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Water-soluble heparin–PTX conjugates for cancer targeting

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ABSTRACT

Targeted drug delivery to cancer cells or tumor vasculature is an attractive approach to treating cancer. We here report the synthesis of an anticancer drug conjugate composed of paclitaxel (PTX) and polysaccharide heparin through the reaction of aminated PTX with the carboxyl group of heparin. The structure of the conjugates was identified by ^{1}H NMR and FT-IR measurements. Heparin–PTX conjugates have high solubility in aqueous solutions. Unlike physically encapsulated drugs, heparin–PTX can self-assemble to form spherical nanoparticles in aqueous solution as characterized by Transmission Electron Microscopy (TEM). Size distribution of the nanoparticles as determined by Dynamic Light Scattering (DLS) was in the range of 200–400 nm depending on the coupling ratio of PTX to heparin molecules. The anticoagulant activity of heparin–PTX conjugates was decreased compared to that of heparin, thereby reducing hemorrhagic side effects. Cellular uptake of the nanoparticles was significantly enhanced compared to heparin as visualized by Confocal Laser Scanning Microscopy (CLSM). Furthermore, heparin–PTX conjugate nanoparticles exhibited higher cytotoxicity against KB cancer cells than did free PTX. The cytotoxicity of nanoparticles was found to depend on the amount of PTX conjugated to heparin as well as the conjugate concentration. Thus, conjugation of PTX to heparin may be useful for the solubilization and targeted delivery of PTX to solid tumors.

1. Introduction

Recent advances in tumor therapy have led to the development of new probes, drugs, and genes for tumor targeting [1,2]. Among these new drug targeting systems, polymeric nanoparticles have been demonstrated to significantly improve the specificity of drug action. This effect is mainly attributed to changes in tissue distribution and pharmacokinetics [3–5]. Furthermore, it has been demonstrated that nanoparticles can escape from the vasculature through the leaky endothelial tissue surrounding the tumor and accumulate in certain solid tumors. Most polymeric nanoparticles display this enhanced permeability and retention effect (EPR effect). Several synthetic as well as natural polymer carriers such as poly(ethylene glycol) (PEG), N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA) and poly(L-glutamic acid) (PG) have been successfully used in improving cancer chemotherapy [6–8].

Heparin is a natural polysaccharide held together by glycosidic linkages. The covalently linked sulfate, hydroxyl and carboxyl groups of heparin are the main functional groups contributing to its anticoagulant activity [9,10]. Other studies have shown that heparin is not just an anticoagulant, but actually a complex set of multifunctional glycosaminoglycan molecules with many other potential biological effects on cancer cells [11,12]. Recently, many reports have shown that the effect of heparin on tumors is associated with the binding of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [13,14]. Heparin is also associated with the inhibition of heparanases enzymes that are thought to be required by tumor cells for invasion of the vascular basement membrane [15]. These features make heparin a promising candidate as a drug carrier for targeted drug delivery.

Paclitaxel (Taxol) is an effective anticancer drug that has shown significant antineoplastic activity against various human cancers, including breast and ovarian tumors [16,17]. However, the clinical use of PTX is limited due to its poor solubility and toxicity. Currently, available PTX formulations include a combination of Cremophor EL and ethanol for solubilization. However, Cremophor EL is toxic and has side effects such as hypersensitivity, nephrotoxicity and neurotoxicity [17]. In order to overcome these limitations, polymeric carriers such as PG, polyethylene glycol (PEG) and albumin have been widely used to conjugate to or encapsulate PTX [18]. These carriers produced desirable pharmacokinetics, enhanced anti-tumor activity and low toxicity. To prepare a stable

