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Research paper

In vivo and *in vitro* anti-cancer activity of thermo-sensitive and photo-crosslinkable doxorubicin hydrogels composed of chitosan–doxorubicin conjugates

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ABSTRACT

Doxorubicin was chemically conjugated to acrylated chitosan in order to obtain sustained-release profiles of doxorubicin from thermo-responsive and photo-crosslinkable hydrogels. Chitooligosaccharide was acrylated with glycidyl methacrylate and subsequently conjugated to doxorubicin via an amide linkage. A mixture of doxorubicin–chitosan conjugates, acrylated Pluronic, and doxorubicin formed physical gels at 37 °C. Photo-irradiation was subsequently performed to chemically crosslink the physical hydrogel at 37 °C. Chitooligosaccharide–doxorubicin conjugates in the doxorubicin hydrogels significantly reduced burst release of free doxorubicin from doxorubicin hydrogels compared hydrogels without the conjugates. Upon incubating doxorubicin hydrogels at 37 °C, chitosan–doxorubicin conjugates were confirmed to be degraded into more hydrophilic oligomers by reversed-phase chromatography. *In vitro* cytotoxicity assay using released media from doxorubicin hydrogels showed that degraded chitosan–doxorubicin had cytotoxicity comparable to free doxorubicin. Athymic nude mice bearing human lung adenocarcinoma were subjected to intra-tumoral injections of physical hydrogels. After photo-crosslinking injected hydrogels using surgical catheters, tumor sizes, body weights, and survivals were measured for 1 month. Released media from doxorubicin hydrogels exerted similar cytotoxicities to free doxorubicin, and the tumor volume was significantly reduced for 1 month compared to other samples. Thus, doxorubicin hydrogels containing doxorubicin conjugates can be employed as a novel injectable anti-cancer drug aiming to achieve sustained release of doxorubicin for several weeks against solid tumors.

1. Introduction

Anti-cancer therapy of human solid tumors has mostly relied on extensive dissection of cancerous tissues followed by intensive chemotherapy with potent anti-cancer agents. Although the surgical methods are still promising and widely-accepted treatments against defined solid tumors, non- and less-invasive treatments against solid tumors have also received much attention with an aim to reduce and eliminate complications after surgical treatments. For this purpose, depot systems of bioactive molecules have received much attention because sustained release of anti-cancer agents could be easily accomplished with those depot systems [1–4]. Doxorubicin, one of the most employed anti-cancer agents, was loaded in various polymeric or natural hydrogels including poly(organophosphazene), human serum albumin (HSA)-tartaric acid derivatives (TAD), chitosan, and photo-crosslinkable chitosan [3,4]. Many researchers employed hydrophobic or ionic interactions between anti-cancer drugs and polymeric molecules in order to prepare thermo-sensitive gels entrapping anti-cancer drugs. Therefore, doxorubicin entrapped in physical gels was released out easily, thus showing initial burst-release profiles within 1–2 days. Burst release of anti-cancer agents was advantageous because it further increased local concentrations of anti-cancer drugs at the injection sites. However, this also increased the necessity of multiple injections in order to obtain a prolonged efficacy of anti-cancer drugs. Therefore, a release profile of anti-cancer drug should be optimized to control tumor growths over several weeks because tumor growth rates are very variable among affected individuals and dependent on types of tumors [5–7].

For decades, thermo-responsiveness of multi-block copolymers was widely studied including poly(ethylene oxide) [PEO]-poly(propylene oxide) [PPO]-poly(ethylene oxide) [PEO] (Pluronic) as injectable depot systems for clinical purposes. Above a low critical solution temperature (LCST), sol–gel transitions of those polymers occur between hydrophobized PPO blocks, thus forming physical gels without any chemical crosslinking. Therefore, many researchers employed Pluronic and Pluronic derivatives to prepare injectable depot system [8,9]. Chung et al grafted Pluronic to chitosan

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and showed feasibility as a novel biomaterial [8]. Dimitrios et al encapsulated doxorubicin in hydrogel nanoparticles composed of PEG and Pluronic. However, the loading efficiency of doxorubicin was relatively low (12–44%) and more than a half of the encapsulated doxorubicin was released out within 3 days [9]. This was attributed that doxorubicin was physically loaded, and hydrophobic interactions were the only force controlling the release rates.

In the current study, we prepared doxorubicin hydrogels composed of chitosan–doxorubicin conjugates and Pluronic for sustained release of doxorubicin. In order to prepare photo-crosslinkable hydrogels, chitosan and Pluronic were separately acrylated and subjected to physical gelation above LCST. Release profiles of doxorubicin from the photo-crosslinked hydrogels were investigated *in vitro*. The released fraction was analyzed by reversed-phase chromatography, and *in vitro* cytotoxicity was also evaluated. Prolonged anti-cancer effects of doxorubicin hydrogels were tested in athymic nude mice with human solid tumors.

