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Biorecognizable Nanoparticles Based On Hyaluronic Acid/Poly(ε-Caprolactone) Block Copolymer

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***Abstract*—**Since hyaluronic acid (HA) receptor such as CD44 is over-expressed at sites of cancer cells, HA can be used as a targeting vehicles for anti-cancer drugs. The aim of this study is to synthesize block copolymer composed of hyaluronic acid and poly(ε-caprolactone) (HAPCL) and to fabricate polymeric micelles for anticancer drug targeting against CD44 receptor of tumor cells. Chemical composition of HAPCL was confirmed using ^{1}H NMR spectroscopy. Doxorubicin (DOX) was incorporated into polymeric micelles of HAPCL. The diameters of HAPHS polymeric micelles were changed around 80nm and have spherical shapes. Targeting potential was investigated using CD44-overexpressing. When DOX-incorporated polymeric micelles was added to KB cells, they revealed strong red fluorescence color while blocking of CD44 receptor by pretreatment of free HA resulted in reduced intensity, indicating that HAPCL polymeric micelles have targetability against CD44 receptor.

***Keywords*—**Hyaluronic acid, CD44 receptor, biorecognizable nanoparticles, block copolymer.

I. Introduction

TARGETED drug delivery based on colloidal carriers such as nanoparticles, polymeric micelles, and polymer conjugates have been extensively investigated in the biomedical field [1]-[3]. Especially, nanoparticles decorating tumor-specific ligand have been spotlighted in recent decades since tumor cells normally express various receptors [3]-[5]. Among them, HA-decorated nanoparticles is regarded as a ideal vehicles for tumor targeting because HA receptor such as CD44 or RHAMM in tumor cells is especially over-expressed on the surfaces of highly malignant and invasive tumor cells [4]-[6]. Furthermore, since HA is a fully biocompatible material, HA can be considered as an ideal candidate for tumor-specific drug targeting. Many scientists reported drug delivery vehicles or nanoparticles having HA on the surface [7]-[10]. We also previously reported that nanoparticles of HA-poly(DL-lactide-co-glycolide) (HALG) block copolymer were prepared for tumor targeting and HALG nanoparticles revealed specificity against CD44 receptor of HCT116 human colon carcinoma cells.

In this study, we fabricated HA-decorated nanoparticles using HAPCL block copolymer. Since PCL is a liphophilic polymer, PCL may compose inner-core of the nanoparticles and HA can form outershell of the nanoparticles. We studied physicochemical properties of HAPCL nanoparticles and their targeting potential against KB human carcinoma cells.

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II. Experimental

A. Materials

HA (molecular weight (M.W.): 8,300g/mol) was purchased from LIFECORE BIOMEDICAL (Chaska, Minesota, USA). PCL (Average M.W. (M_w): 14,000g/mol; Average M_n: 10,000 g/mol), succinic anhydride (SA), dimethylaminopyridine (DMAP), triethylamine (TEA), sodium cyanoborohydride, hexamethylene diamine (HMDA), *N*-hydroxysuccimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide HCl (EDAC) and doxorubicin HCl (DOX) were purchased from Sigma Chem. Co. (St. Louis, USA). The dialysis membranes (M.W. cutoffs: 12,000g/mol) were purchased from Spectra/Pro^{TM} Membranes. Chloroform, methanol and dimethyl sulfoxide (DMSO) were of HPLC grade or extra-pure grade.

