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Hydrotropic hyaluronic acid conjugates: Synthesis, characterization, and implications as a carrier of paclitaxel

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ABSTRACT

Amphiphilic hyaluronic acid conjugates were synthesized as a potential drug carrier by chemical conjugation of an amine-terminated hydrotropic oligomer, which has a unique ability to enhance the solubility of paclitaxel (PTX), to a hyaluronic acid (HA) backbone using carbodiimide chemistry. The physicochemical properties of the hydrotropic hyaluronic acid (HydroHA) conjugates were investigated using ^{1}H NMR, dynamic light scattering, transmission electron microscopy (TEM), and fluorescence spectroscopy. HydroHA conjugates could form self-assembled nanoparticles in an aqueous medium because of hydrophobic interactions among hydrotropic oligomers. Their particle sizes were in the range of 274–356 nm, depending on the degree of substitution (DS) of the hydrotropic oligomer. TEM images showed that particle morphology was spherical in shape. Critical aggregation concentrations of HydroHA conjugates ranged from 0.034 to 0.125 mg/mL, which decreased with an increase in the DS of the hydrotropic oligomer. The HydroHA conjugates were selectively taken up by the cancer cell line (SCC-7) over-expressing CD44, a hyaluronic receptor. The nanoparticles could encapsulate PTX up to 20.7 wt.% by the dialysis method. The *in vitro* release pattern of PTX from nanoparticles was significantly dependent on drug loading content, in which the release rate was lower for the nanoparticles that contained larger amounts of the drug. From the cytotoxicity test, it was found that the drug-loaded HydroHA nanoparticles exhibited stronger cytotoxicity to SCC-7 than to normal fibroblast cell (CV-1). These results suggest that HydroHA nanoparticles have potential as the carrier of PTX for cancer therapy.

1. Introduction

Development of novel polymeric amphiphiles with unique characteristics is of great interest due to their potential applications in pharmaceutical and biomedical industry (Gref et al., 1994; Allen et al., 1999). In particular, polymeric amphiphiles have received attention since they form self-assembled nanoparticles in an aqueous environment. The inner core of the nanoparticle can serve as the reservoir for various bioactive agents and allow their release in a controlled manner *in vivo*, thus increasing water solubility and bioavailability of the drug (Park et al., 2004; Kim et al., 2006). Although polymeric nanoparticles have been widely studied for the solubilization of hydrophobic drugs, their poor loading capacity of the drug and low physical stability with increase in load content are still the challenging issues for drug delivery (Gaucher et al., 2005). In this regard, considerable efforts have focused on the development of polymeric amphiphiles that can accommodate a large quantity of hydrophobic drug molecules, while maintaining high thermodynamic stability.

One approach to increase drug-loading capacity is incorporation of additional functionality to the hydrophobic constituents of amphiphiles to enhance the physical interactions between core-forming constituents and the hydrophobic guest molecules to be encapsulated (Giacomelli et al., 2007). It is well known that hydrotropic agents, including sodium salicylate and nicotinamide derivatives, can increase the aqueous solubility of poorly water-soluble drugs (Rasool et al., 1991). Therefore, construction of polymeric amphiphiles using hydrotropic agents as hydrophobic constituents may significantly increase the solubility of hydrophobic drugs. Of the various hydrotropic agents, the nicotinamide-based hydrotrope, *N*,*N*-diethylnicotinamide (DENA), has been recently identified as a specific candidate to enhance

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water solubility of paclitaxel (PTX), a highly hydrophobic chemotherapeutic agent (Lee et al., 2003). Polymeric micelles constructed with hydrotropic DENA in place of the hydrophobic block increased the aqueous solubility of PTX up to 37.4 wt.% (Lee et al., 2007). In recent years, we also demonstrated the potential of DENA in the self-aggregated nanoparticular system by preparing glycol chitosan derivatives modified using DENA-based oligomers (Saravanakumar et al., 2009). As expected, the self-assembled hydrotropic glycol chitosan nanoparticles exhibited high loading capacity of the drug (23.9 wt.%), which was significantly higher than other conventional polysaccharide-based amphiphiles.

One of the major challenges in cancer chemotherapy is severe toxicity due to the indiscriminate distribution of anticancer drugs. Therefore, selective delivery of these therapeutic drugs selectively to the target tumor cell is desirable. Selective delivery could be achieved using polymeric nanoparticles by a passive targeting mechanism, which exploits the disorganized vasculature of solid tumors (Maeda, 2001). An active targeting mechanism is also useful for cancer therapy, where the carrier or drug is often chemically modified with a targeting moiety capable of being recognized by the specific cell type (Allen, 2002; Byrne et al., 2008). Recently, hyaluronic acid (HA), a naturally occurring biocompatible polyanionic polysaccharide, has been widely used as a targeting constituent in drug carriers since various tumor cells are known to over-express HA receptors, such as CD44 and RHAMM (Platt and Szoka, 2008; Yadav et al., 2008). Our *in vivo* biodistribution study using self-assembled HA nanoparticles (HA-NPs), composed of bile acid as the hydrophobic constituent, also showed preferential accumulation of nanoparticles at the tumor site, which substantiated the potential of HA-NPs as carriers for cancer treatment (Choi et al., 2009).