2. Materials and methods

2.1. Materials

Pluronic® F127 [$(PPO)_{99}(PEO)_{69}(PPO)_{99}$] was donated from BASF (Germany). Chitooligosaccharide (molecular weight range: 1000–3000) was purchased from Kitto Life (Seoul, Republic of Korea). Acryloyl chloride, succinic anhydride, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aldrich (Milwaukee, WI). Irgacure2959 was gifted by Ciba Specialty Chemicals (Tarrytown, NY). 3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and doxorubicin hydrochloride were purchased from Sigma. Glycidyl methacrylate was purchased from Junsei Chemical (Japan). Human lung adenocarcinoma cell line A549 was purchased from Korea Cell Line Bank (Seoul, Republic of Korea). Athymic nude mice were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea).

2.2. Preparation of acrylated chitooligosaccharide–doxorubicin conjugates

Acrylated chitooligosaccharide–doxorubicin conjugates were prepared by conjugating doxorubicin to primary amine groups of acrylated chitooligosaccharide (COS). Acrylated COS was prepared by conjugating glycidyl methacrylate to COS as shown in the literature [10]. COS (4 g) in deionized and distilled water (DDW) was slowly mixed with glycidyl methacrylate (10.5 g) in a round-bottomed flask. The reaction mixture was precipitated in an ice-cold acetone twice and completely dried in vacuum. The degree of acrylation was determined by 400 MHz ^{1}H-NMR at the Central Laboratory, Kangwon National University (DPX400, Brucker). The purified glycidyl methacrylated COS (GM-COS) was then chemically conjugated to doxorubicin via amide bonds as described in the literature with a minor modification [11]. Succinic anhydride (0.11 g, 0.11 mmol) in dioxane (2 ml) was slowly added to doxorubicin (0.06 g, 0.10 mmol) in an ice-chilled 0.1 M phosphate buffer (pH 9.0, 9.5 ml) with a gentle vortexing. The reaction mixture was precipitated with 1 M HCl until opaque red precipitates were formed. The precipitate was centrifuged at 4000*g* and the pellet was re-dissolved in DW (20 ml) and pH was adjusted to pH 8.0. The solution was then added to GM-COS (0.031 g, 0.025 mmol), EDC (0.02 g, 0.10 mmol) and NHS (0.025 g, 0.21 mmol) dissolved in succinyl doxorubicin solution (20 ml). The reaction was performed at 20 °C for 12 h and dialyzed against DDW for three times to remove unreacted doxorubicin (Spectrapor6, molecular cut-off = 1000). The freeze-drying dialyzed solution generated doxorubicin-conjugated GM-COS (COS–DOX). FT-IR spectroscopy quantitatively confirmed the conjugation of DOX to GM-COS via an amide bond (EXCALIBER Series, BIO-RAD, Cambridge, MA).

2.3. Preparation of di-acrylated Pluronic F127

Terminal hydroxyl groups of Pluronic were acrylated with acryloyl chloride as described previously in the literature [10]. Briefly, completely dried Pluronic F127 (55 g) and triethylamine (1.83 ml) were dissolved in dichloromethane (80 ml), and then acryloyl chloride (1.41 ml) was slowly added drop by drop. The reaction was performed in a nitrogen atmosphere at 4 °C for 12 h. After additional incubation at 25 °C, the reaction mixture was precipitated in an ice-chilled diethylether and completely dried in vacuum. A degree of acrylation was determined by ^{1}H-NMR spectroscopy at the Central Laboratory, Kangwon National University.

2.4. Preparation of chemically crosslinked doxorubicin hydrogels

Acrylated Pluronic and COS–DOX were prepared for various hydrogels as shown in Table 1. Total concentration of hydrogel was fixed at 25% (w/v). A blend ratio of COS–DOX to acrylated Pluronic was previously determined, and the ratio was fixed throughout the current study [10]. Acrylated Pluronic (64 mg), COS–DOX (19.5 mg), and a photo-initiator, Irgacure2959 (0.32 mg) were completely dissolved in deionized water (0.236 ml) in a 1.5 ml tube at 4 °C and then physical gels were formed at 37 °C. A long wave UV light (320 nm–500 nm, 18 W/cm^2) was irradiated at a distance of 1.5 cm from the surface of the physical gel (Omnicure®1000, EXFO). Photo-irradiation was performed at 37 °C for 3 min.

