburn, MA 01801, USA).

DexPHS graft copolymer was synthesized previously [7]. Dextran-Succinate: dextran and succinate was dissolved in DMSO and then DMAP was added to this solution. 48h after resulting solution was precipitated into methanol and then dried *in vacuo*. Dextran-succinate was obtained as a white solid. The substitution degree succinic acid was 10.7/100 glucose. DexPHS graft copolymer: 60mg of dextran-succinate was dissolved in 10ml DMSO. Then, 19.2mg EDC and 11.5mg of NHS was added and reacted for 6h. After that, 150mg of PHS was dissolved in 10ml DMSO with trace amounts of TEA. This solution was reacted for 3 days and then dialyzed against distilled water for 2 days following lyophilization.

B. Fabrication of Nanoparticles

20mg of DexPHS was dissolved in 4 ml of DMSO and then 5 mg of DOX was added to this solution with trace amounts of triethylamine. This solution was precipitated into 10ml water and dialyzed against distilled water. Dialyzed solution was used for analysis, cell culture study, or lyophilization.

DOX as a model anticancer agent was incorporated into DexPHS nanoparticles. DOX and DexPHS graft copolymer were dissolved in DMSO and precipitated in water. Free drug was removed by dialysis procedure. Drug contents ((drug weight in the nanoparticles/total weight of nanoparticles)*100) was measured with UV-spectrophotometer at 479 nm (UV-spectrophotometer, UV-1801, Shimadzu Co. Japan).

C. Analysis of DexPHS Nanoparticles

Particle sizes were analyzed with dynamic light scattering ((DLS-7000, Otsuka Electronics Company, Osaka, Japan). Transmission electron microscope (TEM) was used to observe morphology of the nanoparticles (JEOL JEM-2000 FX II, Japan).

D. Cell Culture

CT26 mouse colorectal carcinoma cells were used in this study. CT26 cells were maintained with RPMI1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics at 5% CO_2 incubator (37°C).

Anticancer activity of DOX-incorporated DexPHS nanoparticles were assessed by cytotoxicity test using MTT proliferation analysis method. Briefly, 3×10^4 CT26 cells were seeded in 96-well plates and then incubated overnight at 5% CO_2 incubator (37°C). Free DOX or DOX-incorporated nanoparticles were added to this culture and exposed for 24h. After that, 30μL of MTT (5mg/mL) was added to the 96-well plates and incubated for 4 hours. The formazan crystals formed in living cells were solubilized with DMSO and the absorbance (560nm test/630nm reference) was determined using an automated computer-linked microplate reader (Molecular Device Company, Sunnyvale,CA).

E. Fluorescence Microscopy

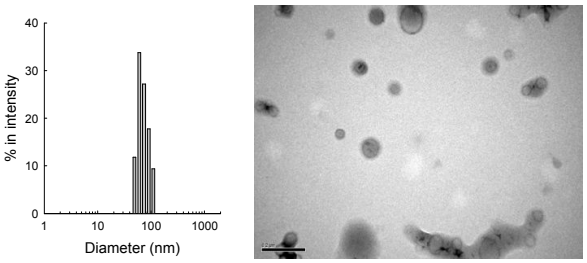
CT26 cells exposed to free DOX or DOX-incorporated DexPHS nanoparticles at various pHs for 1h were washed with PBS (pH 7.4, 0.1M) and then treated with 4% paraformaldehyde. Next, cells were washed with PBS and fixed by immobilization solution (ImmuMount, Thermo Electron Corporation, Pittsburgh, PA). Cells were observed with a confocal laser scanning microscope (CLSM, TCS-SP2; Leica, Wetzlar, Germany). For flow cytometric analysis, cells were treated with free DOX or nanoparticles (DOX concentration was equivakent to 1μg/ml) and harvested to analyze with a flow cytometer.

III. Results

DexPHS graft copolymer was synthesized as reported previously [7]. Since PHS has one amine end group, it was conjugated with carboxylic acid of dextran-succinate. ^1H nuclear magnetic resonance (NMR) spectroscopy was used to confirm synthesis of DexPHS graft copolymer. Specific peaks of dextran and PHS were confirmed at 2~5ppm and 7.4~9.0 ppm, respectively (data not shown). The substitution degree of PHS versus dextran was approximately 2.5.

Nanoparticles of DexPHS were fabricated by nanoprecipitation and dialysis methods, i.e. DexPHS and/or DOX in DMSO was dropped into water to form nanoparticles and then solvents were removed by dialysis procedure. To confirm formation of nanoparticles, particle size was measured by DLS and their morphologies were observed by TEM as shown in Fig. 1. Fig. 1 showed that DexPHS nanoparticles showed narrow distribution in particle size distribution in deionized water and spherical shapes at TEM observation. Average particle size of DexPHS nanoparticles was 84nm. These results indicated that DexPHS can form spherical nanoparticles in aqueous environement. Furthermore, TEM image showed that sizes of DexPHS nanoparticles were less than 100nm, indicating that nanoparticle size at TEM observation was almost similar to the results of particle size measurement.