In this study, we attempted to synthesize hydrotropic HA (HydroHA) derivatives by chemical conjugation of an amine-terminated hydrotropic DENA oligomer to the backbone of HA. A series of HydroHA conjugates were prepared by varying the degree of substitution (DS) of the hydrotropic oligomer, after which their physicochemical characteristics were studied using ^{1}H NMR, dynamic light scattering, transmission electron microscopy (TEM), and fluorescence spectroscopy. Drug release behavior from and cytotoxicity of PTX-loaded nanoparticles were also evaluated.

2. Materials and methods

Sodium hyaluronate (MW = 2.344×10^5) was purchased from Lifecore Biomedical (MN, USA). Paclitaxel was obtained from Samyang Genex Co. (Daejeon, Korea). 1-Hydroxybenzotriazole (HOBt), 2-hydroxynicotinic acid, diethylamine, 4-vinylbenzyl chloride, [1-ethyl-3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC), 2-aminoethanethiol hydrochloride (AET·HCl), *N*,*N*-azobisisobutyronitrile (AIBN), and anhydrous *N*,*N*-dimethylformamide (DMF) were purchased from Sigma–Aldrich Co. (St. Louis, MO). AIBN was used after purification by recrystallization in methanol. Water used in all the experiments was prepared by AquaMax-ultra water purification system (Anyang, Korea). All other reagents were analytical grade and used as received. 2-(4-(Vinylbenzyloxy)-*N*,*N*-diethylnicotinamide) (VBODENA) monomer was prepared, as reported previously (Lee et al., 2007).

2.1. Synthesis of Oligo(VBODENA)–NH_2

Amine-terminated hydrotropic oligomer (Oligo(VBODENA)–NH_2) was synthesized by free radical chain transfer telomerization of VBODENA in the presence of AET as a functional chain transfer agent, according to the scheme shown in Fig. 2. In a typical reaction, VBODENA, AET·HCl, and AIBN as an initiator were dissolved in DMF, followed by degassing by at least three freeze-thaw cycles. The reaction mixture in the flask was transferred to a preheated oil bath maintained at 75 °C. After 15 h, Oligo(VBODENA)–NH_2 was obtained by precipitation in excess of diethylether. The product was dried under vacuum at room temperature. The number-average molecular weight (M_n) of the Oligo(VBODENA)–NH_2 was determined using ^{1}H NMR.

2.2. Synthesis of HydroHA conjugates

HA (100 mg) was dissolved in distilled water at a concentration of 4 mg/mL. EDAC (192 mg) and HOBt (135 mg), dissolved in 2 mL of distilled water/methanol mixture (1/1 (v/v)), was added to the HA solution under stirring. Oligo(VBODENA)–NH_2 (15.63–78.23 mg) dissolved in methanol (25 mL) was added and the pH was adjusted to 6.8. The reaction was then allowed to proceed for 24 h at room temperature. The resulting solution was dialyzed against an excess of water/methanol (1 v/3 v–1 v/1 v) for 2 days and an additional day with distilled water (MW cutoff: 12,000–14,000; spectrum®, Rancho Dominquez, CA). After freeze-drying, the chemical structure of HydroHA conjugates was confirmed using ^{1}H NMR (D_2O/CD_3OD). The conjugates, synthesized in this study, were coded depending on the degree of substitution (DS), defined as the number of the hydrotropic oligomers conjugated to 100 repeating units of HA. For example, HydroHA3.2 indicates the conjugate bearing a hydrotropic oligomer with a DS value of 3.2.

2.3. Preparation and characterization of self-assembled HydroHA nanoparticles

HydroHA conjugates were suspended in phosphate buffered saline (PBS, pH = 7.4) under gentle shaking. The solution was sonicated three times using a probe-type sonifier (VCX-750, Sonics & Materials, CT, USA) at 90 W for 2 min each, and the pulse was turned off for 1 s with a 5 s interval to prevent an increase in temperature. After filtration of each sample through a membrane filter (pore size: 0.45 μm, Millipore), particle size and distribution was measured by dynamic light scattering with a helium laser system (Spectra Physics Laser Model 127-35, CA, USA), which was operated at 633 nm and 25 ± 0.1 °C. The conjugate concentration was kept constant at 1 mg/mL. To observe nanoparticle morphology, transmission electron microscopy (TEM) images were recorded using a Philips CM 30, operated at an accelerating voltage of 200 ekV. The zeta potentials of the HydroHA nanoparticles were measured using a Photal ELS-8000 electrophoretic light scattering spectrophotometer (Otsuka Electronics, Osaka, Japan). The results were expressed as the mean value ± standard deviation (n = 3).

Critical aggregation concentrations (CACs) of HydroHA conjugates were determined by the pyrene fluorescence method (Wilhelm et al., 1991). In brief, a pyrene solution (12×10^{-7} M in distilled water) was mixed with HydroHA conjugates to obtain polymer concentrations from 1.0×10^{-4} to 1.0 mg/mL. The final concentration of pyrene in each sample was 6.0×10^{-7} M, which is nearly equal to its solubility in water at 22 °C. Fluorescence spectra were recorded using an ISS K2 multifrequency phase and modulation fluorometer (ISS, Champaign, IL). The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 336 nm and 390 nm, respectively.

2.4. Preparation of PTX-loaded nanoparticles

HydroHA nanoparticles bearing PTX (HydroHA–PTX) were prepared using a dialysis method. In brief, HydroHA conjugates (10 mg) were dissolved in 3 mL of DMF/H_2O (2:1 (v/v)), to which a predetermined amount of PTX (1–5 mg) in DMF was slowly added.