2.5. In vitro release study

Chemically crosslinked hydrogels were transferred to a cell culture insert with 3.0 μm pore-sized polycarbonate membrane (BD Biosciences, San Jose, CA). The cell culture insert containing hydrogels was incubated in 3 ml of 0.1 M phosphate buffered saline (PBS, pH 7.4). Release study was performed at 37 °C with a gentle shaking. Released medium was collected at a pre-determined time and analyzed at 480 nm. The released amount of doxorubicin conjugates was calculated using a standard curve of free doxorubicin. The released fractions with doxorubicin conjugates were analyzed by a HPLC system equipped with a reversed-phase C18 column (SCL-10AVP, SPD10A, LC10AT-VP, Shimazu, Japan). A mobile phase was a mixture of water/acetonitrile (25:75, v/v) and a flow rate was 1 ml/min. A detector was set for absorbance at 480 nm.

Table 1
Doxorubicin hydrogels composed of doxorubicin–chitosan conjugate and Pluronic.

Hydrogel[a]	GM-COS (DOX) (mg)	Doxorubicin (mg)	Pluronic (mg)	Irgacure2959 (mg)
COS–DOX/Pluronic	19.5 (3.5)[b]	–	64	0.32
COS–DOX/Pluronic + free DOX	19.5 (3.5)[b]	2	64	0.32
GM-COS/Pluronic + free DOX	16	5.5	64	0.32
Pluronic + free DOX	–	5.5	80	0.32

[a] Total polymer concentration in hydrogels is kept at 25% (w/v), and volume of each hydrogel is 0.25 ml.
[b] The actual amount doxorubicin in the conjugate is shown in (), which is based on the conjugation extent of doxorubicin to GM-COS, 17.95% (w/w).

2.6. In vitro cytotoxicity study

Anti-cancer effects of released fractions from doxorubicin hydrogels were determined by a MTT-based cytotoxicity assay in a human lung adenocarcinoma cell line (A549) [12]. A549 cell was cultivated in RPMI1640 medium supplemented with fetal bovine serum (FBS) at 37 °C in 5% CO_2 atmosphere. A549 cell at a logarithm phase was seed on a 96-well culture dish at a cell density of 5×10^4cells/well. After 24 h, released fractions at 21 days (100 μl) from the release experiment of doxorubicin hydrogels were added to each well containing 100 μl of cell culture medium. After 48 h, cell culture medium was removed and 10 μl of a MTT solution was added. After incubation for 4 h at 37 °C, 100 μl of a solubilizing buffer (10% sodium dodecyl sulfate in 0.01 N HCl) was added. Absorbance of each well was measured at 570 nm, and relative cytotoxicities were obtained using a non-treated cell as a control.

2.7. Animal study

Athymic nude mice with human solid tumors were employed to determine *in vivo* anti-cancer effects of doxorubicin hydrogels as described previously in the literature with a minor modification [12]. Female athymic nude mice at 6 weeks (15–18 g) were subcutaneously injected with A549 cell (5×10^7cells/animal) at left and right thighs. One week after the size of the tumors reached approximately 1.0 cm in diameter, doxorubicin hydrogels at sol state (100 μl/tumor, 1.6 mg of doxorubicin equivalent/tumor) were intra-tumorally injected through 22G needles. After 1 h, the animal was kept under anesthesia using diethyether and 5 mm of cut was made at the injection site using a scalpel to crosslink the injected hydrogels. A fine light guide (diameter = 3 mm) was employed to deliver UV light through the cut, and photo-irradiation was performed for 15 s (Omnicure®1000, EXFO). The cut at the injection site was sealed with a nylon suture. The size of tumors from day 1 to day 30 was measured using calipers, and the tumor volume was calculated as (tumor volume = $(a \times b^2)/2$, a = major axis, b = minor axis). The number of animals in a group was nine, and all measurements were performed in triplicate. All animal experiments were carried out in accordance with EC Directive 86/609/EEC.

2.8. Statistical analysis

Statistical significance was determined by Student's test of Sigmaplot 9.0 software (SPSS Inc., Chicago, IL). P value below 0.05 was only considered statistically significant.

3. Results and discussion

Fig. 1 shows a schematic diagram of preparing injectable doxorubicin hydrogels containing doxorubicin–chitosan conjugates. We previously prepared thermo-responsive hydrogels composed of acrylated chitosan and acrylated Pluronic, which showed feasibilities of chitosan/Pluronic as injectable drug reservoirs for bioactive molecules. First, acrylated chitosan was conjugated to doxorubicin and then Pluronic was separately acrylated with acryloyl chloride. Acrylated Pluronic forms micelles in aqueous solution then undergoes physical gelation above LCST because of hydrophobic interactions between Pluronic micelles [13]. This hydrogel could be chemically crosslinked by photo-irradiation for further increasing the mechanical strength of physically crosslinked hydrogels. At a physiological condition, doxorubicin hydrogels could release free doxorubicin by simple diffusion when free doxorubicin was added during physical crosslinking of hydrogels. Conjugated doxorubicin was then released out upon chemical degradation of doxorubicin hydrogels. Chemical degradation is believed to mainly occur at chitooligosaccharide moieties because very low-molecular weight chitosan (1.2 kDa) was employed. Therefore, doxorubicin hydro-