DOX was incorporated into DexPHS nanoparticles and drug content was 8.1 % (w/w). Fig. 2 showed particle size changes of empty nanoparticles and DOX-incorporated DexPHS nanoparticles. Average particle sizes of DOX-incorporated nanoparticles were increased compared to empty nanoparticles of DexPHS. As shown in Fig. 2, particle sizes were increased at acidic environment both of empty nanoparticles and DOX-incorporated nanoparticles. These results indicated that DexPHS nanoparticles have pH-responsiveness in releasing anticancer drugs.



(a) (b)

Fig. 1 Particle size distribution (a) and TEM image (bar = 200 nm) (b) of DexPHS nanoparticles

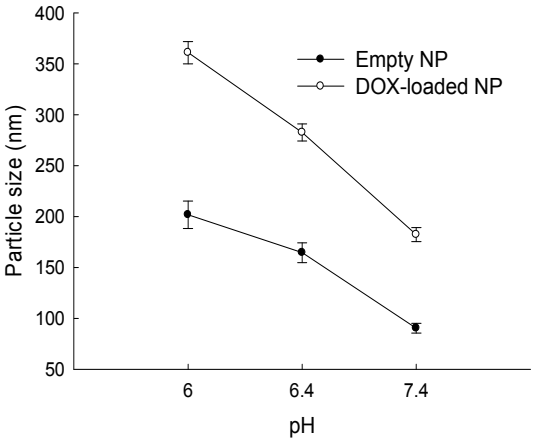


Fig. 2 Particle size changes of empty or DOX-loaded DexPHS nanoparticles according to the variation of solution pH

Anticancer activity of DexPHS nanoparticles was investigated using CT26 colorectal carcinoma cells. Since solid tumor has acidic environment, the delivery capacity of DexPHS nanoparticles to tumor cells was assessed in the acidic media *in vitro*. Free DOX or DOX-loaded DexPHS nanoparticles were added to CT26 cell culture media having various pHs. As shown in Fig. 3, CT6 cells revealed higher fluorescence intensity at basic pH (pH 7.4) when free DOX was treated. However, DOX-loaded DexPHS nanoparticle treatment showed stronger fluorescence intensity at acidic pH. These results indicated that DexPHS nanoparticles can deliver the anticancer agents to tumor cells according to the pH variations. Furthermore, flow cytometric analysis of CT26 cells also suppoted these results. Fig. 4 showed that fluorescence intensity of CT26 cells wuth treatment of CTDexPHS is

increased at acidic pHs compared to basic pH while CT26 cells treated with free DOX revealed decreased fluorescence intensity at acidic pHs. These results suppoted the results of Fig. 3, i.e. DexPHS nanoparticles can control the drug release behavior by pH variation and stimulate the delivery of the anticancer drug at acidic pHs.

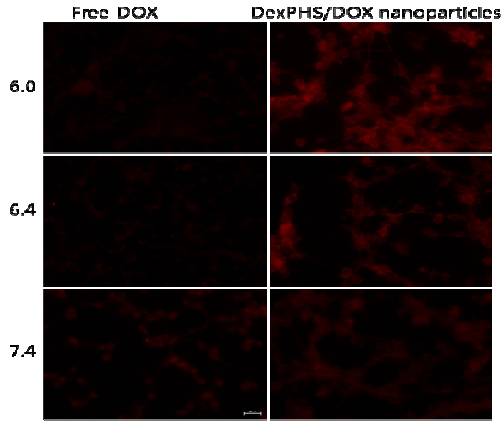


Fig. 3 Fluorescence images of CT26 cells. Free DOX or DOX-loaded DexPHS nanoparticles (DOX concentration was equivalent to 1 microgram/ml) were treated to CT26 cells for 1h at various pH conditions. (Resolution: 400×)

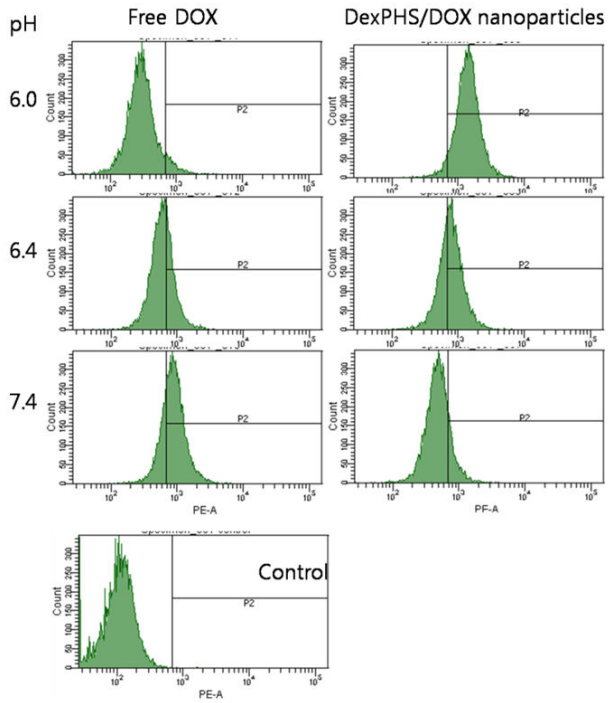


Fig. 4 Flocytometric analysis of CT26 cells. Free DOX or DOX-loaded DexPHS nanoparticles (DOX concentration was equivalent to 1 microgram /ml) were treated to CT26 cells for 1h at various pH conditions

