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In vitro and in vivo anti-tumor activities of nanoparticles based on doxorubicin–PLGA conjugates

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Abstract

Doxorubicin was chemically conjugated to a terminal end group of poly(D,L-lactic-co-glycolic acid) [PLGA] by an ester linkage and the doxorubicin–PLGA conjugate was formulated into nanoparticles. A carboxylic acid end group of PLGA was conjugated to a primary hydroxyl group of doxorubicin. The primary amine group of doxorubicin was protected during the conjugation process and then deprotected. The nanoparticles containing the conjugate exhibited sustained doxorubicin release profiles over a 1-month period, whereas those containing unconjugated free doxorubicin showed a rapid doxorubicin release within 5 days. Doxorubicin release patterns could be controlled by conjugating doxorubicin to PLGA having different molecular weights. The conjugated doxorubicin nanoparticles showed increased uptake within a HepG2 cell line, which was quantitated by a flow cytometry and visualized by confocal microscopy. The nanoparticles exhibited slightly lower IC_{50} value against the HepG2 cell line compared to that of free doxorubicin. In vivo anti-tumor activity assay also showed that a single injection of the nanoparticles had comparable activity to that of free doxorubicin administered by daily injection. The conjugation approach of doxorubicin to PLGA was potentially useful for the formulation of nanoparticles that requires targeting for cancer cells as well as sustained release at the site.

Keywords: PLGA; Doxorubicin; Conjugation; Sustained release; Anti-cancer therapy

1. Introduction

A conjugation approach of drugs to natural and synthetic polymers has been widely used for the purposes of long circulating in blood stream, targeting to a specific organ, and sustained release at the injection site [1–3]. Doxorubicin is one of the most widely used anti-cancer drugs that exert its cytotoxic activity by inhibiting the synthesis of nucleic acids within cancer cells [4,5]. In spite of its potent anti-cancer activity, nonspecific action of doxorubicin causes serious side effects to the patients, which has been a major problem to solve. It was proposed that doxorubicin conjugation to hydrophilic synthetic polymers resulted in an increased cytotoxic effect relative to free doxorubicin therapy [3,4]. This is caused by the 'enhanced permeation and retention (EPR)' effect, which plays a critical role in accumulating polymer–drug conjugates in solid tumors, while minimizing a lymphatic drainage to the surrounding tissue [2,5–8]. While endothelial cells in blood capillaries existing in normal tissue are

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tightly packed, those in tumor tissue are loosely packed for the greater uptake of nutrients and oxygen. The different cell junction structures between normal and tumor capillaries permit the polymer–drug conjugates to be targeted passively to the tumor site [9,10]. Additionally, the development of multi-drug resistance of cancer cells, which results from the expression of p-glycoprotein pump in the cell membrane, hampers the drug action by pumping out drug molecules from cytosol to extracellular area [2–4]. Thus, the polymer conjugation approach, along with formulations with polymeric micelles and liposomes, is an attractive means to selectively suppress the tumor growth [6,11–13]. However, most of the high molecular weight synthetic polymers used in the drug conjugation are non-degradable and they tend to eventually accumulate in the body because they are macromolecules whose molecular weight is larger than the cutoff value (~5000) of glomerular filtration capacity in the kidney [14–17]. Thus, it is desirable to use biodegradable polymers for the drug conjugation. In the case of using biodegradable polymers such as a family of poly(lactic acid-co-glycolic acid) [PLGA], it was shown that drug–PLGA conjugates could be achieved by coupling of a drug to the terminal end group of PLGA due to the lack of reactive functional groups in the polymer backbone [7]. These conjugates could be easily processed into nanoparticles having up to the size of 200 nm in diameter. In our previous papers, it was reported that various drug molecules could be conjugated to the terminal end group of poly(D,L-lactic-co-glycolic acid) [PLGA] and they could be formulated into microspheres or nanoparticles [7,18]. They exhibited high drug loading efficiency and showed a near zero-order release profile without showing any burst effect. The drug–PLGA conjugation approach has several advantages. Due to the biodegradable nature of PLGA, the nanoparticles can be degraded into glycolic acid and lactic acid that are non-toxic to the human body. Besides, changing the co-monomer ratio in PLGA to be conjugated permits control of the release rate of drug from nanoparticles. Furthermore, the formulation of PLGA nanoparticles with doxorubicin–PLGA conjugate reveals that the released doxorubicin fraction from nanoparticles had comparable cytotoxic activity compared to free doxorubicin. However, the conjugation was accomplished by a non-cleavable carbamate linkage, which cannot be easily broken under physiological conditions.

In this study, doxorubicin was conjugated to PLGA by an ester linkage that is expected to be more readily cleavable under the physiological conditions. The nanoparticles containing doxorubicin–PLGA conjugates were characterized in terms of size, zeta potential, and drug loading. In vitro sustained release profiles of doxorubicin from nanoparticles were examined by conjugating two PLGA samples of different molecular weights. In vitro anti-cancer activity of doxorubicin nanoparticles was determined using a HepG2 cell line. Uptake of nanoparticles into HepG2 cells was quantitated by a flow cytometry method and visualized by using confocal microscopy. Lastly, in vivo anti-tumor activity was assessed by a single subcutaneous injection of the nanoparticle formulation into an animal tumor model to see the sustained release effect, which was compared to daily injection of doxorubicin.

2. Materials and methods

2.1. Materials

Two samples of poly(D,L-lactic-co-glycolic acid), having a lactic/glycolic molar ratio of 50/50 with different nominal molecular weights of 5000 and 10 000, were obtained from Wako Chemicals (Japan). [PLGA5005 and PLGA5010]. They have a free hydroxyl and a carboxylic group at their terminal ends. Doxorubicin, 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc–Osu), *N*,*N*-diisopropylethylamine and 3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. Human hepatoblastoma cell line (HepG2) was obtained from Korean Cell Line Bank (KCLB). All other chemicals were of analytical grade.