The resulting mixture was dialyzed for 1 day against water using a membrane tube (MW cutoff = 12,000–14,000). Thereafter, the solution was centrifuged at 10,000 × *g* for 30 min to remove insoluble PTX, and the supernatant was filtered through a 0.8 μm membrane filter and freeze-dried to obtain a white powder. The amount of PTX in the nanoparticles was measured using high performance liquid chromatography (HPLC, Aglient Technologies, Willmington, DE). The mobile phase, composed of an acetonitrile/water (45:55 (v/v)) co-solvent, was delivered at a flow rate of 1.0 mL/min. Eluted compounds were detected at 227 nm using a Spectra 100 UV–vis detector.

2.5. In vitro drug release studies

The *in vitro* drug release profile of PTX from HydroHA–PTX nanoparticles was evaluated using a 0.1 M sodium salicylate medium as reported previously (Huh et al., 2005). Lyophilized HydroHA–PTX nanoparticles were dispersed in PBS (pH = 7.4), and the solutions were transferred to a cellulose membrane tube (MW cutoff = 12,000–14,000). The dialysis tube was placed in PBS containing 0.1 M sodium salicylate and gently shaken in a 37 °C water bath at 100 rpm. At various time intervals, the medium was refreshed. The concentration of PTX, from the samples collected at predetermined times, was determined using HPLC as described earlier (Lee et al., 2007).

2.6. In vitro cellular uptake and cytotoxicity test

SCC7 (squamous carcinoma) and CV-1 (kidney fibroblast) cell lines, obtained from the American Type Culture Collection (Rockville, MD), were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% (v/v) fetal bovine serum and 1% (w/v) penicillin–streptomycin at 37 °C in a humidified 5% CO_2–95% air atmosphere.

To determine cellular uptake behavior of HydroHA conjugates, Cy5.5-labeled HydroHA conjugates were exposed to cancer cells (SCC7) and to normal cells (CV-1), followed by visualization using the microscope. In brief, after cell attachment to the 96-well plates, the medium was replaced with 2 mL of serum-free culture medium containing Cy5.5-labeled HydroHA conjugates (100 μg/mL), followed by incubation for 30 min. The cells were then washed twice with PBS (pH 7.4) and fixed with a 4% paraformaldehyde solution. For nuclear staining, the cells were incubated with 4,6-diamino-2-phenylindole (DAPI, 3 μmol) for 10 min at room temperature, followed by washing with PBS (pH 7.4). The intracellular localization of HydroHA nanoparticles was observed using an IX81-ZDC focus drift compensating microscope (Olympus, Tokyo, Japan), in which the excitation and emission wavelengths were 673 nm and 692 nm, respectively.

The cytotoxicity of HydroHA nanoparticles, HydroHA–PTX, and Cremophor-based PTX (Cremophor–PTX) formulation was evaluated using the methylthiazol tetrazolium (MTT) assay. In brief, cells were seeded at a density of 1×10^4 cells/well in 96-well flat-bottomed plates. After 1 day of growth, the cells were washed twice with PBS and incubated for 2 days with various concentrations of either HydroHA nanoparticles, HydroHA–PTX, or Cremophor–PTX. The cells were then washed twice with PBS to remove any remaining drug and fresh culture medium was added. 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and the cells were incubated for an additional 4 h at 37 °C. Subsequently, the medium was removed and the cells were dissolved in DMSO. Absorbance at 570 nm was measured using a microplate reader (VERSAmax™, Molecular Devices Corp., Sunnyvale, CA).

3. Results and discussion

In an attempt to develop a carrier of PTX, amphiphilic HydroHA conjugates were synthesized since they could form self-assembled nanoparticles in the physiological condition, composed of hydrotropic inner core as the container of PTX and hydrophilic HA shell as the targeting moiety of cancer (Fig. 1).

3.1. Synthesis

HydroHA conjugates were prepared by chemical conjugation of an amine-terminated hydrotropic oligomer (Oligo(VBODENA)–NH_2) to the backbone of HA, as shown in Fig. 2. The oligomer was synthesized by radical telomerization of VBODENA in the presence of 2-aminoethanethiol as the chain transfer agent. The chemical structure of the Oligo(VBODENA)–NH_2 was readily confirmed by its ^{1}H NMR spectrum (Fig. 3a). The number-average molecular weight of Oligo(VBODENA)–NH_2 was 2944 Da, which was determined from the integration ratio of the two methyl peaks at 1.0–1.2 ppm [6H, –N–(CH_2–C$\underline{H}_3$)$_2$] of VBODENA to the methylene peak at 2.9 ppm [2H, –C$\underline{H}_2$–] of the AET moiety.