HAPCL block copolymer was synthesized as described previously with brief modification [8]. Aminated HA: 80 mg of HA was dissolved in 10ml H_2O/DMSO mixtures (3/7) with sodium cyanoborohydride and stirred for 3h. To this solution, HMDA was added and stirred for 1 days following dialysis against distilled water, purification, and lyophilization. Succinylated PCL: 100mg of PCL was dissolved in DCM with trace amounts of SA and DMAP. This solution was stirred for 24h and then solvent was evaporated. After that, solid was precipitated into methanol for purification and then filtered (succinylated PCL). Succinylated PCL was reacted with EDAC and NHS for 6h to obtain NHS-activated PCL in the carboxylic acid end group. NHS-activated PCL was dissolved in 10ml DMSO and mixed with aminated HA (HA dissolved in 10ml H_2O/DMSO mixtures (3/7)). This solution was reacted for 3 days and, then dialyzed against water for 2 days to remove byproducts and unreacted HA following lyophilization. To remove unreacted PCL, lyophilized solid was precipitated into chloroform and dried *in vacuo*.

B. Fabrication of Nanoparticles

40mg of HAPCL dissolved in 4ml of DMSO was mixed with 5mg of DOX in 1ml DMSO (with trace amounts of TEA). This solution was dropped into 10ml of water and then dialyzed against water for 1day. After that, dialyzed solution was used to

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analyze or lyophilize. DOX contents in the nanoparticles were checked with UV-spectrophotometer at 479nm (UV-spectrophotometer, UV-1801, Shimadzu Co. Japan). Drug contents were calculated as following equation:

Drug contents (%, w/w) = (Drug weight in the nanoparticles/Total weight of nanoparticles)*100)

C. Analysis of DexPHS Nanoparticles

Diameter of the HAPCL nanoparticles was analyzed with dynamic light scattering ((DLS-7000, Otsuka Electronics Company, Osaka, Japan). Morphological observation was performed with transmission electron microscope (TEM, JEOL JEM-2000 FX II, Japan).

D. Cell Culture

KB cells were maintained in RPMI1640 (10 % fetal bovine serum, 1% antibiotics) at 5% CO_2 incubator (37°C).

Cytotoxicity of free DOX or DOX-incorporated HAPCL nanoparticles was evaluated by MTT proliferation analysis method. Briefly, 3×10^4 KB cells seeded in 96-well plates were incubated overnight at 5% CO_2 incubator (37°C). After that, Free DOX or DOX-incorporated nanoparticles (DOX concentration was equivalent to 10μg/ml) were added to this culture and then cultured in 5% CO_2 incubator for 8h. After that, medium was removed and fresh serum-free medium was added. After that, plates were further incubated for 24h. After 24h 30μL of MTT (5mg/mL) was added to the 96-well plates and incubated for 4 hours. After that, 0.1ml DMSO was added and the absorbance (560nm test/630nm reference) was determined using an automated computer-linked microplate reader (Molecular Device Company, Sunnyvale, CA).

E. Fluorescence Microscopy

KB cells were treated with free DOX or DOX-incorporated nanoparticles for 1h. 1h before treatment of DOX or DOX-incorporated nanoparticle, free HA was pre-treated to block CD44 receptor of tumor cells. Cells 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

HAPCL block copolymer was synthesized as reported previously with brief modification [8]. Reductive end of HA was conjugated with HMDA to endow amine group in the end of the polymer chain. Carboxylic acid end group of PCL was conjugated with aminated HA by aid of water-soluble carbodiimide (EDAC) and then HAPCL was obtained by purification. Synthesis scheme of HAPCL block copolymer was shown in Fig. 1 (a). Specific peaks of PCL block was observed at 1.4, 1.6, and 2.3 ppm while specific peaks of HA was shown in 3~5 ppm. these results indicated the successive synthesis of HAPCL block copolymer.