2.2. Preparation of doxorubicin–PLGA conjugate by an ester linkage

Fifty mg of doxorubicin was dissolved in 2 ml of *N*,*N*-dimethylformamide (DMF) and 30 mg of

Fmoc–Osu was added, followed by 30 μl of *N,N*-diisopropylethylamine. After 3 h, the solvent was evaporated under vacuum, and the residue was crystallized by triturating from 0.1% aqueous trifluoroacetic acid (TFA) (v/v). The crystals were collected by filtration and washed once with cold diethyl ether to remove the trace of excess Fmoc–Osu. After freeze drying, 55 mg of 98% pure *N*-Fmoc–DOX was obtained (84% yield). PLGA (0.18 g) and Fmoc–DOX (20 mg) were dissolved in 10 ml of methylene chloride, followed by the addition of 15.6 mg of bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP), 20 μl of triethylamine (TEA), and 4.5 mg of dimethylaminopyridine (DMAP) under nitrogen. After 15 h, the reaction mixture was diluted with methylene chloride (20 ml). The organic layer was washed with 5% aqueous HCl solution and brine twice, and then concentrated under vacuum. The conjugation process was monitored by GPC with a UV–vis detector (480 nm for doxorubicin, 267 nm for Fmoc, 230 nm for PLGA). Fmoc–DOX–PLGA conjugate was dissolved in 3 ml of DMF and 300 μl of piperidine was added. After 5 min, the reaction mixture was placed in an ice bath and acidified by the addition of a mixture containing a 300 μl of TFA, 700 μl of pyridine, and 2 ml of DMF. The solvent was evaporated under vacuum. The residue was solidified by triturating by the addition of diethyl ether in DMF. The obtained solid was dissolved in methylene chloride, washed with 5% HCl aqueous solution and brine, and concentrated under vacuum, and was further purified by precipitation in diethyl ether and then dried.

2.3. Preparation and characterization of nanoparticles

Two types of nanoparticles containing doxorubicin–PLGA conjugate and free doxorubicin were prepared by a spontaneous emulsion solvent diffusion method [7]. One hundred mg of doxorubicin–PLGA conjugate dissolved in 10 ml of acetone, was slowly added to 100 ml of deionized water containing 0.1% (w/v) Pluronic F-127 under vigorous stirring. For the encapsulation of free doxorubicin into nanoparticles, 98 mg of PLGA and 2 mg of doxorubicin were co-dissolved in acetone and then used. The nanoparticles formed in the aqueous solution were collected by ultracentrifugation (Beckman, USA) at 15 000 rpm for 1 h and re-suspended in phosphate buffered saline (PBS) solution. The resuspended nanoparticles were stored under frozen condition (−20°C) until use. The loading amount of doxorubicin within nanoparticles was determined by a spectroscopic method. A known amount of freeze-dried doxorubicin encapsulated nanoparticles was completely dissolved in dimethylsulfoxide (DMSO) and then the absorbance was measured at 480 nm according to the aforementioned method. Encapsulation efficiency was calculated based on the percentage ratio of the amount of doxorubicin incorporated into nanoparticles to the initial amount used. Size distribution was measured by using a laser light scattering technique (ZetaPlus, Brookhaven Instrument Corp., USA).

2.4. Release experiment

Twenty mg of nanoparticles, suspended in 20 ml of PBS buffer, was sealed in a dialysis bag (M.W. cutoff: 10 000, Spectra/Por). The dialysis bag was incubated in 30 ml of PBS buffer at 37°C. The released doxorubicin in the incubation medium was collected at pre-determined time intervals and stored frozen for quantitative analysis. The release amount was analyzed at 480 nm. The released product was analyzed by reversed phase HPLC using a C_{18} column.

2.5. Cytotoxicity assay

Cytotoxicities of free doxorubicin and doxorubicin nanoparticles were determined by measuring the inhibition of cell growth using a tetrazolium dye (MTT) assay according to the previously established method [21]. Dulbecco's Modified Eagle's Medium (DMEM) was used as a major cell growth medium and a humidified atmosphere (5% CO_2) was maintained for cell culture. HepG2 cells harvested in a logarithmic growth phase were seeded on 96 wells at a cellular density of 5×10^3 cells/ml. After incubating the cells in a logarithmic phase with various concentrations of free doxorubicin and nanoparticles for 48 h at the end of experiment, the MTT assay was performed and the percentage of cell viability was then determined.

2.6. Determination of DOX–PLGA nanoparticles transported within HepG2 cells.

HepG2 cell line was used as a model cancer cell for the examination of endocytosis. HepG2 cells, at a density of 5×10^3 cells/ml, were seeded onto a 100-mm culture dish or a cover glass pre-soaked in culture medium and after 24 h, DOX–PLGA NP and free doxorubicin were added to the culture medium, both of which had the same doxorubicin concentration of 170 μM. The loading amount of doxorubicin in DOX–PLGA NP was taken into account to adjust the doxorubicin concentration. After 6 h, the cells on the cover glass were washed three times with PBS and examined by confocal microscopy (Carl Zeiss LSM5100, Germany) at an excitation wavelength of 488 nm and an emitting signal was detected at 515 nm. In order to quantify the amount of doxorubicin within the cell, the cells were harvested and analyzed by flow cytometry (FACSCalibur, USA), and the gate was arbitrarily set at for the detection of green fluorescence (FL1-H>200, 535 nm, linear scale). The relative fluorescence intensity of HepG2 cells incubated with DOX–PLGA NPs was calculated using those incubated with free doxorubicin as a control, 100%.