Anticancer activity of DOX-loaded DexPHS nanoparticles were evaluated with CT26 cells using MTT assay. Free DOX or DOX-loaded DexPHS nanoparticles were exposed to CT26cells at various pHs. Then, viability of CT26 cells were checked after 24h. As shown in Fig. 5, DOX-loaded DexPHS nanoparticles showed increased anticancer activity at acidic pHs. The viability of CT26 cells was decreased at acidic pHs compared to basic pH whereas free DOX showed opposite results. These results indicated that DexPHS nanoparticles can deliver the anticancer drug according to the variation of pH and selectively kill the tumor cells at acidic environment.

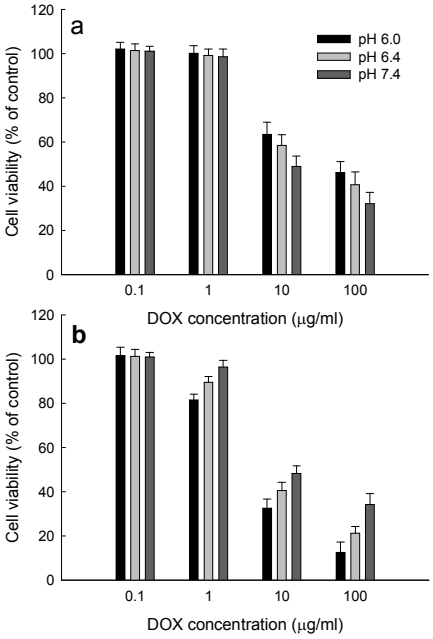


Fig. 5 The viability of CT26 cells by treatment of free DOX (a) or DOX-loaded DexPHS nanoparticles (b)

IV. Discussion

Tumor tissues are known to have strong acidic environment [9] and this fact stimulated development of stimuli-sensitive vehicles for tumor targeting of drugs. Since imidazole group has pH-responsiveness, histidine derivatives and PHS have been extensively investigated pH-sensitive delivery of anticancer agents and gene medicine [4]-[8]. Especially, polymer conjugates or copolymers based on PHS is to be a promising vehicles because they are able to can form nano-aggregates in aqueous environment and can used as a pH-sensitive drug delivery vehicles [6], [7]. Furthermore, PHS also can be used as gene delivery vehicles. For example, block or graft copolymer composed of PHS and poly(ethylene glycol) showed equal transfection efficiency compared to polylysine/DNA complexes [5]. However, their intrinsic toxicity against normal cells were lower than polylysine or polyethyleneimine. Histidine derivatives such as lipid-histidine conjugates or stearylated octahistidine conjugates were also known to be a effective delivery vehicles for genetic medicine [10], [11]. We also previously reported that nanoparticles composed of PHS-dextran or PHS-Poly(2-hydroxyethyl

methacrylate) block copolymer have superior pH-sensitivity and have capacities to deliver the anticancer drug to tumor cells with pH-responsiveness [6]-[12]. Nanoparticles of PHS-based block copolymer has small diameter around 100~300nm and particle sizes were increased at acidic pH. Furthermore, substitution number of histidine in conjugates or PHS chain length in copolymer are known to have significant effect on the efficacy of drug delivery to tumor tissues [13]-[15].

We previously reported that the number of PHS significantly affect to the physicochemical properties of nanoparticles, drug release rate and particle size [6]. Then, in this report, we evaluate the pH-responsive delivery capacity of DexPHS nanoparticles using CT26 cells *in vitro*. pH-sensitive nanoparticles using comb-shaped graft copolymer composed of dextran/PHS were to fabricate for pH-responsive tumor targeting. Nanoparticles of DexPHS graft copolymer has pH-sensitive changes in particle size, i.e. particle sizes of DexPHS nanoparticles were increased at acidic pH. Furthermore, uptake of DexPHS nanoparticles was increased at acidic pH compared to basic pH as shown is Figs. 3 and 4. Anticancer activity was also higher at acidic environment compared to basic pH. Nanoparticles of DexPHS graft copolymer showed improved uptake by tumor cells at acidic media.

V. Conclusion

We demonstrated that nanoparticles of DexPHS graft copolymer have pH-sensitive particle size changes and antitumor activity. DexPHS nanoparticles showed increased particle sizes and improved anticancer activity at acidic pH compared to basic pH. We suggest that DexPHS nanoparticles are promising vehicles for pH-sensitive drug targeting against tumor.

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