Oligo(VBODENA)–NH_2 was reacted with the carboxylic acid of HA in the presence of EDAC and HOBt, resulting in a series of HydroHA conjugates that were obtained by varying the feed mole ratio of the oligomer to HA. The chemical structure of the HydroHA conjugate was characterized using ^{1}H NMR, as shown in Fig. 3b. Successful conjugation was supported by the presence of aliphatic and aromatic peaks of Oligo(VBODENA)–NH_2 in addition to the characteristic peaks of HA. The DS value, defined as the number of the hydrotropic oligomers conjugated to 100 repeating units of HA, was quantitatively estimated from the integration ratio between the characteristic peak of HA at 1.9 ppm [3H, –NH–CO–C$\underline{H}_3$] and that of VBODENA at 0.8–1.0 ppm [6H,

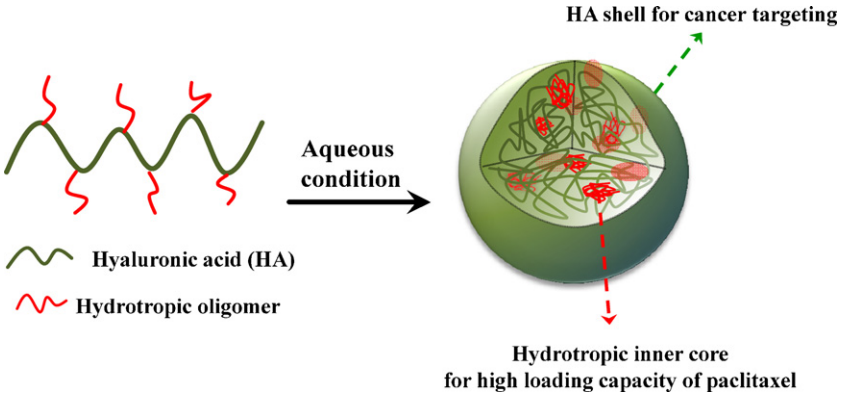

Fig. 1. Formation of self-assembled nanoparticles based on hydrotropic hyaluronic acid.

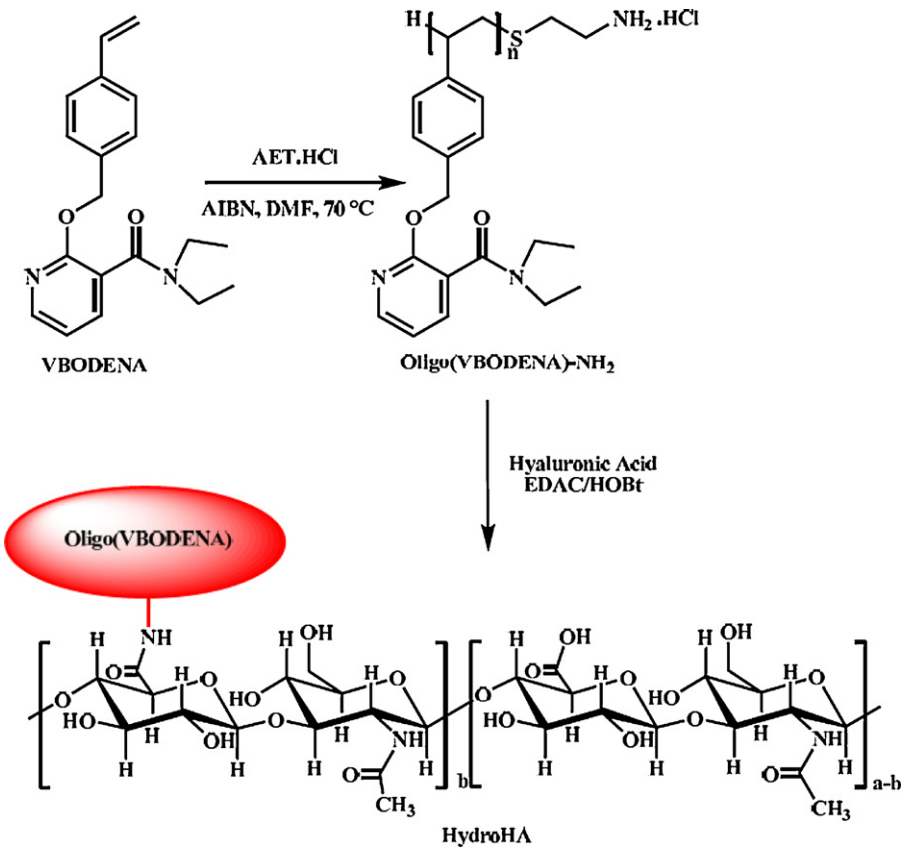

Fig. 2. Synthetic route of hyaluronic acid derivatives bearing the hydrotropic oligomer.

–N–(CH_2–C$\underline{H}_3$)$_2$]. The results indicated that the DS increased as the feed ratio of Oligo(VBODENA)–NH_2 to HA increased (Table 1). Since HydroHA conjugates with a DS higher than 7 were poorly water-soluble due to high hydrophobicity, they were not tested in further experiments.

3.2. Characteristics of HydroHA conjugates

The HydroHA conjugates, composed of HA and hydrotropic oligomer as the hydrophilic and hydrophobic segments, respectively, could self-assemble to form nano-sized particles in an aqueous condition. Table 1 shows the physicochemical properties of HydroHA nanoparticles with different DS values. The mean diameters of HydroHA nanoparticles were in the range of 274–356 nm, depending on the DS value. The size of the HydroHA nanoparticle decreased as the DS value increased, suggesting that a large amount of hydrophobic segment in the conjugate allowed for formation of compact hydrophobic cores. This result is consistent with the results obtained from other polymeric amphiphiles such as bile acid-modified HA and chitosan (Kwon et al., 2003; Choi et al., 2009). The zeta potentials of HydroHA nanoparticles ranged from −23.82 to −22.14 mV, indicating that the ionized carboxylic groups of HA covered the surface of the nanoparticles. All the HydroHA nanoparticles had a fairly narrow size distribution (Fig. 4). From the TEM images of nanoparticles, it was found that the shape of nanoparticles, constructed by the HydroHA conjugates, was spherical. All the HydroHA nanoparticles with different DS values maintained physical integrity in PBS (pH = 7.4) and their mean diameters were not significantly changed for a week, implying high stability (Fig. 5). Similarly, polymeric micelles and self-assembled chitosan nanoparticles, using hydrotropic DENA as the hydrophobic constituent, have also exhibited excellent physical stability (Lee et al., 2007; Saravanakumar et al., 2009). High stability of HydroHA nanoparticles might result from the presence of strong hydrophobic inner cores to enhance the integrity of the nanoparticle. In addition, the negatively charged HA shells may prevent inter-particular aggregation by electrostatic repulsion.