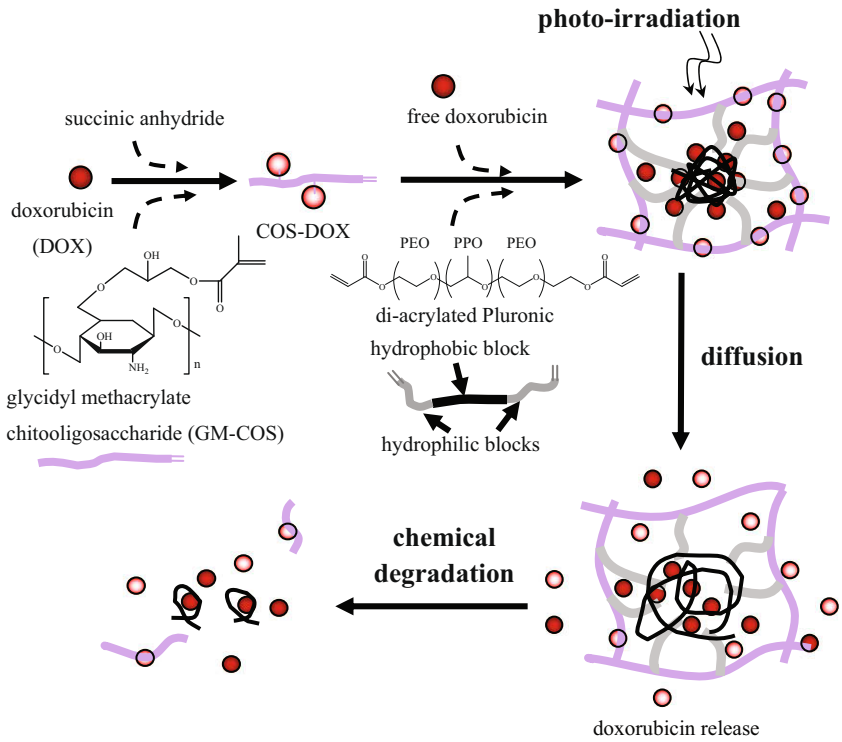

Fig. 1. Schematic diagram of GM-COS/Pluronic hydrogel for anti-cancer therapy.

gels with conjugated doxorubicin was expected to release doxorubicin in a sustained manner.

As we previously reported, chitooligosaccharide was reacted with glycidyl methacrylate to prepare glycidyl methacrylated chitooligosaccharide (GM-COS) [10]. ^{1}H-NMR spectroscopy determined that the degree of acrylation was 62.5% (w/w). This result suggests that 0.6 acrylated groups were conjugated to one molecule of chitooligosaccharide. Terminal hydroxyl groups of Pluronic were reacted with acryloyl chloride to prepare di-acrylated Pluronic. ^{1}H-NMR spectroscopy confirmed that 0.8 acrylated groups were conjugated to one molecule of Pluronic (degree of acrylation = 81.0%).

Doxorubicin was chemically conjugated to GM-COS to prepare doxorubicin-conjugated chitooligosaccharide as shown in Fig. 2A, and the conjugation was confirmed by FT-IR and NMR spectroscopy as shown in Fig. 2B and C, respectively. FT-IR spectrum quantitatively confirmed the conjugation between doxorubicin and GM-COS because of amide bonds from the synthesized COS–DOX. FT-IR spectrum of COS–DOX clearly showed two distinguishable peaks, thus confirming the formation of COS–DOX conjugates [14–17]. Amide bond formation between doxorubicin and COS at 1573 cm^{-1} and a methyl group of doxorubicin at 1232 cm^{-1} (COS–DOX) clearly suggested that doxorubicin and COS were conjugated by amide linkages compared to chitooligosaccharide without conjugated doxorubicin (GM-COS) because secondary amine peaks (1573 cm^{-1}) appeared instead of primary amine peaks of chitosan [14–16]. NMR spectroscopy also revealed the conjugation of doxorubicin to COS by three distinctive peaks of doxorubicin (Fig. 2C, a–c). This could be attributed that carboxylic groups of succinic anhydride doxorubicin were reacted with primary amine groups of COS, subsequently forming an amide bond. Other studies using doxorubicin conjugates employed similar methods for characterizing synthesized doxorubicin conjugates [8,18,19]. The conjugation amount of doxorubicin in COS–DOX was 17.95% (w/w), which was determined by measuring the absorbance of COS–DOX solution at 480 nm.