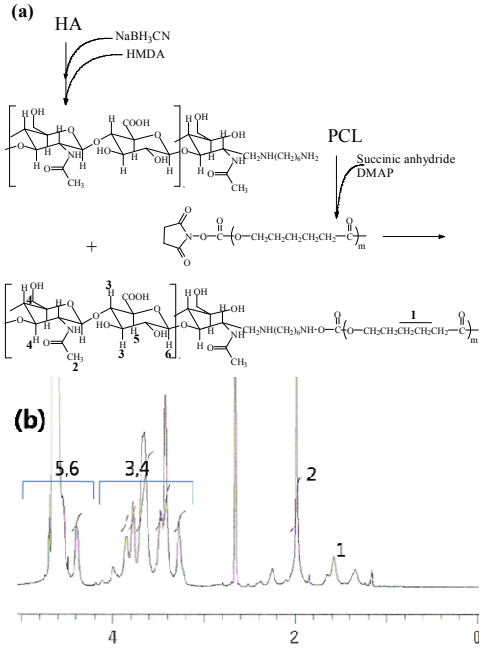


Fig. 1 Synthesis scheme (a) and ^{1}H NMR spectra (b) of HAPCL block copolymer in DMSO

Self-assembled nanoparticles of HAPCL block copolymer was prepared by nanoprecipitation and dialysis method. DOX was used as a model anticancer drug. Since HAPCL block copolymer has amphiphilic properties, hydrophobic domain, PCL, formed core of the nanoparticles and act as a drug reservoir. HA, a hydrophilic domain, formed outershell of the nanoparticles and act as a targeting moiety for tumor targeting [5]. DOX contents in HAPCL nanoparticles were approximately 8.1 % (w/w).

Fig. 2 showed the morphologies of HAPCL nanoparticles. When DOX was loaded into the core of the nanoparticles, particle size was higher than empty nanoparticles. Both empty and DOX-loaded nanoparticles have spherical shapes. Diameter of empty nanoparticles was less than 100nm while DOX-loaded nanoparticles were higher than 100nm.

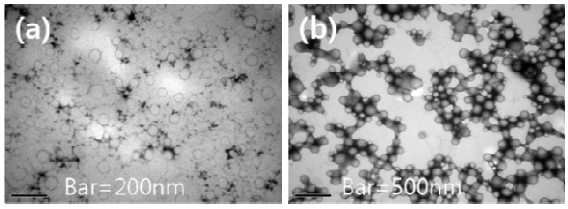


Fig. 2 TEM images of empty (a) and DOX-loaded (b) HAPCL nanoparticles

Fig. 3 showed the changes of particle size according to the increase of drug contents. As shown in Fig. 3, average particle

sizes of DOX-loaded nanoparticles higher than empty nanoparticles. According to drug loading, particle sizes were slightly increased from 90.5 nm to 172.1 nm.

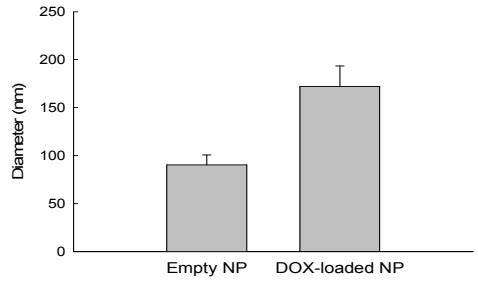


Fig. 3 Average particle size of empty and DOX-loaded HAPCL nanoparticles

The targeting potential of DOX-loaded HAPCL nanoparticles was investigated using KB human carcinoma cells because KB cells is known to express CD44 receptors [11]. To assess targetability, KB cells were pre-treated with free HA to block CD44 receptor 1h prior to treatment of DOX or DOX-loaded nanoparticles. As shown in Fig. 4, DOX treatment revealed strong fluorescence intensity regardless of CD44 receptor blocking. However, DOX-loaded nanoparticle treatment showed significantly decreased fluorescence intensity when CD44 receptor was blocked with free HA. These results indicated that HAPCL nanoparticles have targeting potential to cancer cells and have delivery of anticancer agents via CD44 receptor.

