Carbohydrate Polymers 87 (2012) 1105–1111

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Thermo-sensitivity and triggered drug release of polysaccharide nanogels derived from pullulan-*g*-poly(L-lactide) copolymers

Seogjin Seo, Chung-Sung Lee, Young-Seok Jung, Kun Na*

Department of Biotechnology, The Catholic University of Korea, Bucheon 420-743, Republic of Korea

ARTICLE INFO

Article history:
Received 19 July 2011
Received in revised form 15 August 2011
Accepted 19 August 2011
Available online 26 August 2011

Keywords:
Thermo-responsive
Nanogels
Pullulan
Phase transition temperature
Doxorubicin

ABSTRACT

Thermo-responsive nanogels from poly(L-lactide)-*g*-pullulan (PLP1 and 2) copolymers with different lactide contents were investigated as an anticancer drug delivery carrier. The phase transition temperature of PLP 1 with lower lactide content in distilled water showed around 35 °C. Upon adding 0.15 M NaCl to PLP 1, a significant difference in the transmittance was observed when comparing the non-additive salt condition. The total amount of released doxorubicin (DOX) from the DOX-loaded PLP nanogels increased with increasing temperature for 50 h. A noticeable difference in the initial release by PLP 1 was observed between 37 and 42 °C. In the 50% inhibitory concentration (IC_{50}) analysis, the IC_{50} values of DOX released from PLP 1 were approximately 5.9 and 9.3 μg/mL at 37 and 42 °C, respectively. The results suggest that self-assembled PLP nanogels, by means of a triggering temperature, can be used as a long-term drug delivery system in cancer treatments.

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1. Introduction

Stimuli-responsive biomaterials are of great interest because of their properties and applications. These materials can be tuned by altering the molecular structure or the environmental conditions, such as pH, temperature, ionic strength and light (Butun et al., 2001; Butun, Billingham, & Armes, 1998; Klaikherd, Nagamani, & Thayumanavan, 2009). Among the internal and external stimuli, high temperature is one of the best signals in terms of ease and safety in medical applications. The merits of high temperature are due to the substantial strides that medical researchers have made in the applications of hyperthermia therapy for the treatment of solid tumors.

Poly(N-isopropylacrylamide) (PNIPAAm) is the most widely used synthetic temperature-responsive material in biomaterial and intelligent material studies concerning hydrogels and bioconjugates (Kaneko et al., 1995; Matsukata et al., 1996; Yoshida et al., 1995). One reason for its frequent use is that its phase transition occurs approximately at body temperature. PNIPPAm exhibits a sharp phase transition in water with a lower critical solution temperature (LCST) of 32 °C. Due to its resilient LCST, PNIPPAm has been used for targeting solid tumors with local hyperthermia (Meyer, Shin, Kong, Dewhirst, & Chilkoti, 2001), in thermo-sensitive coatings or micelles for controlled drug release (Gutowska et al., 1995) and as a cell attachment/detachment surface (Okano, Yamada, Okuhara, Sakai, & Sakurai, 1995). Scaffolds consisting of PNIPAAm can support the regeneration of nerve fibers in cell-delivery systems (Shimmura et al., 2003), enable rapid wound healing (Wang, Su, & Chen, 2008) and limit foreign body reactions after implantation (Zhou et al., 2007). These properties of PNIPAAm and its ease of preparation have made this polymer very attractive for use in many types of drug delivery systems, including hydrogels, block copolymers and liposomes. However, its clinical application in drug delivery systems is limited due to its non-biodegradability.

For this reason, biodegradable thermo-responsive copolymers have been used in clinical applications (Malmsten & Lindman, 1992). Thermoreversible block copolymers composed of biodegradable polyesters, such as poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLA), poly[(lactic acid)-co-(glycolic acid)] (PLGA) and poly[(D,L-LA)-co-(ε-caprolactone)] (CL), were studied as controlled-release drug carriers for surgical implantation and wound treatment because of their non-toxicity and biocompatibility (Johnston, Punjabi, & Froelich, 1992). Jeong et al. reported that the PLLA-based triblock polyester copolymers demonstrated a thermoreversible sol–gel transition as a function of both the concentration and the composition of the block copolymers (Jeong, Bae, & Kim, 1999; Jeong, Bae, Lee, & Kim, 1997).