In vitro-release profile of doxorubicin hydrogels was investigated as shown in Fig. 3 and summarized various doxorubicin hydrogels for *in vitro* study and *in vitro* cytotoxicities. Pluronic hydrogel containing free doxorubicin (Pluronic + free doxorubicin hydrogel) and chitosan/Pluronic hydrogel containing free doxorubicin (GM-COS/Pluronic + free doxorubicin) showed a burst-release profile among other groups. Within 3 days, more than 70% and 90% of encapsulated doxorubicin was released out, respectively. No changes were then observed in the release profile after 5 days. This could be explained that doxorubicin was physically encapsulated within Pluronic hydrogel. Because no covalent linkage was employed to encapsulate doxorubicin within those hydrogels, the encapsulated doxorubicin was released out by simple diffusion. In addition, it was of interest that the hydrogel composed of a mixture of GM-COS and Pluronic showed a fast release of doxorubicin compared to the hydrogel without GM-COS. This could be attributed that GM-COS interfered physical crosslinking among acrylated Pluronic chains. In our previous study that employed similar polymers, we observed that increasing blend ratios of GM-COS in Pluronic hydrogels significantly increased a half-life of the hydrogel at 37 °C, suggesting that GM-COS played an important role as a crosslinking center [10]. However, the UV-crosslinking time was much longer compared to the current study (10–15 min); thus, most acrylated chitosan participated in chemical crosslinking. It should be mentioned that exposure to UV light for a long time is associated with degradation of doxorubicin, thereby decreasing cytotoxicity of doxorubicin [20,21]. Thus, we here minimized UV-crosslinking time to avoid inactivation of doxorubicin, and it is noticeable that optimizing chemical crosslinking time is important for maintaining cytotoxicity. On the contrary, doxorubicin hydrogels containing COS–DOX (COS–DOX/Pluronic and COS–DOX/Pluronic + free doxorubicin) showed a sustained-release profile compared to those without COS–DOX. In case of COS–DOX/Pluronic hydrogel, within 3 days, only about

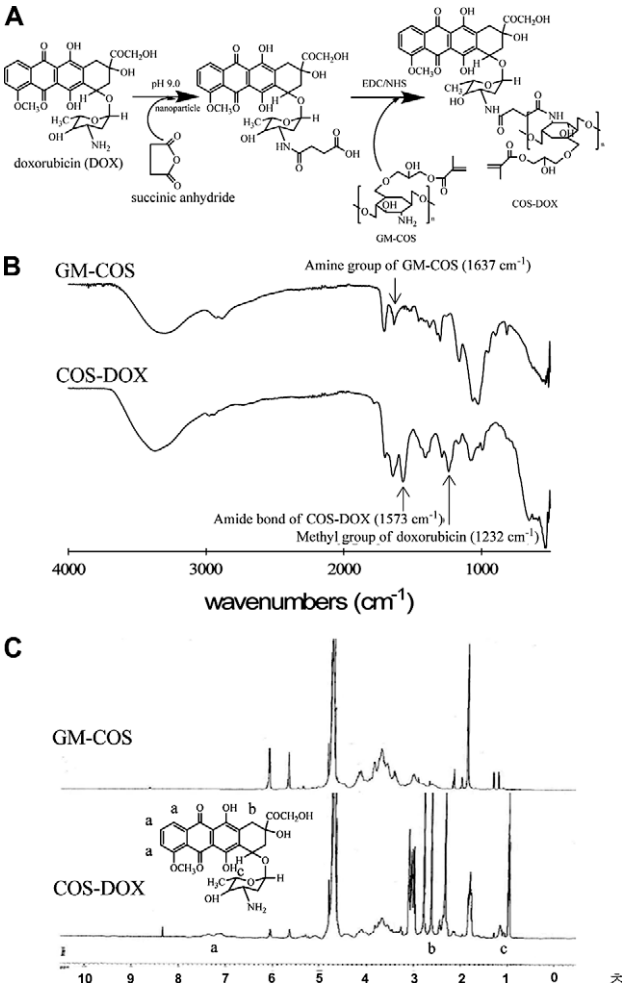

Fig. 2. Synthetic route of COS–DOX conjugates (A), FT-IR spectra (B), and ^{1}H-NMR spectra of COS–DOX in comparison with GM-COS (C).

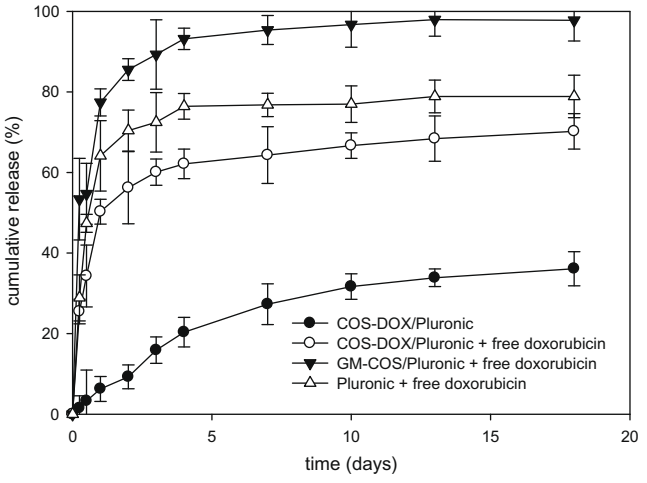

Fig. 3. *In vitro*-release profile of doxorubicin hydrogels at 37 °C. Each point is shown as means ± standard deviation (SD) (n = 3).