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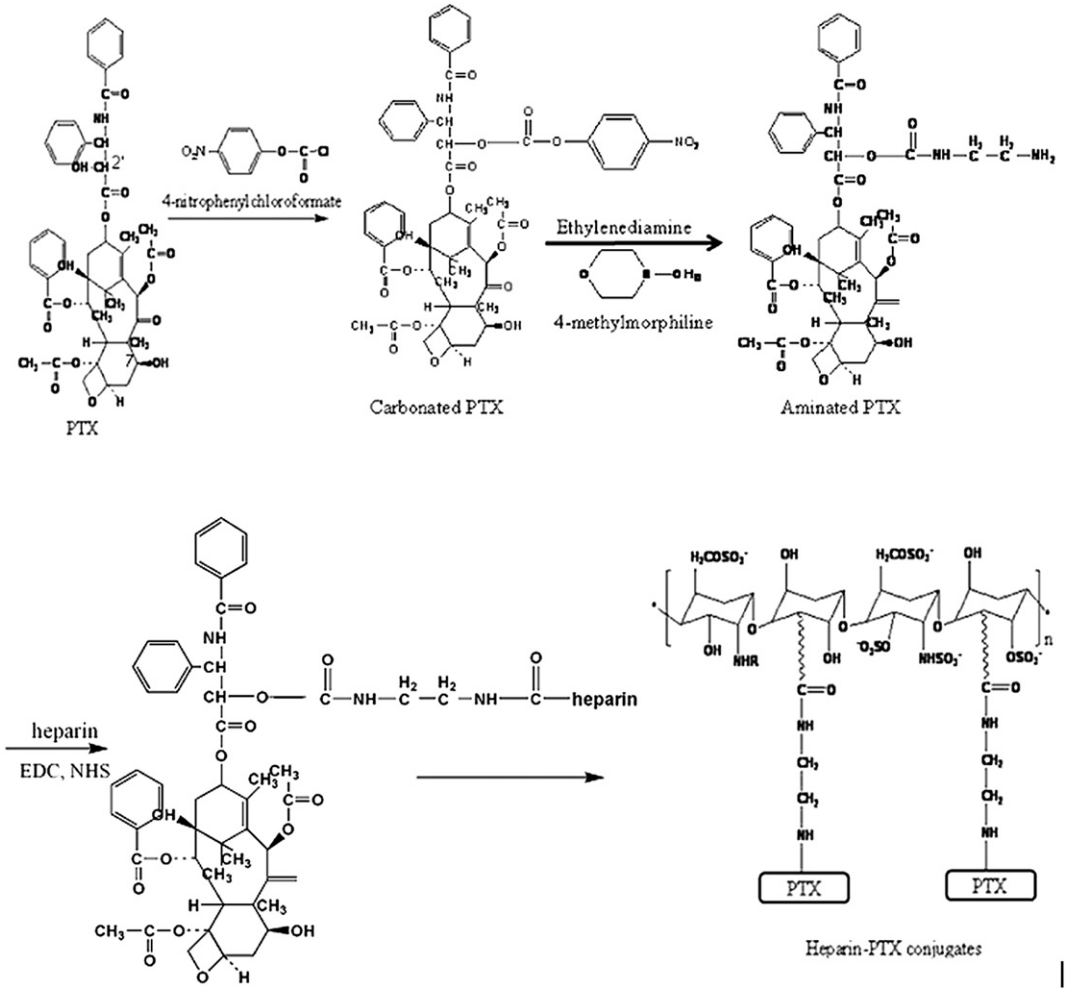

Fig. 1. Synthesis and schematic structure of heparin–PTX conjugate.

water-soluble conjugate exerting anti-tumor effects, we selected the natural polysaccharide heparin due to its following advantages: (a) it is highly hydrophilic due to the presence of sulfate, hydroxyl and carboxyl groups; (b) it is non-toxic *in vivo* and readily neutralized by antagonists; and (c) it acts as an anticancer agent.

Several strategies for the conjugation of drugs to polymer carriers have been developed to promote cancer cell targeting. In these approaches, drugs are directly conjugated to polymer carriers or to a linker for cleavage by lysosomal enzymes. This strategy can endow derivatives with enhanced water-solubility and chemical stability, an improved pharmacokinetic and distribution profile, and reduced side effects [4,5,18]. In this paper, we report the synthesis, characterization and *in vitro* experiment of heparin–PTX conjugate nanoparticles. We expect that our strategy will be applied to passive targeted delivery of therapeutic agents to tumor tissues.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (114.1 IU/mg, heparin) of average molecular weight ca. 5000 Da was purchased from Glaxosmithkline (Brentford, Middlesex, UK). Paclitaxel (PTX) was provided by Samyang Genex Co. (Daejeon, Korea). 4-nitrophenyl chloroformate (4-NPC), triethylamine, N-(3-dimethylaminopropyl)-N′-ethylcarboimide hydrochloride (EDC), 4-methylmorpholine, N-hydroxysuccinimide (NHS), ethylenediamine, dimethyl sulfoxide (DMSO), methanol, and n-hexane were purchased from Sigma Chemical CO. (St. Louis, MO). Penicillin-streptomycin, fetal bovine serum (FBS), 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution, and EMGM medium were purchased from American Type Culture Collection (Rockville, MD). RPMI-1640 medium (without FA) was obtained from Invitrogen (Carlsbad, CA). All reagents were analytical grade and used as received without further purification.