2.7. In vivo anti-tumor activity assay

Anti-tumor activities against subcutaneoulsy implanted solid tumor induced by mouse EL4 thymoma cells in mice were evaluated for free doxorubicin, blank PLGA nanoparticles, nanoparticles containing free doxorubicin, and nanoparticles containing doxorubicin conjugates. EL4 cells (2×10^4 cells in 0.1 ml of PBS (pH 7.4)) were transplanted into C57BL16 female mice (10 weeks old) subcutaneously at day 0 and the peri-tumoral drug injection started at day 10, when the tumor diameter reached approximately 2 mm [22]. The mice were divided into five different groups, consisting of six mice in each group. The first group, as a control, was daily injected with 100 μl of PBS (pH 7.4) over the following 12 days, and the second group of free doxorubicin (240 μg/kg body weight) was injected in the same way. The rest of mice groups was peri-tumoraly injected on the first two days (days 10 and 11) by blank PLGA nanoparticles (240 mg of PLGA/kg body weight) (third group), by nanoparticles containing doxorubicin conjugates (2.4 mg of equivalent doxorubicin/kg body weight) (fourth group), and by nanoparticles containing free doxorubicin (1.2 mg of equivalent doxorubicin/kg body weight) (fifth group). The tumor diameters were measured daily and used as an index for in vivo anti-tumor activity of released doxorubicin from nanoparticles.

3. Results and discussion

3.1. Conjugation of doxorubicin to PLGA

Doxorubicin has two major functional groups in its structure: a primary amine group in a sugar moiety and a primary hydroxyl group of $-C{=}OCH_2OH$ group in the aliphatic chain ring. Both of them can be utilized for the conjugation of PLGA. For the generation of cleavable conjugation linkage, the primary hydroxyl group was reacted with a terminal carboxylic acid of PLGA by using a pair of coupling agents, PyBroP/DMAP, to yield an ester bond between doxorubicin and PLGA. The primary amino group was protected with Fmoc and deprotected after the conjugation. The conjugation scheme is illustrated in Fig. 1. Gel permeation chromatography (GPC), with monitoring at 480 nm, was used to confirm the doxorubicin conjugation as previously reported [7].

3.2. Formulation and characterization of nanoparticles

PLGA nanoparticles were prepared by a spontaneous emulsion solvent diffusion method using the prepared doxorubicin–PLGA conjugate. The size of nanoparticles was around 200 nm in diameter with a narrow distribution, as determined by the dynamic light scattering method. Table 1 shows doxorubicin loading efficiency and loading amount in the formulated nanoparticles with and without conjugation. The nanoparticles containing the conjugates have far greater loading efficiency and amount compared to those encapsulated with free doxorubicin, which exhibited over 95% encapsulation efficiency. This is because the doxorubicin–PLGA conjugate had a limited water solubility, suppressing the tendency of

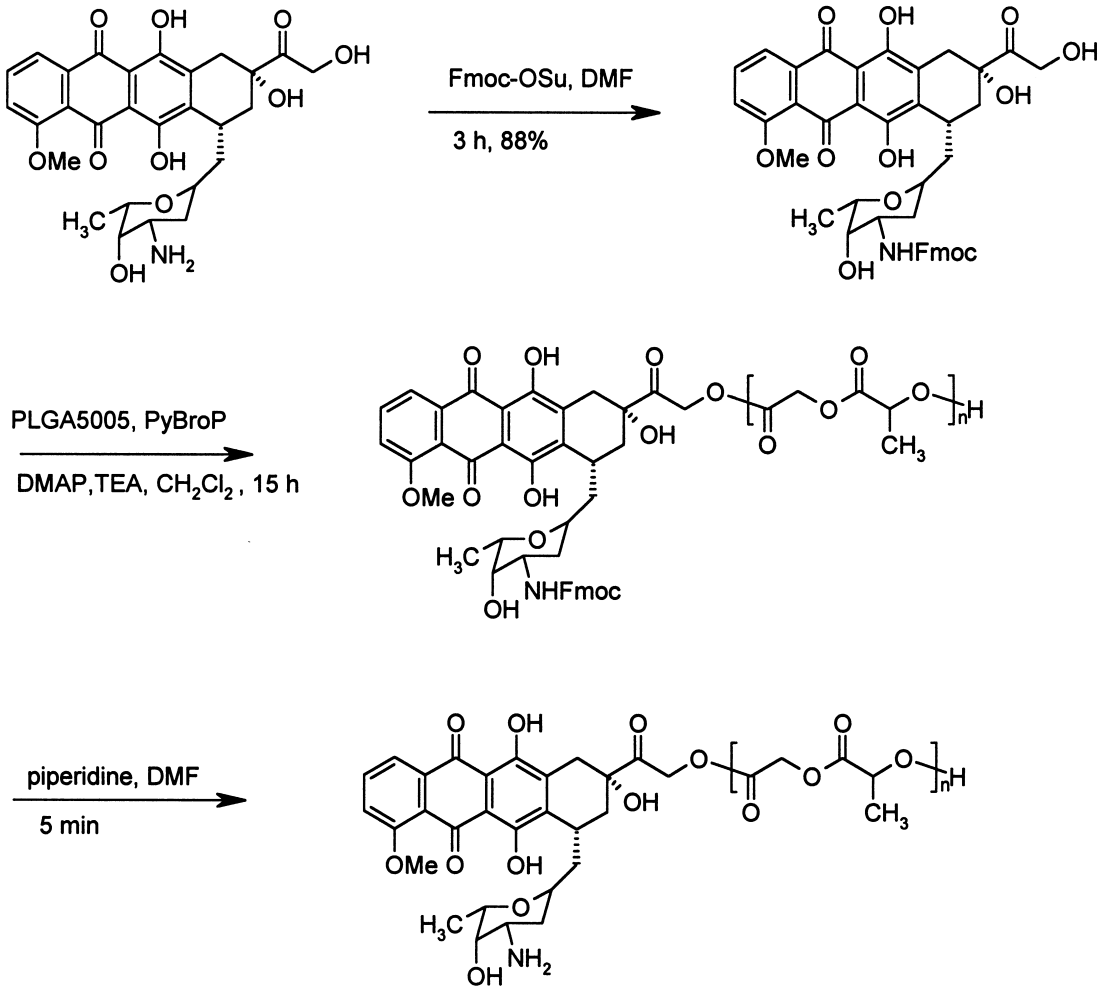

Fig. 1. Synthetic route of DOX–PLGA conjugate by an ester linkage.

the conjugate to escape out of nanoparticles into aqueous medium during the formulation process. On the other hand, the nanoparticles formulated with free doxorubicin exhibited lower loading efficiency and amount due to its greater aqueous solubility.