10% of encapsulated doxorubicin was released out and a sustained-release profile was thereafter observed for 18 days. This could be explained that doxorubicin was covalently attached to chitosan, which was also crosslinked in the hydrogel networks. Therefore, a release profile of doxorubicin or doxorubicin moieties were controlled by a degradation rate of the hydrogel and not by a diffusion rate of doxorubicin in the hydrogel. The doxorubicin hydrogel containing both doxorubicin and COS–DOX showed a decreased initial burst compared to those without COS–DOX and a controlled release profile over 18 days. This could be attributed that conjugated doxorubicin to chitosan controlled a release pattern of free doxorubicin although free doxorubicin was encapsulated in the hydrogel. Many researchers found that doxorubicin formed a dimer in an aqueous environment by a π–π interaction between two anthracycline rings of doxorubicin [22–24]. Furthermore, this interaction was often employed to control a burst release of doxorubicin in many drug delivery devices including micelles and nanoparticles [25]. In those studies, doxorubicin micellar nano-aggregates composed of free doxorubicin and doxorubicin-polymer conjugates showed high loading efficiencies when hydrophobized doxorubicin was encapsulated within the aggregates. However, in this study, a π–π interaction between two anthracycline rings of free doxorubicin and COS–DOX oligomers did not attenuate free doxorubicin in COS–DOX hydrogels. This could be attributed that a local concentration of free doxorubicin was too low to have the interaction (0.9%, w/w). However, in the cases of doxorubicin micellar nano-aggregates, a local concentration of doxorubicin was relatively high because a hydrophobic interaction between doxorubicin and doxorubicin conjugates was increased by adding triethyamine. Doxorubicin, COS–DOX, and a released fraction from COS–DOX/Pluronic were analyzed by reversed-phase chromatography as shown in Fig. 4. Upon chemical conjugation of doxorubicin to chitooligosaccharide, the conjugate became hydrophobic compared to intact doxorubicin with a trace amount of unreacted doxorubicin (4.2%, w/w) as shown in Fig. 4A and C. It could be attributed to the hydrophobic chitooligosaccharide in COS–DOX conjugates. However, compared to intact COS–DOX (Fig. 4C), a retention time of the released fraction for 18 days (Fig. 4B) was shifted from 11.5 min to 10.2 min. This result clearly suggested that COS–DOX degraded into more hydrophilic products. Considering that doxorubicin was covalently conjugated to chitooligosaccharide by an amide linkage, which is not hydrolyzed under physiological conditions, the released fraction from hydrogels containing COS–DOX was not intact doxorubicin but doxorubicin conjugates composed of chitooligosaccharide or Pluronic oligomers. Interconnected networks of Pluronic-COS–DOX formed after chemical-crosslinking of doxorubicin hydrogels and hydrolysis of this biodegradable polymeric networks rendered doxorubicin oligomers composed of degradation products of Pluronic and chitooligosaccharide. However, it should be noticed that a molecular weight of chitooligosaccharide is approximately ten times smaller than that of Pluronic. Thus, degradation rates of Pluronic would be significantly slower than that of chitooligosaccharide, and released fraction from doxorubicin hydrogels containing COS–DOX would generate doxorubicin conjugated to chitooligosaccharide oligomers.

Cytotoxicity of released fractions from each hydrogel was determined in A549 as shown in Fig. 5. Released fractions from a blank hydrogel (GM-COS/Pluronic) without doxorubicin or doxorubicin conjugates showed negligible cytotoxicity, clearly suggesting a fair biocompatibility of Pluronic and chitooligosaccharide. Low cytotoxicity of these materials has also been demonstrated in other literatures, and many researchers have employed these materials for injectable and implantable devices [25,26]. Compared to free doxorubicin (positive control), the released fractions from doxorubicin hydrogels showed slightly decreased cytotoxicity. IC_{50} values of COS–DOX/Pluronic + free doxorubicin, GM-COS/Pluronic + free doxorubicin, and Pluronic + free doxorubicin were 29.5 μM, 21.9 μM, and 20.4 μM, respectively, while that of doxorubicin was 17.8 μM. Although the differences in IC_{50} values were not high, reduced cytotoxicity of the released fraction from COS–DOX/Pluronic + free doxorubicin hydrogel could be attributed to doxorubicin–chitooligosaccharide oligomers. Many researchers previously conjugated doxorubicin to synthetic and natural polymers for the purpose of preparing nanoparticulated anti-cancer drugs or increasing a biological half-life [29–31]. At physiological conditions, biodegradable polymer–drug conjugates spontaneously degraded into low-molecular weight polymer–drug conjugates, and the conjugated oligomers were confirmed to slightly decrease the cytotoxicities of the anti-cancer drugs. However, it should be mentioned that they still showed comparable cytotoxicities in comparison with intact anti-cancer drugs as proven in many studies [27,28,31]. In those studies, doxorubicin oligomers usually showed inferior cytotoxicities compared to intact doxorubicin for a short period of time (2–3 days). However, these showed enhanced cytotoxicities *in vivo* or *in vitro* cytotoxicities when they were incubated for a long period of time. This was attributed to enhanceendocytic uptake of these oligomeric forms by cells because endocytosed doxorubicin was not easily pumped out of the cells as many researchers previously showed [27,28]. In addition,