Recently, self-organized nanogels derived from polysaccharide-*g*-poly(L-lactide) copolymers have been investigated as a drug delivery carrier due to their biocompatibility and perfect degradability (Ohya, Maruhashi, & Ouchi, 1998). In the cases of block polymers with longer PEO chains, PEO was readily to accumulate in the body after polyester degradation for targeted amphiphilicity (Bae, Huh, Kim, & Park, 2000). Our group already reported the

* Corresponding author. Tel.: +82 2 2164 4832; fax: +82 2 2164 4865.
E-mail address: kna6997@catholic.ac.kr (K. Na).

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doi:10.1016/j.carbpol.2011.08.061

physicochemical characterization and *in vitro* doxorubicin release of self-organized nanogels from pullulan-*g*-poly(L-lactide) copolymers using the fluorescence probe method (Cho, Park, & Na, 2009). However, the dramatic thermo-sensitivity of the nanogels has not been reported including our groups. Akiyoshi, Deguchi, Tajima, Nishikawa, and Sunamoto (1997) investigated colloidal stability of hydrophobized polysaccharide hydrogel, cholesterol-bearing pullulan, upon heating. However, there was no the dramatic phase change upon heating with respect to sol–gel transition of the self-aggregated nanoparticles.

In this study, we attempted to show that the thermo-sensitivity of PLP nanogels makes them a promising anticancer drug agent that can be used in clinical applications. We conducted an *in vitro* release study of PLP nanogels loaded with doxorubicin (DOX) as a model drug for cancer treatment in a physiological environment to confirm the potential of the compounds. We hypothesized that PLP nanogels triggered by higher temperatures may have increased rates of drug release, resulting in the effective killing of cancer cells. To check the physicochemical properties of DOX-loaded PLP nanogels, their phase-transition temperature and CAC were examined with increasing temperature. Additionally, we investigated the temperature effects of DOX-loaded PLP nanogels on HeLa cells.

2. Methods and materials

2.1. Materials

Pullulan (MW: 10^5 g/mol) was purchased from Hayashibara (Okayama, Japan) and was purified as follows. The pullulan (10 g) was dissolved in distilled water (100 mL) and precipitated into ethanol (1 L). The precipitate was redissolved in distilled water. The solution was dialyzed for 1 day by using a dialysis membrane (molecular weight cut-off (MWCO): 10,000) to remove the solvent and the small molecules in the raw materials. The resultant solution was lyophilized for 3 days and stored in a dry desiccator. L-Lactide, triethylamine (TEA) and dimethyl sulfoxide (DMSO) were acquired from Aldrich. L-Lactide was recrystallized from ethyl acetate before use. TEA and DMSO were used without purification. All of the other chemicals and solvents were used as received. DOX was supplied by Sigma–Aldrich (St. Louis, MO) and used as received.

2.2. Synthesis and characterization of PLP copolymer

The syntheses were accomplished by stirring for 12 h in DMSO at 75 °C with TEA and pullulan as the catalyst and macroinitiator, respectively (Cho et al., 2009). Briefly, the previously purified pullulan was dissolved in DMSO by stirring at room temperature under dry N_2. To this pullulan solution, L-lactide was added to reach a concentration of 10% (w/v). When the mixture was clearly soluble, it was stirred in a preheated oil bath adjusted to 70–75 °C. TEA was introduced into this solution when the ratio of TEA to DMSO was 1.67% (v/v). All of these processes were conducted for 2 h under N_2. After 12 h of reaction, the reactant solution was filtrated through a 0.5-μm syringe filter. The filtrate was then dialyzed for 2 days against distilled water by the use of a dialysis membrane (MWCO: 10,000) with the distilled water being exchanged every 2 h during the first 12 h. The resultant product was then lyophilized for 3 days and stored at −20 °C until use. The synthetic copolymers were characterized by ^{1}H-nuclear magnetic resonance (^{1}H NMR, Varian, 300 MHz) spectroscopy. By observing the new peaks and comparing them with the peaks of raw pullulan, the formed chemical bonds were verified. The ratios of the integrated peaks at 1.4 ppm (the lactide methyl protons, 6H) and 5.2 ppm (3H) were used to determine the degree of substitution (DS) for the polymers (Donabedian & McCarthy, 1998).