3.3. Release behavior of doxorubicin from doxorubicin nanoparticles

Fig. 2 shows the release profiles of doxorubicin from the nanoparticles. Nanoparticles containing free

Table 1
Characteristics of doxorubicin nanoparticles

	Loading efficiency (w/w, %)	Loading amount (w/w, %)
DOX–PLGA NP[a]	95.0±7.5	1.90±0.25
DOX/PLGA NP[b]	33.3±4.3	0.66±0.19

[a] DOX–PLGA NP: nanoparticles containing DOX–PGLA conjugate.
[b] DOX/PLGA NP: free DOX encapsulated particles.

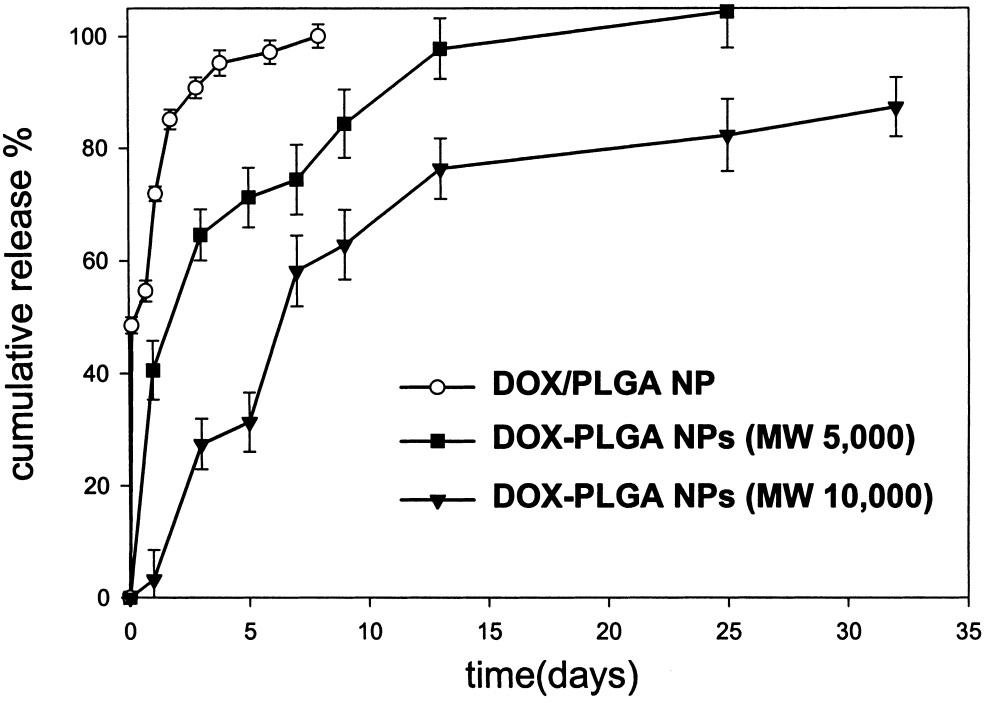

Fig. 2. Release profiles of DOX–PLGA nanoparticles.

doxorubicin show a large burst effect at day 1 and subsequent doxorubicin release duration for the next 5 days. This relatively short release duration was attributed to the fact that the drug release occurred by a simple diffusion process through the matrix of nanoparticles. On the other hand, doxorubicin release profiles from the nanoparticles containing the conjugate exhibits more sustained release patterns. To see the effect of PLGA molecular weight on the release profile, two different nominal molecular weights of PLGA 50/50, 5000 and 10 000, were conjugated to doxorubicin and the resultant conjugates were used for the preparation of nanoparticles. It can be seen that the nanoparticles formulated with higher M.W. PLGA exhibit a more sustained release pattern than those with lower M.W. PLGA. The higher M.W. PLGA degrades slower than the lower M.W. PLGA, which resulted in the slower water solubilization process of doxorubicin–PLGA oligomer conjugates [18]. Since the water solubilization process of hydrolyzed PLGA fragments takes place when the critical molecular weight of PLGA oligomers was approached around 1000, it is highly conceivable that the doxorubicin release profile follows the water solubilization process of cleaved PLGA fragments containing a terminal doxorubicin molecule [19,20]. These release results directly suggest that M.W. of PLGA to be conjugated is an additional critical variable in controlling the doxorubicin release rate besides PLGA co-monomer composition. It can also be found that the nanoparticles containing the conjugates do not show any significant initial burst effect in contrast to those formulated with free doxorubicin as previously reported [7].