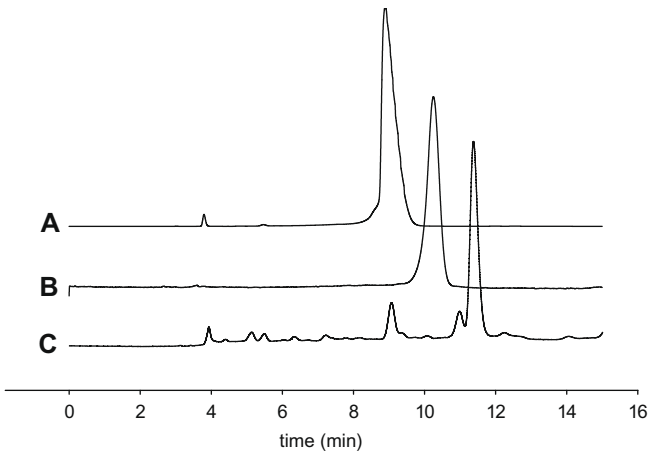

Fig. 4. Reversed-phase chromatography of doxorubicin (A), released fraction for 18 days (B), and intact COS–DOX (C).

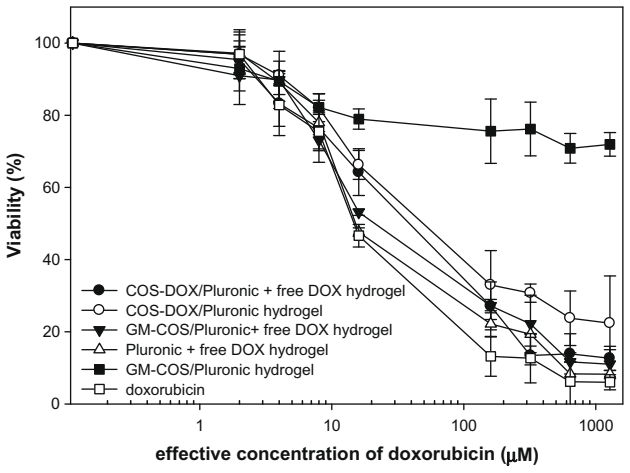

Fig. 5. *In vitro* cytotoxicities of released fractions at 18 days from doxorubicin hydrogels and free doxorubicin against A549 cells. Each point is presented as means ± SD ($n = 3$).