2.2. Preparation of aminated PTX

The synthetic methods for aminated PTX and heparin–PTX conjugates were similar to the method described in a previous report [19]. For amination of PTX, PTX (0.026 mmol) in 5 mL of DMSO was reacted with triethylamine (0.174 mmol) and 4-nitrophenyl chloroformate (4-NPC, 0.174 mmol) for 12 h at room temperature. The reactant was then extracted with 20 mL of methanol and 20 mL of n-hexane. The crude product was washed with 10 mL of n-hexane for 3 times to obtain PTX carbonate. To obtain aminated PTX, PTX carbonate was reacted with 4-methylmorpholine (1.73 mmol) and ethylenediamine (86.4 mmol) overnight at room temperature. The reactant was concentrated by

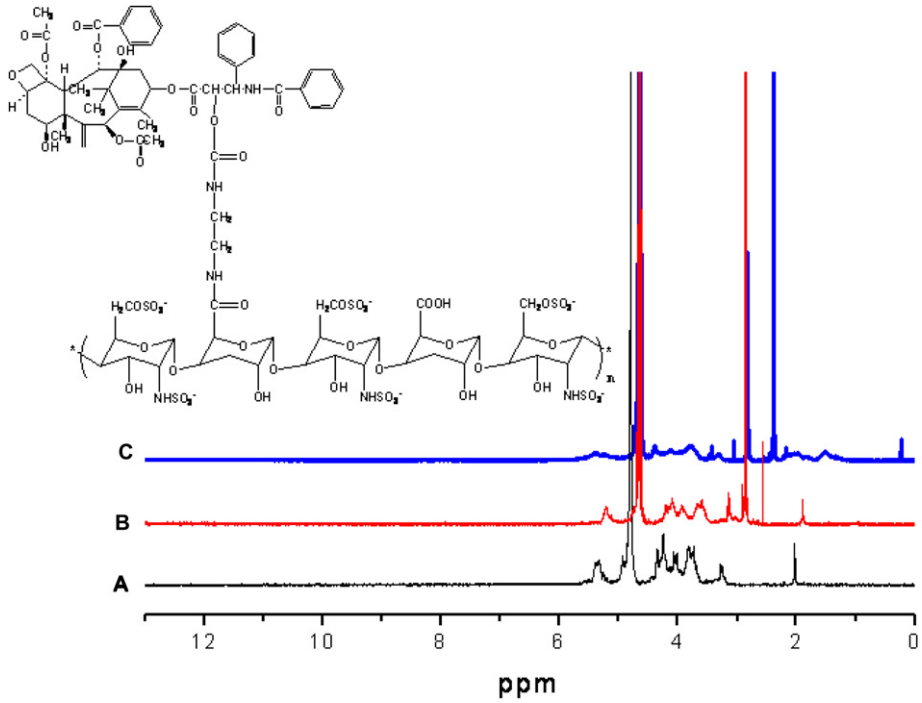

Fig. 2. ^{1}H NMR spectra of heparin in D_2O (A), heparin–PTX in D_2O (B) and heparin–PTX in D_2O/DMSO (0.7/0.3, v/v) (C) at 25 °C.

evaporation and unreacted chemicals were removed by adding 20 mL of n-hexane. The final product was dried under vacuum for 24 h.

2.3. Preparation of heparin–PTX conjugates

Heparin–PTX conjugates were synthesized by coupling reaction of heparin with aminated PTX. For example, heparin (0.01 mmol) was dissolved in water after which the pH was adjusted to pH 5.0–6.0 by the addition of 0.1 M HCl solution. EDC (0.1 mmol) was mixed and stirred with heparin solution, followed by the addition of NHS (0.024 mmol) and aminated PTX (0.026 mmol). After reaction at room temperature for 12 h, the solution was dialyzed (MWCO: 2000) against water to remove unreacted EDC, NHS, and aminated PTX. Heparin–PTX was obtained after freeze-drying. The product was confirmed by ^{1}H NMR (JNM-AL400, Jeol Ltd, Akishima, Japan). The values for ^{1}H NMR of PTX ($CDCl_3$) were: δ 1.11 [s, $^{17}CH_3$], 1.20 [s,$^{16}CH_3$], 1.69 [s, $^{19}CH_3$], 1.97 [s, $^{18}CH_3$], 2.2 [m, OAc], 2.4 [m, OAc], 3.78 [d, ^{3}CH], 4.17 [d, $^{20}CH_2$], 4.27 ppm [H-α, PG], 4.3 [d, $^{20}CH_2$], 4.39 [dd, ^{7}CH], 4.96 [d, ^{5}CH], 4.78 and 5.63 [d, $^{2'}$CH], 5.67 [d, ^{2}CH]. The values for ^{1}H NMR of heparin (D_2O) were: δ 5.38 [H1 of glucosamine residue(A)], δ 5.04 [H1 of iduronic acid residue(I)], δ 4.84 [I-5], δ 4.36–4.23 [A-6], δ 4.12–4.40 [I-3], δ 4.08 [I-4], δ 4.02 [A-5], δ 3.78 [I-2], δ 3.71 [A-4], δ 3.65–3.69 [A-3], δ 3.24 [A-2]. The values of ^{1}H NMR of heparin–PTX (D_2O) were δ 1.11–2.4 [CH_3 or OAc of PTX], δ 3.24–5.38 [A or I of heparin].