Fig. 5 showed the flow cytometric analysis of KB cells after treatment with DOX or DOX-loaded nanoparticles. Results of flow cytometer supported results of Fig. 4, indicated that KB cells with DOX treatment showed increased fluorescence intensity regardless of HA receptor blocking. However, fluorescence intensity was significantly decreased at treatment of DOX-loaded nanoparticles when CD44 receptor was blocked by pre-treatment with free HA. These results indicated that HAPCL nanoparticles can be used targeting carriers against CD44 receptor of tumor cells.

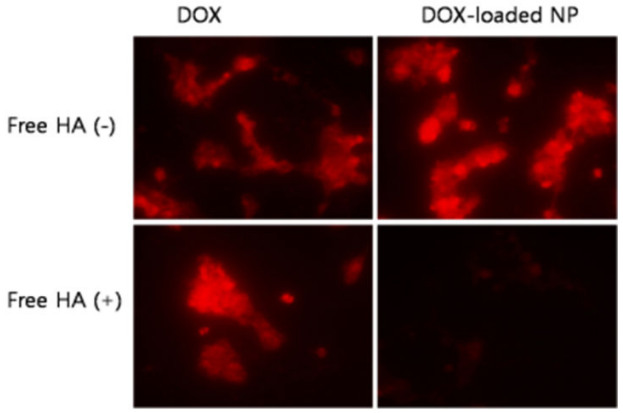


Fig. 4 Fluorescence images of KB cells. Free DOX or DOX-loaded DexPHS nanoparticles (DOX concentration was equivalent to 5 microgram/ml) were treated to KB cells for 1h at various pH conditions. (Resolution: 400×)

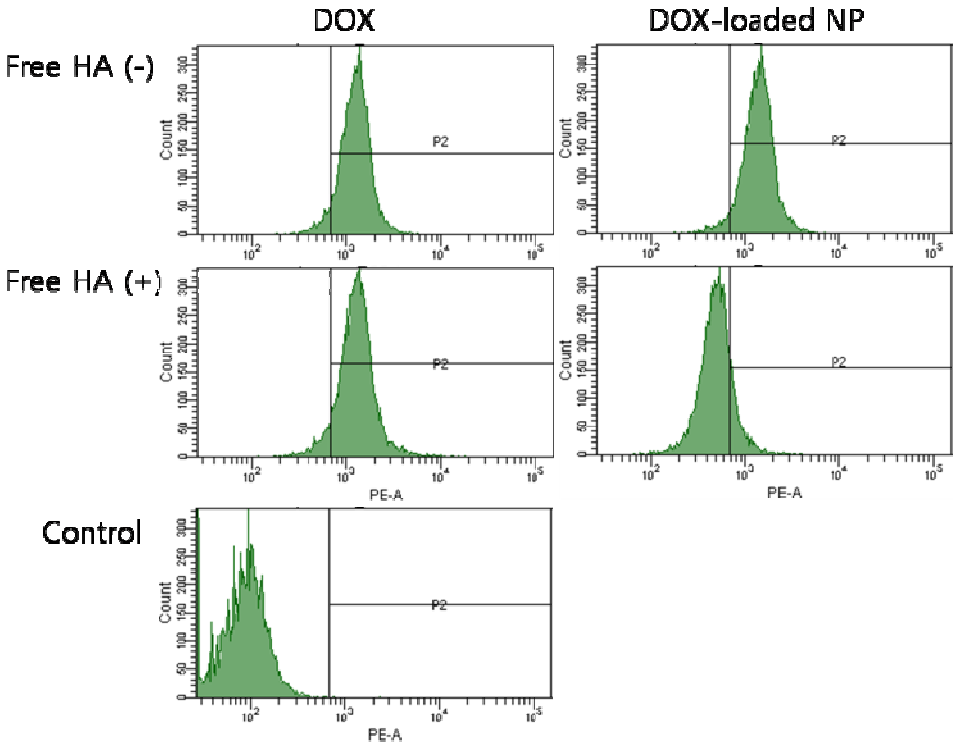


Fig. 5 Flocytometric analysis of KB cells. Free DOX or DOX-loaded HAPCL nanoparticles (DOX concentration: 5 microgram/ml) were treated