3.4. Analysis of released fractions from doxorubicin nanoparticles

It should be mentioned that the release fraction as shown in Fig. 2, contained a mixture of water soluble doxorubicin–PLGA oligomer species that could be detected at 480 nm, a typical wavelength for monitoring doxorubicin regardless of whether doxorubicin was still conjugated or not. Reversed phase HPLC analysis of the released fraction, collected for a 7-day incubation period, is shown in Fig. 3. A major

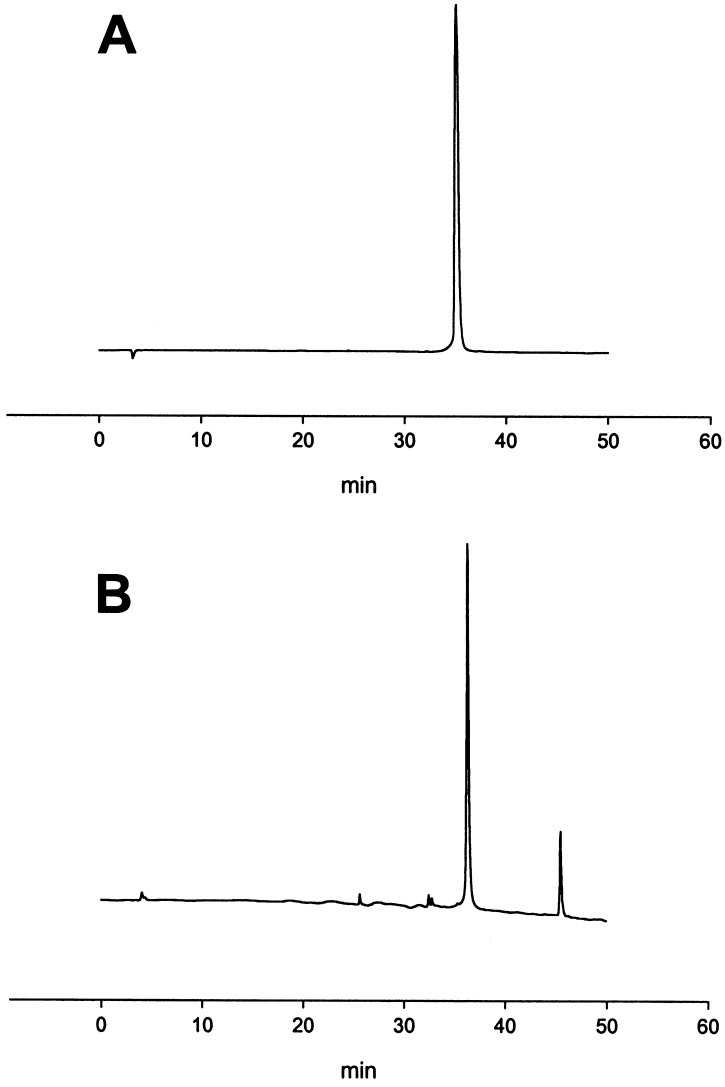

Fig. 3. Reversed phase HPLC chromatography of (A) free doxorubicin and (B) released fraction from nanoparticles for 7 days.

peak of the released fraction appears at 36 min with other small peaks, indicating that this is not an intact doxorubicin molecule eluting at 35 min, but PLGA oligomer conjugate species. The collected fraction of the major peak in HPLC was subjected to mass spectroscopy (data not shown). It was found that two major species appeared at 563.2 and 673.7 mass units. They are supposed to be a slightly modified doxorubicin and its PLGA oligomer conjugate, respectively. It was hard to define the chemical structure of these two species based on their molecular weights. It is highly probable that a majority of doxorubicin species were still conjugated to PLGA oligomers, possibly due to the inherently slow hydrolytic rate of the ester linkage, although a cleavable linkage was introduced between doxorubicin and PLGA.