2.4. Characterization of heparin–PTX conjugates

UV–Vis absorption spectra were analyzed on a Sinco PDA UV–Vis spectrometer. The content of PTX conjugated to heparin was calculated from UV–Vis measurements based on a standard curve of PTX ($\lambda = 228$ nm). The IR spectra of heparin–PTX conjugates were analyzed by Fourier Transform infrared spectroscopy (FT-IR) using a MAGNA 560 spectrometer after which samples were made as KBr pellets. The average size, size distribution and particle shapes of heparin–PTX conjugate nanoparticles were analyzed using Electronic Light Scattering (ELS, Otsuka Electronics, ELS-Z series) and a Transmission Electron Microscope (TEM, JEM 1010, JEOL, Japan).

2.5. Anticoagulant activity of heparin–PTX conjugates

To obtain mixtures of heparin–PTX conjugates–ATIII complexes, heparin–PTX conjugates (100 μL) at 100 μg/mL were mixed with 100 μL of anti-thrombin III (ATIII) solution (1 IU/mL). The mixture solution was incubated at 37 °C for 5 min, after which FXa (100 μL) was added to the mixture solution which was incubated at 37 °C for 1 min. The substrate (200 μL) was then added, followed by incubation at 37 °C for 3 min. The reaction was terminated by the addition of 300 μL of 20% acetic acid. The anticoagulant activity of heparin–PTX conjugates were calculated from the absorbance at 405 nm using UV–Vis spectrometry.

2.6. Confocal microscopy study

To visualize the cellular uptake and intracellular distribution of FITC-labeled heparin–PTX conjugates, confocal laser scanning microscopy (Zeiss LSM510, Germany) was performed on KB cells grown on a Lab-Tek® II chamber slide (Nalge Nunc, Napevillem,

Table 1
Coupling ratio and bioactivity of heparin–PTX conjugates.

	Heparin–PTXI	Heparin–PTXII	Heparin–PTXIII
Heparin (mol)	1	1	1
Feed mole ratio of PTX (mol)	1	2.6	5
Coupling ratio of PTX (mol)	0.8	1.7	4.1
Anticoagulant activity (IU/mg)	107.9	104.8	95.2

Anticoagulant activity of heparin is 114.1 IU/mg.

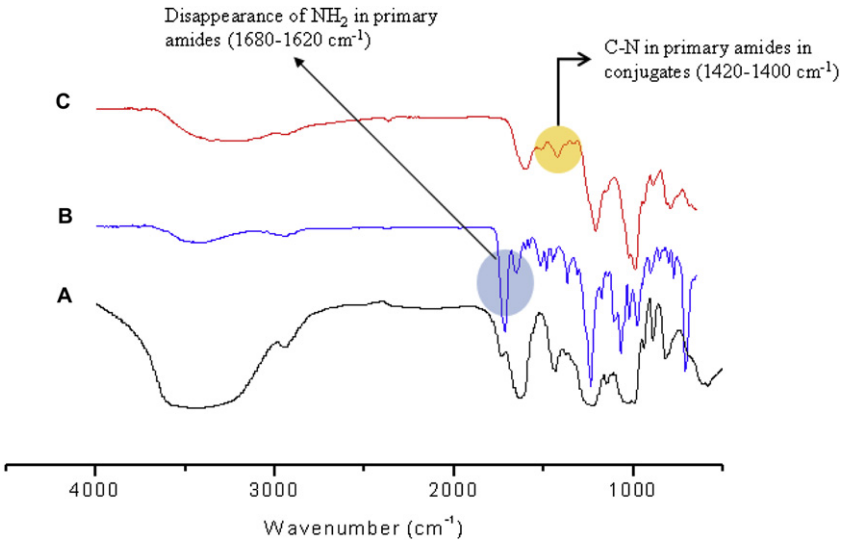

Fig. 3. FT-IR spectra of heparin (A), PTX (B) and heparin–PTX (C).

IL). FITC (64 mg) was conjugated with heparin or heparin–PTX (200 mg) in sodium bicarbonate buffer (pH 8.5) for 6 h at 25 °C. Heparin–FITC or heparin–PTX–FITC were precipitated by ethanol and then dialyzed against water for 4 days. The cells were incubated with 1 μg/mL FITC-labeled heparin or 1 μg/mL FITC-labeled heparin–PTX conjugates in RPMI-1640 medium for 3 h at 37 °C and 5% CO_2 atmosphere. The cells were then washed three times with PBS buffer (pH 7.4) and finally 200 μL of 4% formaldehyde in phosphate buffer saline was added. Cell images were taken under low power magnification (10× lens) with conventional fluorescence microscopy and a FITC filter. The FITC-labeled areas were then scanned with a confocal microscope (excitation/emission wavelengths: 488 nm and 510 nm) at higher magnification (63× lens).