Aggregation behavior of HydroHA conjugates under aqueous conditions was investigated using a fluorescence technique with pyrene as the fluorescent probe, which has been widely utilized to monitor self-aggregation behavior of various surfactants and amphiphilic polymers (Lee et al., 1998). Pyrene exhibits little fluorescence intensity in polar environments due to its poor solubility and self-quenching. Upon self-association or the formation of hydrophobic microdomains, pyrene molecules preferably locate inside or close to hydrophobic microdomains of the self-aggregate rather than the aqueous phase, resulting in different photophysical characteristics. Fig. 6 shows the intensity ratio (I_{343}/I_{334}) of the pyrene excitation spectra as a function of HydroHA concentration. At the low concentrations of HydroHA conjugates, no significant change in the total fluorescence intensity was observed. As the concentration increased, the intensity increased markedly, reflecting the partitioning of pyrene molecules into the hydrophobic microdomains of the self-aggregate. The critical aggregation concentration (CAC), the threshold concentration of self-aggregate formation by intra- or inter-molecular association, was determined from the cross-over point at the low concentration ranges. The CAC values of HydroHA conjugates were in the range of 0.034–0.125 mg/mL, which was significantly lower than those of low molecular surfactants such as sodium dodecyl sulfate in water (2.3 mg/mL). Also, these CAC values were lower than other polysaccharide-based amphiphiles such as alkylated derivatives of HA (~0.5 mg/mL) (Creuzet et al., 2006) and *N*-alkyl modified sulfated chitosan (0.45 mg/mL) (Zhang et al., 2004). HydroHA conjugates with higher DS values had lower CAC values, which indicated that an increase in the amount of hydrotropic segment

Table 1
Physical properties of hydrotropic hyaluronic acid conjugates.

Samples[a]	Feed ratio[b] (%)	DS[c]	M_n[d]	x[e]	d (nm)[f]	ζ (mV)[g]
HA	–	–	234,400	–	–	–
HydroHA1.6	0.02	1.59	263,328	0.109	356 ± 5.03	−23.82 ± 0.26
HydroHA3.2	0.04	3.17	292,074	0.197	301 ± 0.88	−22.29 ± 0.45
HydroHA7.1	0.10	7.10	363,576	0.355	274 ± 2.89	−22.14 ± 0.13

[a] HydroHA, in which the number indicates the DS of Oligo(VBODENA)–NH_2.
[b] Mole ratio of Oligo(VBODENA)–NH_2 to repeating unit of HA.
[c] Degree of substitution of Oligo(VBODENA)–NH_2 moiety.
[d] Number-average molecular weight, estimated using ^{1}H NMR.
[e] Weight fraction of Oligo(VBODENA)–NH_2 in the conjugate.
[f] Mean diameter of nanoparticles in PBS (pH 7.4), measured by dynamic light scattering.
[g] Zeta potential of HydroHA nanoparticles in distilled water.