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to KB cells for 1h. Prior to drug treatment, free HA was added to block CD44 recptor of tumor cells

Anticancer activity of DOX or DOX-loaded HAPCL nanoparticles were assessed by cytotoxicity test as shown in Fig. 6. Prior to drug treatment, HA receptor was blocked to inhibit internalization of HAPCL nanoparticles. Free DOX or DOX-loaded HAPCL DexPHS nanoparticles were treated to KB cells and then viability was checked with MTT assay. As shown in Fig. 6, cell viability was not significantly changed at DOX treatment regardless of CD44 receptor blocking. However, viability of KB cells was significantly changed at nanoparticle treatment when CD44 receptor of KB cells was blocked with pretreatment with free HA. These results indicated that HAPCL nanoparticles have targetability to CD44 receptor and selectively deliver the anticancer drug to the tumor cells.

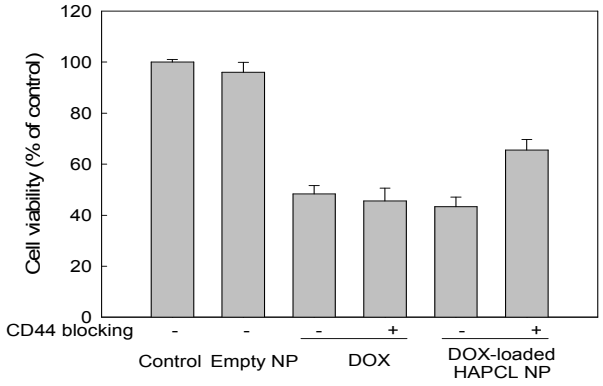


Fig. 6 The viability of KB cells by treatment of free DOX or DOX-loaded HAPCL nanoparticles (NP). 1h before drug treatment, free HA was added to block CD44 receptor of KB cells. DOX concentration was equivalent to 5 microgram/ml. At lest, 5 times higher amount of free HA compared to nanoparticle weight was added to cell culture to inhibit nanoparticle uptake

IV. Discussion

HA is a linear polysaccharide having disaccharide units composed of D-glucuronic acid and Nacetyl-D-glucosamine units [8]. HA is known to have key roles in cancer cell such as adhesion, spreading, invasion/metastasis, and proliferation [4], [5], [11]. From these advantages, many scientists employed HA for conjugation of anticancer drugs or polymers to target tumor cells [6]-[10]. Luo *et al*. reported that N-(2-hydroxypropyl) methacrylamide (HPMA)-HA conjugates selectively delivered DOX to ovarian cancer while HPMA-DOX and HPMA-HA-DOX conjugates showed minimal cytotoxicity against NIH 3T3 mouse fibroblast cells [10]. Furthermore, HA is useful macromolecule for delivery for delivery of extremely hydrophobic drugs such as paclitaxel [6], [9]. HA did not only increase solubility of hydrophobic drug but also enable to target tumor cells.

In this study, we synthesized HAPCL block copolymer and fabricated nanoparticles for tumor targeting. We demonstrated that HAPCL nanoparticles can target tumor cells with CD44 receptor specificity, i.e. targetability of HAPCL nanoparticles was inhibited by blocking of CD44 receptor of tumor cells. Furthermore, DOX-loaded HAPCL nanoparticles preferentially suppressed the viability of tumor cells.

We suggest that HAPCL is a promising candidate for tumor targeting via CD44 receptor.

V. Conclusion

We fabricated HAPCL nanoparticles for tumor targeting. HAPCL nanoparticles showed specificity CD44 receptor of KB cells at inhibition test. HAPCL nanoparticles have potential to deliver the anticancer drug by interaction with CD44 receptor of tumor cells. We suggest that HAPCL nanoparticles are promising candidates for anticancer drug targeting against tumor.

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