2.7. Cytotoxicity of heparin–PTX conjugates

KB cells (human nasopharyngeal epidermoid cancer cells) were used to observe the cytotoxicity of PTX or heparin–PTX. The KB cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in FA-deficient medium RPMI-1640 containing 10% fetal calf serum. The cells grown as a monolayer were harvested by 0.25% trypsin–0.03% EDTA solution. The cells were seeded at 100 000 cells/well in a 96-well plate in RPMI-1640 and preincubated at 37 °C for 24 h before the assay. MTT assay was performed on KB cells by incubation at 37 °C for 1 day of different concentrations of each compound in quadruplicate. Control was incubated at 37 °C for 1 day without any drug. This assay is based on the reduction of yellow tetrazolium component (MTT) to insoluble purple-colored formazan produced by the mitochondria of viable cells. After 48-h of incubation, 100 μL of medium containing 20 μL of MTT solution was added to each well and the plate was incubated for an additional 4 h, followed by the addition of 100 μL of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol, Sigma, Milwaukee, WI) to each well. The solution was gently mixed to dissolve the MTT formazan crystals. The absorbance of each well was read with a microplate reader at a wavelength of 570 nm. The background absorbance of the wells at 690 nm was measured and subtracted from the 570 nm measurement. The results are expressed as % cell viability, and were obtained by dividing the optical density values (OD) of the treated groups (T) by the OD of the controls (C) ([T/C × 100%]). Statistical analysis was done using ANOVA. $p < 0.01$ was accepted as statistically significant. Error bars represent standard error of mean (SEM).

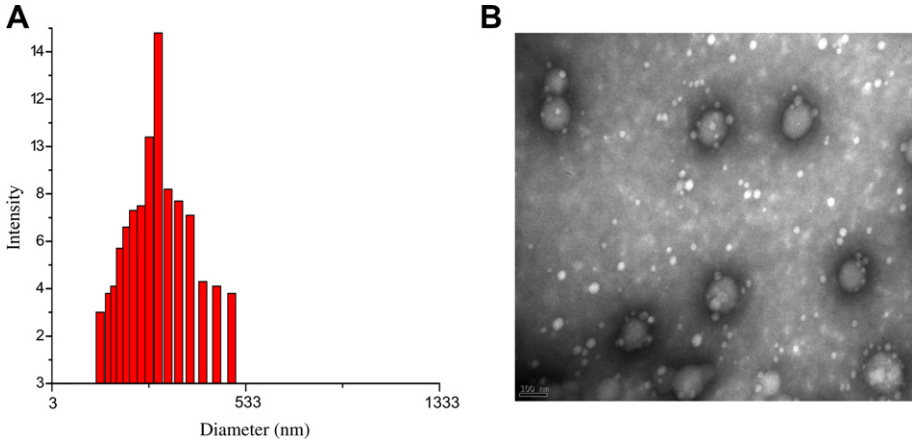

Fig. 4. Size distribution (A) and shape (B) of heparin–PTXII conjugates using ELS and TEM, respectively. Samples were measured at a concentration of 10 wt% in water.

3. Results and discussion

3.1. Synthesis and characterization of heparin–PTX conjugates

We synthesized heparin–PTX nanoparticles for cancer targeting by conjugating the polysaccharide heparin with PTX. To introduce an amine group to PTX, ethylenediamine (EDA) was covalently linked to the hydroxyl group of PTX in the presence of EDC and NHS as shown in Fig. 1. The presence of an amine group on the PTX structure was confirmed by characteristic peaks at 2.0 ppm in the NMR spectrum of NH_2–PTX. However, the specific conjugation site in PTX could not be identified. Cavallaro et al. also reported that the succinylation of PTX takes place preferentially at the 2′-OH position under mild conditions [20]. Thus, the amidation of PTX might preferentially occur at the 2′-hydroxyl group over the 7-hydroxyl group due to less steric hindrance [21]. The amide linkage between heparin and aminated PTX was confirmed by the presence of signals at δ 1.5–3 ppm. The presence of C–N in primary amide conjugates was further confirmed by a specific band at 1420–1400 cm^{-1} in the FT-IR spectra, indicating the conjugation between heparin and PTX (Fig. 3). The weak intensity of C=C stretching of PTX at 710 cm^{-1} in the heparin–PTX spectrum might result from the interference of the stretching of functional groups in heparin. The coupling ratio of PTX to heparin was determined by UV-spectroscopy at 228 nm using 1% (w/w) heparin–PTX conjugate. As shown in Table 1, the amount of PTX conjugated to heparin was increased (from 0.8 to 4.1 mol) by increasing the feed molar ratio of PTX to heparin (from 1 to 5). There are 10 COOH groups in the heparin molecule (molecular weight: 5000 Da). Thus, the modification of 5 COOH groups by PTX may produce water-soluble heparin–PTX (Fig. 2).