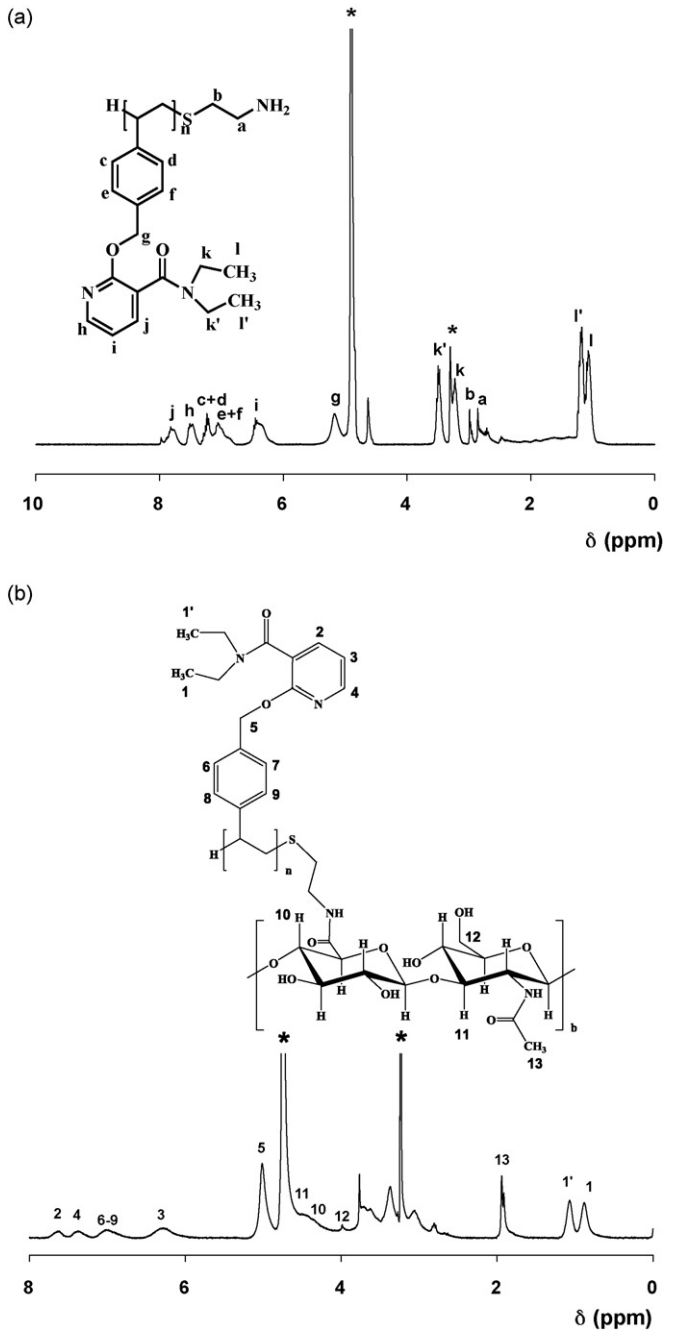

Fig. 3. ^{1}H NMR spectra of (a) Oligo(VBODENA)–NH_2 in CD_3OD and (b) HydroHA7.1 in CD_3OD/D_2O. The asterisks indicate solvent residual peak.

facilitated self-association of the conjugates even at low concentrations due to enhanced interactions among the hydrotropic segments. The low CAC value is one of the essential parameters for the use of self-assembled nanoparticles as a drug carrier, since conjugates with low CAC values may have resistance to dissociation of the assembled structure at highly diluted conditions in the body.

3.3. In vitro release behavior of PTX from HydoroHA nanoparticles

PTX-loaded HydroHA (HydroHA–PTX) nanoparticles were prepared by the dialysis method, in which the feed ratio of PTX to the conjugate was 0.5. HydroHA1.6, HydroHA3.2, and HydroHA7.1 encapsulated 12.1 wt.%, 20.7 wt.%, and 14.6 wt.% of PTX, respectively. This result indicated that larger amounts of hydrotropic oligomer in the conjugate were not always important to increase the loading content of PTX. An increase in the amount of hydrotropic oligomer may enhance hydrophobicity of nanoparticles, thus rendering them susceptible to precipitation during the loading process of the drug. Of the conjugates, HydroHA3.2 showed an excellent ability to encapsulate the drug, which was superior to other polysaccharide-based amphiphiles (Kim et al., 2006; Lee et al., 2009; Zhao et al., 2009). Therefore, HydroHA3.2 was chosen as the representative candidate for the further experimentations.

The *in vitro* release test of PTX from HydroHA–PTX nanoparticles was carried out in the presence of sodium salicylate in PBS (pH 7.4), which had been successfully used as the hydrotropic agent to test the release behavior of PTX from self-assembled nanoparticles (Cho et al., 2004). By varying the feed ratio of PTX to HydroHA3.2, the loading content of PTX was controlled from 3.2 wt.% to 20.7 wt.%. The *in vitro* drug release patterns from HydroHA3.2–PTX (3.2 wt.%), HydroHA3.2–PTX (12.3 wt.%) and HydroHA3.2–PTX (20.7 wt.%) are shown in Fig. 7. PTX was rapidly released from all the nanoparticles at the initial period of time. For example, the amount of PTX released for 6 h from the HydroHA3.2–PTX (3.2 wt.%), HydroHA3.2–PTX (12.3 wt.%) and HydroHA3.2–PTX (20.7 wt.%) nanoparticles were found to be 18.49%, 60.32%, and 69.18%, respectively. At later time points, the release rate gradually decreased. In particular, HydroHA3.2–PTX (20.7 wt.%) exhibited a sustained drug release profile for up to 1 week. This suggests that an increase in the amount of PTX enhances hydrophobicity of the nanoparticle core, resulting in slower release of the drug.

3.4. In vitro cellular uptake and cytotoxicity

In order to monitor cellular uptake behavior of HydroHA nanoparticles, Cy5.5-labeled HydroHA nanoparticles were incubated with cancer cells (SCC7) over-expressing CD44 or normal fibroblast cells (CV-1). As shown in Fig. 8, strong fluorescent signals were detected in the cytoplasm for Cy5.5-labeled HydroHA nanoparticles tested in SCC7 cancer cells, indicating that nanoparticles were readily internalized into SCC7 cells. In contrast, when the