3.5. Changes in size and zeta potential of nanoparticles

Fig. 4 shows time course changes in effective

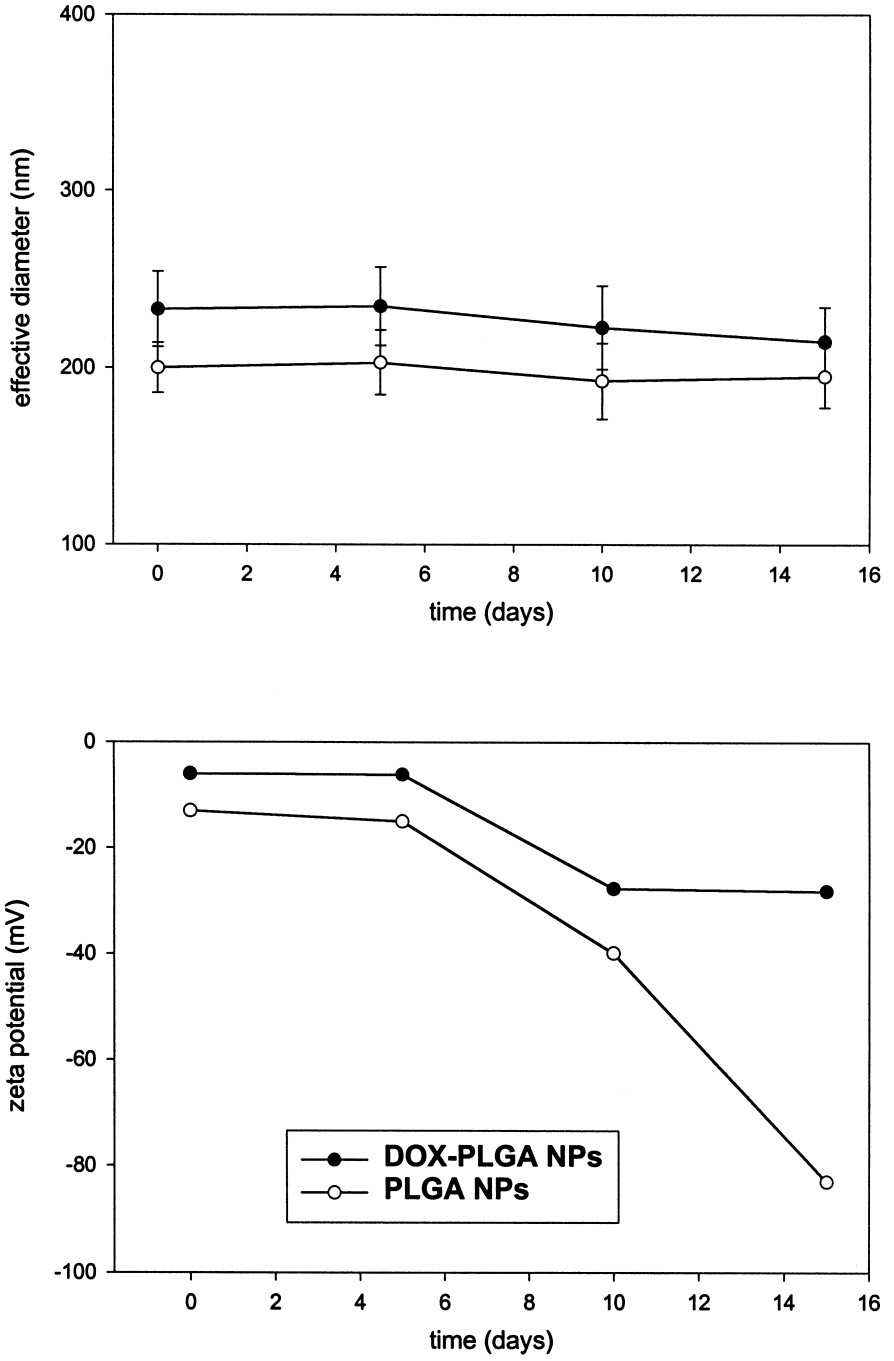

Fig. 4. Changes in size and zeta potential of doxorubicin nanoparticles.

diameter and zeta potential of nanoparticles containing the doxorubicin–PLGA conjugate that was prepared using PLGA with a nominal M.W. of 10 000. Freshly prepared nanoparticles containing the conjugate were around 230 nm in size and had a zeta potential of -7.5 mV . The slightly increased size and zeta potential values compared to that of blank PLGA nanoparticles were caused by doxorubicin encapsulation and the presence of a positively charged primary amine in doxorubicin structure, respectively. There were no significant changes in PLGA nanoparticle size throughout the incubation period regardless of doxorubicin conjugation to PLGA. This confirms the previous finding that PLGA degrades by a bulk erosion mechanism [20]. The zeta potential value of blank PLGA nanoparticles decreases with the incubation time, probably due to the cumulative generation of negatively charged terminal carboxylic acid group in PLGA fragments as a result of PLGA chain scission. On the other hand, the nanoparticles having the conjugate show higher zeta potential values during the incubation time, which was due to the ionic interaction of positively charged doxorubicin with the negatively charged PLGA fragments. GPC analysis revealed that the weight average molecular weight of PLGA, being initially 8300, reached around 1500 after 15 days of incubation.

3.6. In vitro uptake of doxorubicin nanoparticle

Uptake of nanoparticles into HepG2 cells in comparison to free doxorubicin was quantitatively analyzed by a flow cytometry method. Fig. 5 shows that the nanoparticles containing doxorubicin in the form of conjugate were internalized within cells to a greater extent relative to free doxorubicin. This can be attributed to the fact that nanoparticles were more readily internalized by an endocytosis mechanism, while free doxorubicin was transported into cells by a passive diffusion mechanism. The confocal microscopic picture as shown in Fig. 6, illustrates the distribution of internalized nanoparticles within cells. Hepatocytes, incubated with free doxorubicin, were faintly stained with inherent emitting fluorescence of internalized doxorubicin within cells, whereas those with nanoparticles were more strongly stained with clear visualization of internalized nanoparticles. The enhanced uptake of nanoparticles containing doxorubicin conjugates suggests that a multi-drug resistance problem, effluxing of doxorubicin out of cells by p-glycoprotein pump existing within cell membrane, might be circumvented by the nanoparticle approach presented in this study [23,24]. Fig. 7 exhibits cell viability results, which were measured after 48 h of incubation of cells treated with free doxorubicin and nanoparticles containing the conjugate. It appears that free doxorubicin has more potent activity than nanoparticles containing the doxorubicin conjugate, which was likely caused by a sustained release property of doxorubicin from nanoparticles after the internalization within cells. It was possible that although the greater cellular uptake of the nanoparticles within cells occurred, chemical hydrolytic chain scission of PLGA should proceed for the sustained release of water soluble doxorubicin–PLGA oligomer species into cell cytoplasmic area. Thus, 48 h of cell incubation time given for the viability assay, seemed an insufficient period for the release of doxorubicin from the nanoparticles located within cells.

3.7. In vivo anti-tumor activity of doxorubicin nanoparticle

To assess anti-tumor activity in vivo, nanoparticles containing doxorubicin conjugate were directly injected into surrounding tissues of subcutaneously implanted solid tumor in mice. As shown in Fig. 8, a single-shot of the nanoparticles shows similar in vivo anti-tumor activity to daily-injected free doxorubicin, whereas PBS and blank PLGA nanoparticles exhibit no measurable effect on tumor growth. As the size of tumor was suppressed over 12 days after a single shot injection of doxorubicin nanoparticles at the tumor site in contrast to daily-injected free doxorubicin, it was confirmed that a mixture of doxorubicin–PLGA oligomers released from the nanoparticles had significant anti-tumor activities. It remains to be seen whether intravenously-injected nanoparticles demonstrate a passive targeting anti-tumor activity against solid tumors by the EPR effect.