Anticoagulant activities of heparin–PTX conjugates were reduced as the amount of PTX in heparin–PTX increased as shown in Table 1. Anticoagulant activity of heparin and heparin–PTXIII conjugate measured by chromogenic assay were 114.1 and 95.2 IU/mg, respectively. This might be attributed to the introduction of PTX, leading to a conformational change in heparin structure that decreases the affinity to ATIII and affects the anticoagulant mechanism [22]. Although some clinical trials have indicated beneficial effects of heparin in cancer patients, its application is still limited due to high anticoagulant activity leading to hemorrhagic complications. It was reported that modification of the carboxyl group of heparin by chemical conjugation diminishes its affinity to factor Xa and/or anti-thrombin and thus its anticoagulant activity. However, PTX–heparin conjugates maintain the sulfate groups of heparin, which bind endothelial cells or block FGF-heparan sulfate binding, and may therefore decrease FGF activity. Thus, the anti-adhesion, inhibition of angiogenesis, metastasis and tumor growth may be still conserved [23–25]. Conjugated heparin holds several advantages as an anticancer drug carrier: i) More heparin–PTX conjugates can access cancer cells with higher water-solubility than can free PTX ii) Conjugation of an anticancer drug to heparin reduces the number of negative charges of heparin, thereby decreasing side effects such as thrombocytopenia (HIT) or bleeding that arise from the charge and size of heparin [26]. iii) Through the intact sulfate group, PTX conjugated heparin retains its ability to bind with

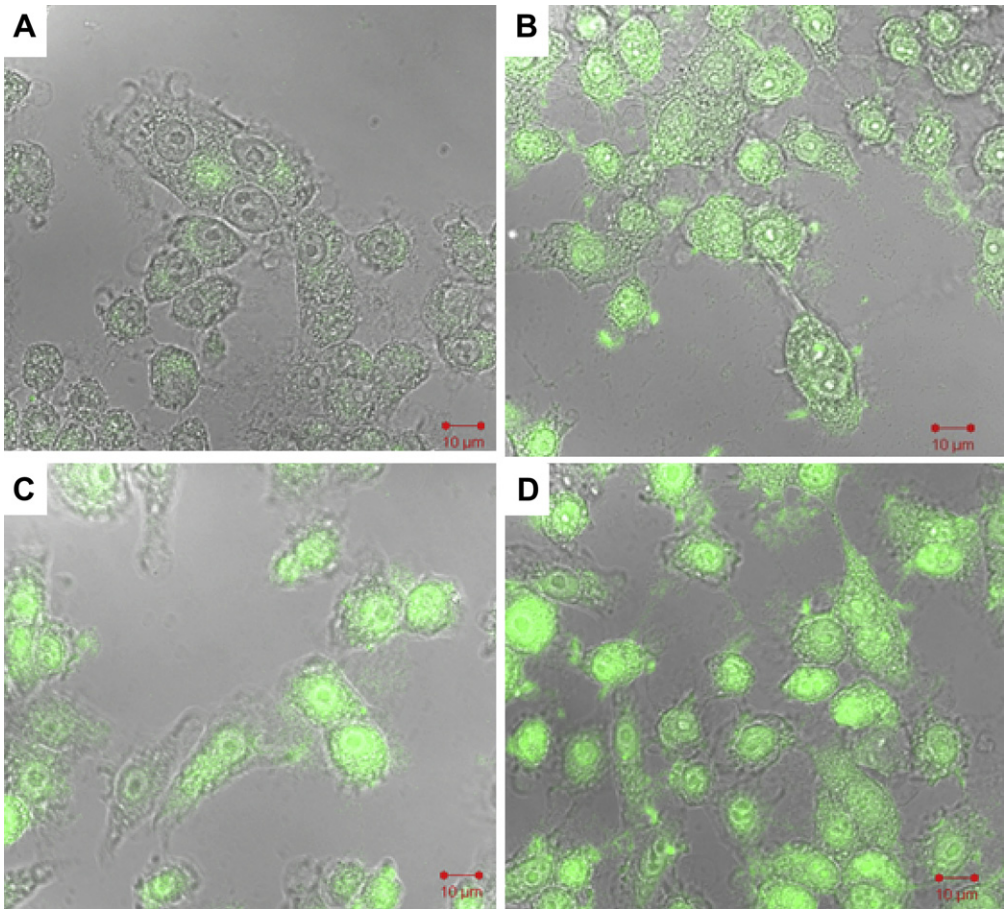

Fig. 5. Confocal microscopy image on KB cell lines after treating with (A) Heparin–FITC, (B) Heparin–PTXI–FITC, (C) Heparin–PTXII–FITC, (D) Heparin–PTXIII–FITC conjugates.

angiogenic factors as well as anti-heparanase activity [27]. Consequently, heparin–PTXIII conjugate with reduced anticoagulant activity could be a safe and effective prodrug that warrants further investigation.