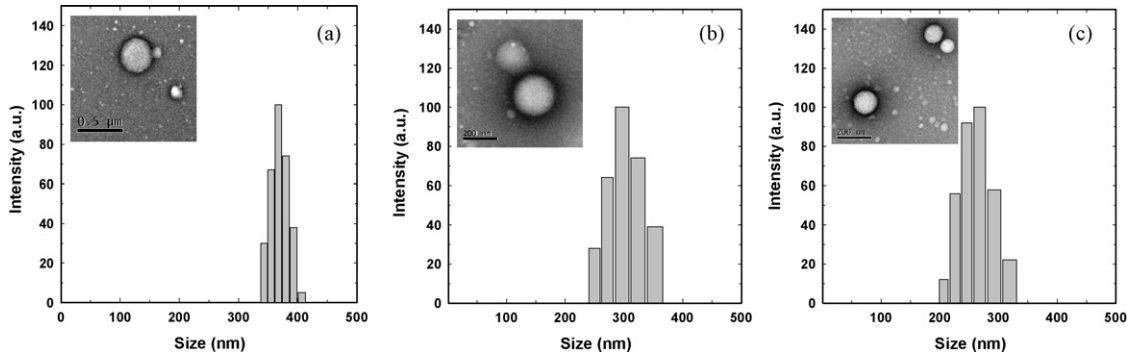

Fig. 4. Size distribution of HydroHA nanoparticles (a) HydroHA1.6, (b) HydroHA3.2 and (c) HydroHA7.1. The inset is for the TEM image of each nanoparticle, where the scale bars for (a), (b) and (c) are 500 nm, 200 nm and 200 nm, respectively.

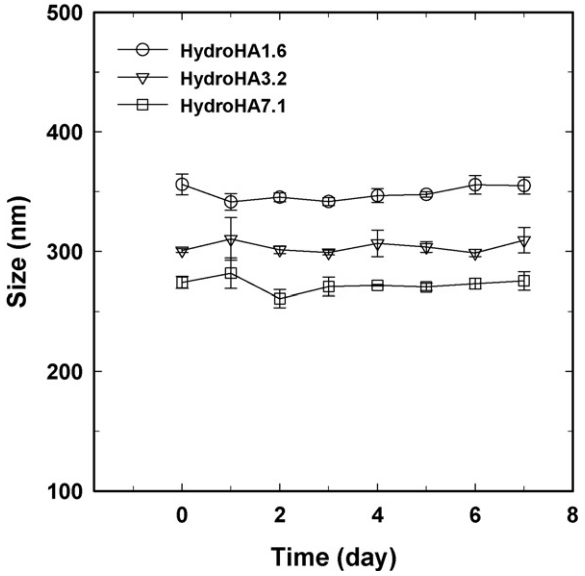

Fig. 5. Particle size of self-assembled HydroHA nanoparticles at 37 °C in PBS (pH = 7.4) as a function of time. The error bar represents the standard deviation ($n = 3$).

nanoparticles were incubated with CV-1 cells, which do not over-express CD44, no considerable fluorescent signals were observed. These results suggest that the HydroHA nanoparticles, prepared in this study, could selectively bind to CD44 on the cancer cells, followed by internalization via receptor-mediated endocytosis.

The cytotoxic effects of HydroHA–PTX and Cremophor–PTX on CV-1 and SCC7 cells were evaluated using the MTT colorimetric assay, as shown in Fig. 9. Cremophor–PTX exhibited dose-dependent cytotoxicity to both type of cells. It is of interest to note that cytotoxicity of HydroHA–PTX to CV-1 cells was significantly lower than Cremophor–PTX and was not dependent on the dose, applied in this study. HydroHA–PTX might not be taken up by CV-1 cells, which could be expected from the cellular uptake results shown in Fig. 8. However, HydroHA–PTX exhibited a dose-dependent cytotoxicity to SCC7 cells, which was comparable to Cremophor–PTX. This could be explained by receptor-mediated endocytosis of HydroHA–PTX, as SSC7 cells are known to over-express CD44.

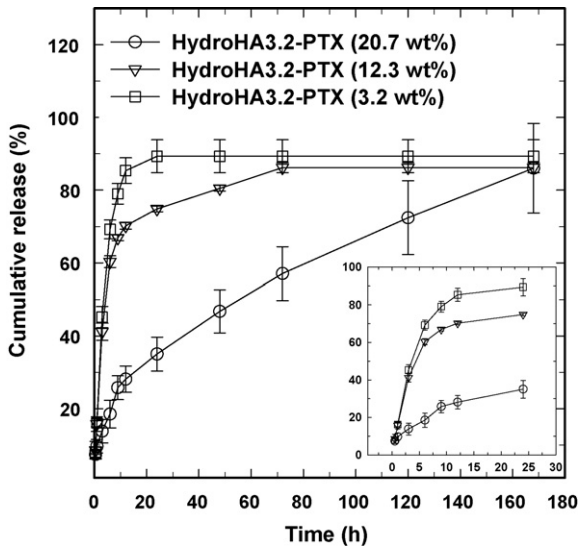

Fig. 7. Release pattern of PTX from HydroHA3.2 nanoparticles. The error bar represents the standard deviation ($n = 3$).

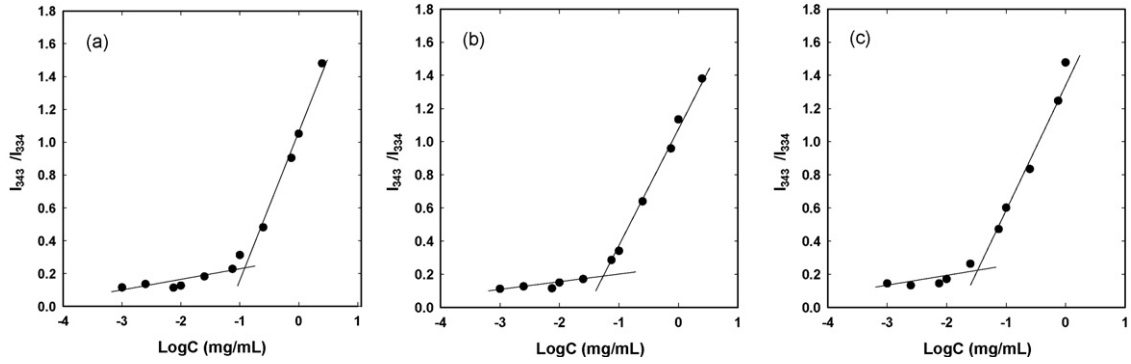

Fig. 6. Intensity ratio of I_{343}/I_{334} from pyrene excitation spectra as a function of HydroHA concentration in distilled water: (a) HydroHA1.6, (b) HydroHA3.2, and (c) HydroHA7.1.