4. Conclusions

Doxorubicin conjugated biodegradable polymeric nanoparticles could be formulated with high loading

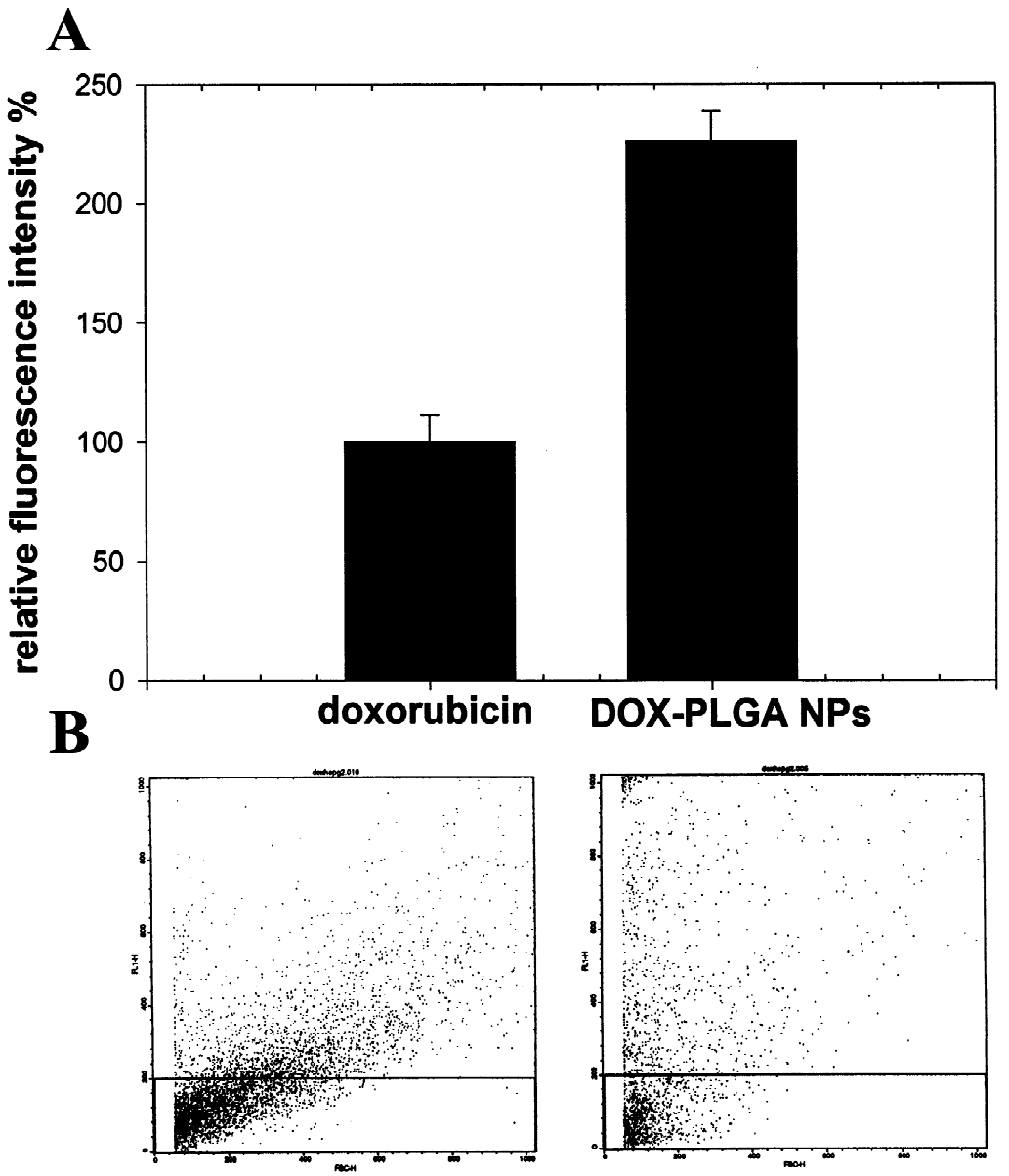

Fig. 5. (A) Relative fluorescence intensity of HepG2 cells incubated with doxorubicin conjugated nanoparticles and free doxorubicin as determined by a flow cytometry. (B) Dot plot of flow cytometry, of free doxorubicin, (left) and of nanoparticles, (right). Relative fluorescence intensity is the cellular fluorescence ratio of doxorubicin nanoparticles to that of doxorubicin. Cell count was 5×10^3.

efficiency and amount. The nanoparticles showed more sustained doxorubicin release behavior with increasing molecular weight of PLGA. Although a cleavable ester linkage was introduced between doxorubicin and PLGA, an intact doxorubicin was not regenerated after release from nanoparticles. The doxorubicin nanoparticles were taken up much more by HepG2 cells than free doxorubicin. The cytotoxicity of doxorubicin conjugated nanoparticles was slightly lower than that of free doxorubicin because the sustained release of doxorubicin could not be examined by MTT assay. In vivo anti-tumor activity