3.2. Size and morphology of heparin–PTX nanoparticles

We found that heparin–PTX conjugates are soluble in water, indicating that the water-solubility of PTX was significantly enhanced through conjugation with water-soluble heparin. Importantly, heparin–PTX conjugates readily self-assembled in aqueous solution to form nanoparticles due to hydrophobic interactions between PTX molecules conjugated to the heparin backbone. Fig. 4 shows the size distribution and morphology of heparin–PTX self-assembled nanoparticles. The mean diameter of heparin–PTX ranged from 200 to 400 nm and decreased with increasing PTX coupling ratio due to the formation of more compact hydrophobic inner core (Fig. 4A). TEM micrograph of heparin–PTX nanoparticles shows uniform spherical shapes with diameters of 200 nm, which is smaller than the size measured by DLS (Fig. 4B). This was mainly due to the process involved in the preparation of samples. In the case of the TEM method, TEM images depicts the size at the dried state of the sample, whereas DLS method involves the measurement of size in the hydrated state. In other words, the size determined by TEM is an actual diameter (dry state) of the nanoparticles, whereas the size measured by the laser light scattering method is a hydrodynamic diameter (hydrated state), and therefore the nanoparticles will have a larger hydrodynamic volume due to solvent effect in the hydrated state; hence, the size measured by DLS method was larger than the size observed by TEM method. Heparin–PTX conjugate nanoparticles likely have a core/shell structure composed of a hydrophobic inner core containing aggregated PTX molecules and a hydrophilic heparin shell layer. Thus, heparin–PTX nanoparticles (200–400 nm in diameter) are considered to be suitable for passive delivery of PTX to targeted tumors due to the Enhanced Permeability Retention (EPR) effect [28,29].

3.3. Fluorescence images of heparin–FITC, Heparin–PTX–FITC conjugates

To evaluate the extent of cellular uptake of heparin–PTX nanoparticles, FITC was conjugated with heparin and heparin–PTX conjugate and confocal laser microscopy was used. Fig. 5 shows KB cancer cells viewed by confocal laser scanning microscopy after 3 h incubation with heparin–FITC, heparin–PTXI–FITC, heparin–PTXII–FITC, and heparin–PTXIII–FITC at 1 μg/mL FITC. We observed that heparin–PTX–FITC was highly internalized. The green fluorescence coming from the nanoparticles was detected within the cell cytoplasm, whereas weak fluorescence intensity was observed in KB cells treated with heparin–FITC. Confocal microscopy clearly indicates that heparin–PTX underwent greater cellular uptake than heparin, whereas heparin–FITC was hardly taken-up by the cells. Their different physicochemical properties offer different cellular uptake capacity. The increase in cellular uptake of heparin–PTX might also result from the possibility that the nanoparticles interact with the cell membrane to a greater extent and thereby experience endocytosis. On the other hands, it is difficult for heparin to interact with the cell membrane because of its highly negative charge and large size [30]. Cellular uptake is an important factor in any nanoparticle targeting system since circulating nanoparticles can diffuse into tumor tissue through the leaky vessel structure without being uptaken and therefore can easily diffuse back into the main bloodstream without accumulating. Thus, the result suggests that more efficient cellular internalization and retention of PTX and heparin in tumor tissue might occur *in vivo* via formation of the water-soluble conjugate.

3.4. Cell viability of heparin–PTX conjugates

To compare the cytotoxicities between free PTX and heparin–PTX nanoparticles, we performed MTT assay using the KB cell line. Fig. 6 showed the cell viability of KB cells treated with different drug and conjugate concentrations after 24 h and 48 h. After 24 h of incubation, cell viability was decreased from ~80%–~50% when the concentration of heparin–PTX nanoparticles was increased from 0.01% to 1% (w/w). At the same concentration, cell viability was slightly diminished when the coupling ratio of PTX to heparin was increased. Decreasing cell viability is in the following order: heparin–PTXI > heparin – PTXII > heparin–PTXIII (Fig. 6A). Treatment for 48 h decreased cell viability to 25% at 1% (w/w) and 50% at 0.01% (w/w) of heparin–PTXIII (Fig. 6B). Heparin–PTXIII showed similar cytotoxicity to free PTX at the same concentrations despite lower PTX content. Thus, heparin–PTX nanoparticles are more potent for killing cancer cells than free PTX, suggesting that heparin–PTX can provide more efficient and less toxic effects for treating cancer. PTX is supposed to be transported into cells by passive diffusion, but heparin–PTX nanoparticles are likely to be internalized within cells via endocytosis. The higher toxicity of heparin–PTX conjugate nanoparticles might be attributed to its greater uptake and accumulation in cancer cells compared to free