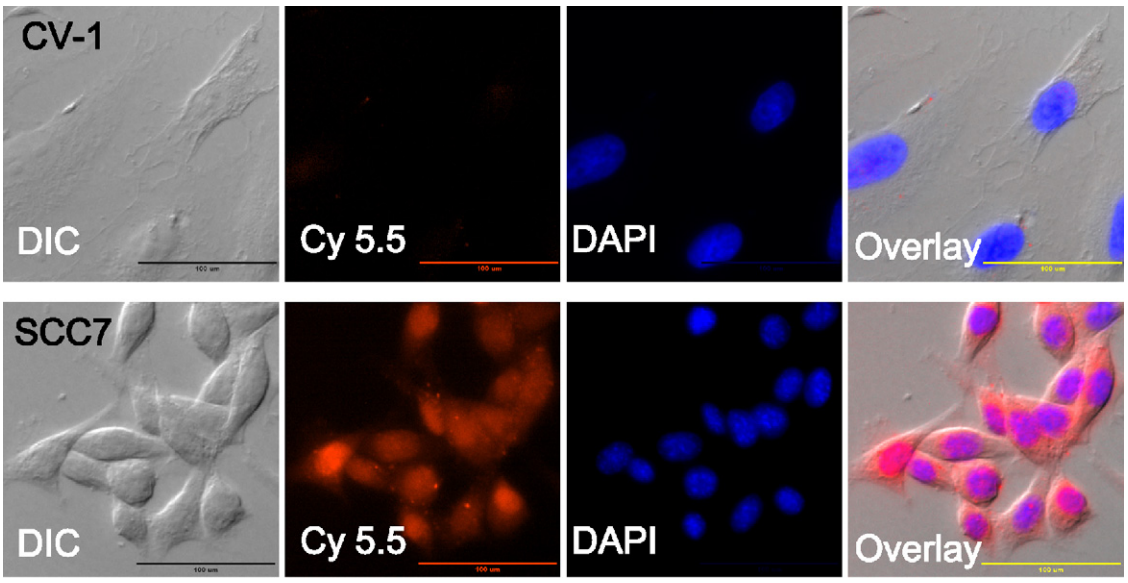

Fig. 8. Confocal microscopic images of SCC7 tumor cells and CV-1 cells treated with Cy5.5-labeled HydroHA nanoparticles.

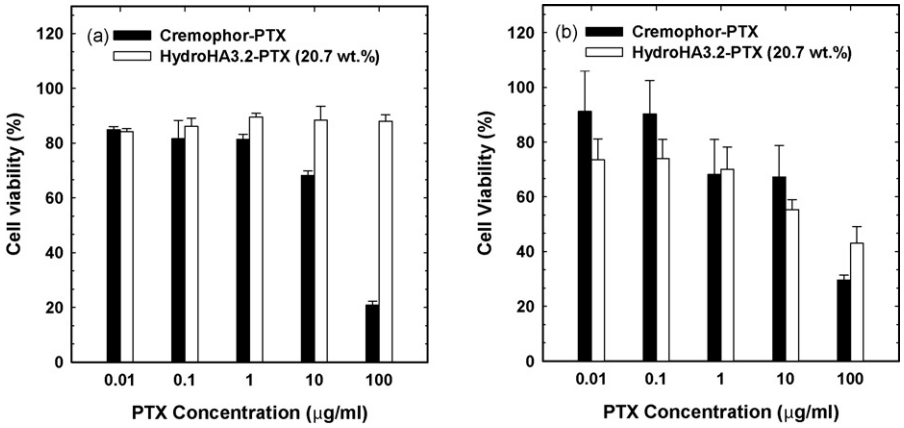

Fig. 9. *In vitro* cytotoxicity of Cremophor–PTX and HydroHA–PTX to (a) CV-1 and (b) SCC7 cells. The error bar represents the standard deviation ($n = 3$).

Overall, it is evident that HydroHA–PTX exhibited specific cytotoxicity to cancer cells, which may lead to effective cancer therapy without serious side effects. Along this line of research, *in vivo* biodistribution of HydroHA–PTX and its anticancer effects are currently under investigation.

4. Conclusion

In summary, a series of novel HydroHA conjugates, which formed self-assembled nanoparticles, were synthesized and their physicochemical properties were explored. These HydroHA conjugates were able to effectively imbibe PTX, a highly hydrophobic drug, up to 20.7 wt.%. The PTX-loaded HydroHA nanoparticles exhibited sustained release behavior of the drug for at least 1 week. In addition, the nanoparticles showed selective cytotoxicity to cancer cells over-expressing CD44, a specific receptor for HA. These results imply that HydroHA conjugates have potential as a carrier for PTX in cancer therapy.

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