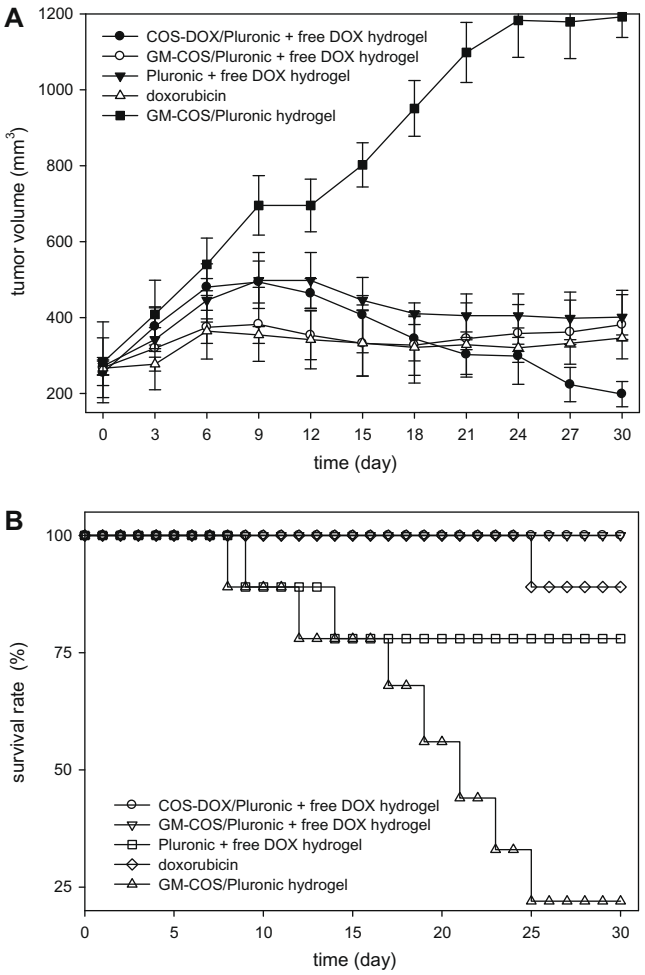

Fig. 6. Tumor volume changes (A) and survival rates (B) of athymic nude mice bearing human solid tumors after intra-tumoral injections of doxorubicin hydrogels ($n = 9$).

it should be noticed that statistical evaluation of cytotoxicity results revealed that doxorubicin hydrogels with COS–DOX had slightly lower cytotoxicities than those with free doxorubicin with statistical significances ($p < 0.05$).

Athymic nude mice bearing human solid tumors were subjected to an *in vivo* efficacy test for doxorubicin hydrogels, and the tumor sizes and the survival rates were measured as shown in Fig. 6. While the tumor size with the blank hydrogel (GM-COS/Pluronic hydrogel) was steeply increased approximately by 423%, the tumor size with the doxorubicin hydrogels showed relatively suppressed tumor growths for 1 month (Fig. 6A). Free doxorubicin showed superior anti-tumor effect to other formulations by the third week. A blank hydrogel with free doxorubicin (GM-COS/Pluronic + free DOX hydrogel) showed similar patterns of tumor volume changes compared to free doxorubicin. This can be explained that doxorubicin physically encapsulated within the hydrogel, therefore, burst release of doxorubicin significantly reduced tumor sizes at the initial stage of tumor growth. However, no change was observed after 3 weeks, suggesting that most doxorubicin was released out right after an intra-tumoral injection. This result coincides with the result from *in vitro* release experiments shown in Fig. 3, which showed that burst release of doxorubicin was observed within 3 days and reached a plateau after that. However, the doxorubicin hydrogel containing COS–DOX showed the most interesting results. After 9 days, a significant decrease in tumor volumes was observed and the tumor volume finally decreased by 22% of the original tumor size at day 30. This dramatic decrease in tumor size can be attributed to sustained release of doxorubicin and doxorubicin–chitooligosaccharide oligomers as shown in Figs. 4 and 5. Free doxorubicin encapsulated within the COS–DOX/Pluronic hydrogel was released out in a controlled manner and played a cytotoxic role at the initial stage. At the later stage, doxorubicin–chitooligosaccharide oligomers from the hydrogel further increased the cytotoxicity of the doxorubicin hydrogel in addition to anti-cancer effects of free doxorubicin. Considering that the amount of encapsulated doxorubicin was the same for all cases, it was of interest how different release profiles of doxorubicin affected tumor regression for 1 month. According to the result from Fig. 6, it was very clear that sustained release of doxorubicin was more advantageous than initial burst release of free doxorubicin because local concentration of doxorubicin could be maintained for the 1-month period. Fig. 6B showed survival rates of treated animals for 1 month. Except those with a blank hydrogel, all treated animals showed a survival rate of over 75%. It should be noticed that statistical significances could not be determined for a control group after 18 days because more than half of the group died. Therefore, this result clearly showed that doxorubicin hydrogels and doxorubicin were potent in a similar level. In other studies employing drug-polymer conjugates, reduced cytotoxicity of anticancer drug-polymer oligomers was not disadvantageous because *in vivo* fates of drug-polymer conjugates were largely controlled by pharmacokinetics of drug delivery devices [32–34]. Undesirable side-effects due to systemic circulation were reduced because anticancer drugs could be localized in specific sites. In fact, depot systems including thermo-responsive hydrogels have been shown to be advantageous over conventional free drugs because local concentration at specific sites can be significantly elevated although administrating hydrogels is slightly more complex than administrating free doxorubicin. In addition, systemic circulation of doxorubicin could be suppressed because doxorubicin and doxorubicin oligomers were slowly released from hydrogels. Thus, doxorubicin hydrogels composed of doxorubicin conjugates can be promising in situ gelation depot system aiming to control release of doxorubicin at tumor sites for an extended period.

4. Conclusion

Doxorubicin hydrogel containing COS–DOX conjugates showed a sustained-release profile of doxorubicin and doxorubicin oligomers. A released fraction composed of doxorubicin and chitosan–doxorubicin oligomers showed comparable *in vitro* cytotoxicities to free doxorubicin. Doxorubicin hydrogels containing chitosan–doxorubicin conjugates showed superior *in vivo* anti-cancer effects in human solid tumors compared to free doxorubicin or hydrogel containing free doxorubicin after 3 weeks.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2009.04.010.

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