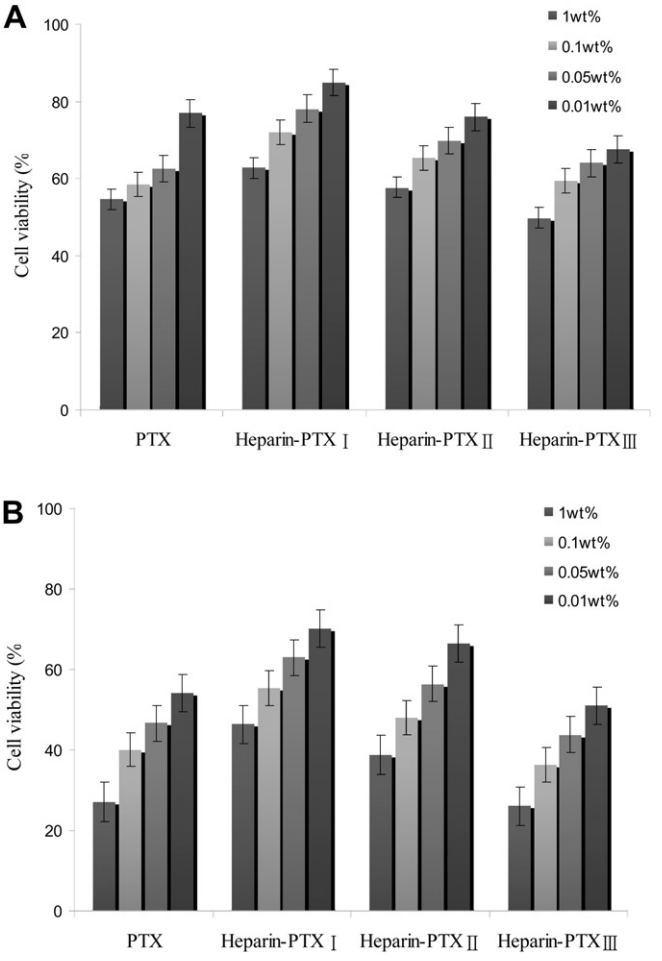

Fig. 6. *In vitro* cytotoxicity of free PTX and heparin–PTX nanoparticles against KB cancer cells after incubation for 24 h (A) and 48 h (B).

PTX. Heparin is highly hydrophilic due to negatively charged groups such as sulfonyl, carboxyl and hydroxyl groups within its structure [23,31]. Therefore, to treat a tumor with heparin, new structure designs that achieve the same therapeutic response with a reduced dose in order to decrease systemic toxicity and side reactions are needed. In the previous study, we found that heparin conjugated with a hydrophobic moiety retained its ability to inhibit binding with angiogenic factor, showing a significant decrease in endothelial tubular formation [16,32]. Furthermore, heparin–PTX conjugates showed improved water-solubility. PTX is a microtubule-stabilizing agent that promotes polymerization of tubulin, causing cell death by disrupting the dynamics necessary for cell division. It is currently used for treatment of ovarian, metastatic breast, lung, head, and neck cancer [33]. Thus, nanosized self-assembled heparin–PTX conjugate nanoparticles could be an efficient all-in-one carrier not only for the solubilization of PTX, but also for its anti-angiogenesis and passive targeted delivery to tumor tissues.

4. Conclusion

Paclitaxel was aminated to conjugate with heparin via an amide linkage. The conjugation of PTX with heparin significantly increased the water-solubility of PTX. A higher coupling ratio of PTX to the heparin backbone could be obtained by increasing the PTX feed ratio. Heparin–PTX conjugates readily self-assembled to form spherical nanoparticles (200 nm) in aqueous solution. Heparin–PTX conjugate nanoparticles demonstrated greater cytotoxicity to KB cancer cells than did free PTX. Furthermore, the enhanced cellular uptake and reduced anticoagulant activity of heparin were achieved by conjugation with PTX. This simple synthetic strategy with increased drug solubility and enhanced cell cytotoxicity suggests that heparin–PTX conjugates could be an efficient system for cancer treatment. Further studies in animals should be carried out to confirm the anti-tumor effect of heparin–PTX *in vivo*.

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