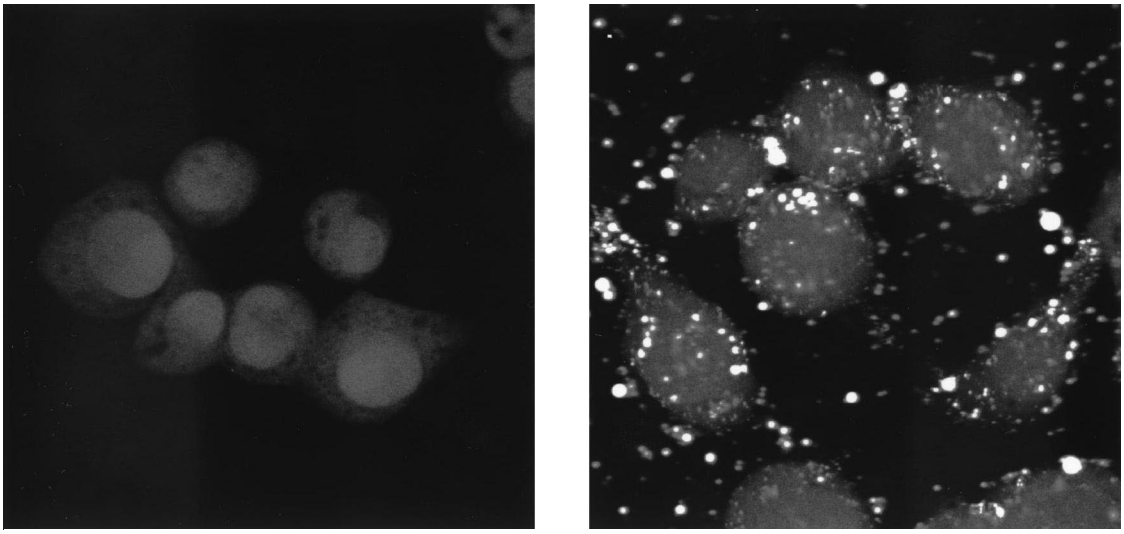

Fig. 6. Confocal microscopy of HepG2 cells containing doxorubicin nanoparticles. The left picture is HepG2 cells incubated with doxorubicin and the right is HepG2 cells incubated with nanoparticles.

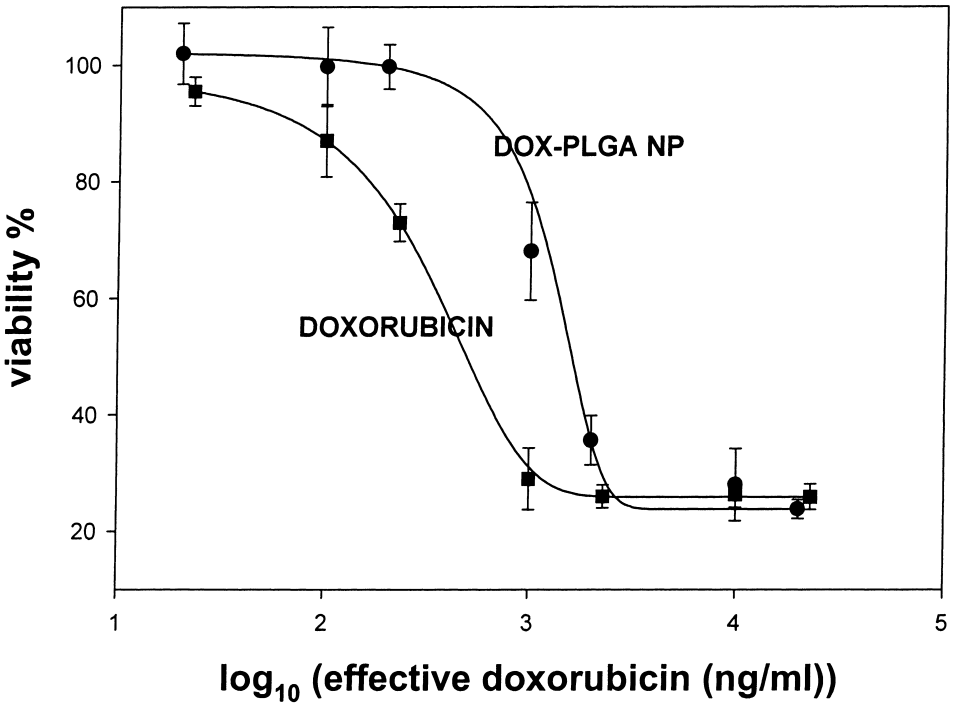

Fig. 7. In vitro cytotoxicity of doxorubicin nanoparticles against HepG2 cells.

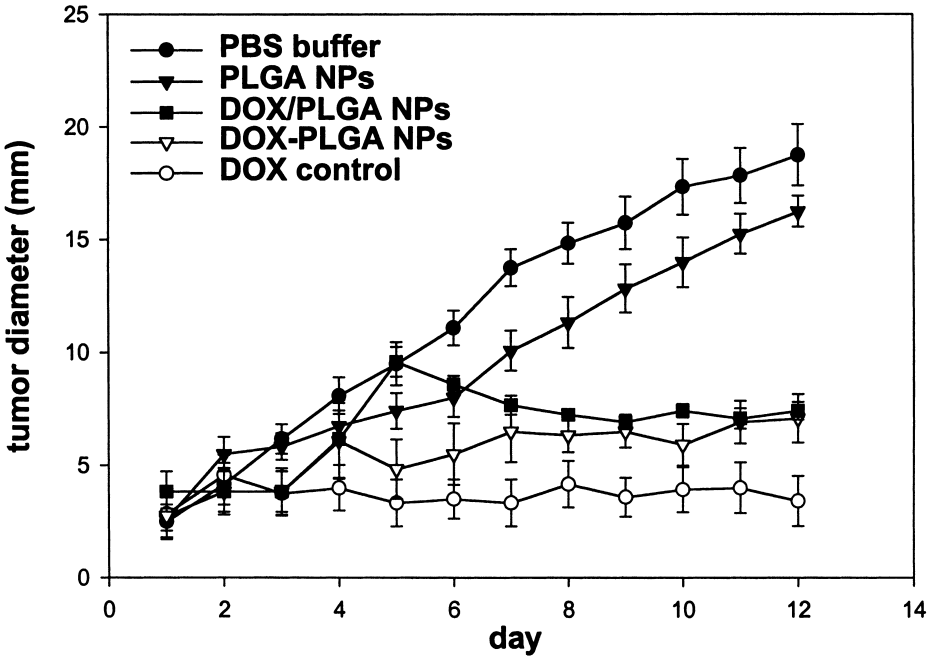

Fig. 8. In vivo anti-tumor activity assay. DOX–PLGA NP is doxorubicin-conjugated PLGA nanoparticles and DOX/PLGA NP is free doxorubicin encapsulated nanoparticles.

of the nanoparticles was almost the same as that of daily-injected free doxorubicin.

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