Dynamic laser scattering measurements were conducted in distilled water at 25 °C on a Zetasizer (Malvern, Worcestershire, UK). Their size was measured at a concentration of 1 g/L.

2.3. Preparation of self-organized PLP nanogels

The self-assembled nanogels were prepared via a dialysis method, resulting in a narrow size distribution. Briefly, PLP (25 mg) was dissolved in DMSO (10 mL) at a concentration of 2.5 mg/mL. The solutions were filtered through a 0.5-μm syringe filter and dialyzed against distilled water by the use of a dialysis membrane (MWCO: 1000) for 2 days with the distilled water being exchanged every 2 h during the first 24 h. The resultant nanoparticle mixture was stored at 4 °C until characterization.

2.4. Measurements by fluorescence spectroscopy (Hoechst 33342)

The critical association concentration (CAC) was measured using a previously reported method with some modification (Jumpertz et al., 2011). Briefly, a stock solution of Hoechst 33342 (1.4×10^{-3} M) in double-distilled H_2O was prepared and stored at 5 °C until use. The Hoechst 33342 solution was mixed with solutions containing polymers at concentrations of 1×10^{-4} mg/mL to 1.0 mg/mL. The final concentration of Hoechst 33342 in each sample solution was 7.0×10^{-6} M. The resultant fluorescence was measured on a RF-5301PC (Shimadzu, Japan) with λ_{ex} = 355 nm and λ_{em} = 457 nm at 37 and 42 °C, and the slit widths were 3 and 3 nm, respectively.

2.5. Drug loading and in vitro release test

For the drug release test, the PLP polymers (40 mg) and predesalted DOX (2 mg) were dissolved in 10 mL of DMSO. The solution was then stirred at room temperature and dialyzed against phosphate buffer solution (pH 7.4) with the use of a dialysis membrane (MWCO: 1000) for 1 day. After filtration through a 0.45-μm syringe filter, the filtrates were stored in a refrigerator until the release test. To determine the drug-loading efficiency of the PLP nanogels, the filtrate was lyophilized and dissolved in DMSO. The filtrate was vigorously stirred for 2 h and then sonicated for 3 min (VCX 750, Sonics & Materials, Newtown, CT). The resultant solution was then centrifuged for 30 min at 20,000 × *g* (Combi-514R, Hanil Science Industry, Incheon, Korea). The supernatant was analyzed at 490 nm using UV-spectroscopy (UV-2450, Shimadzu, Kyoto, Japan). The *in vitro* release test was conducted as follows. One milliliter of the DOX-loaded nanoparticle solution was pipetted into a dialysis membrane and introduced into 10 mL of phosphate-buffered saline (PBS), followed by stirring at 50 rpm at 37 °C. At the predetermined times, the medium was removed and exchanged with fresh PBS. The quantity of DOX in PBS was detected via UV spectroscopy and determined using a standard curve at 365 nm. Due to the light sensitivity of DOX, all of the above tests were conducted in darkness.

2.6. In vitro cytotoxicity and measurement of DOX-loaded PLP nanogels and cellular uptake of PLP nanogels

The *in vitro* cytotoxic activity of DOX-loaded PLP nanogels was measured using cultured HeLa cells. The cells were plated in 96-well plates at a density of 3×10^4 cells/well and were then exposed to free DOX and DOX-loaded PLP nanogels for 6 h at 37 and 42 °C, respectively. After drug exposure, the cells were washed three times with PBS and then cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin sulfate in a humidified atmosphere (5% CO_2, 37 °C). The chemosensitivity

Table 1
Synthetic results of PLP copolymers.

Sample	Feed ratio[a]	Degree of polymerization of lactic acid[b]	Appearance
PLP 1	11:1	0.81	White powder
PLP 2	13:1	1.42	White powder

[a] Feed ratio means the molar ratio of L-lactide to repeating units of pullulan in the reactants.
[b] Degree of substitution and degree of polymerization of lactic acid were calculated from ^{1}H NMR spectroscopy.

was assessed using tetrazolium salt (MTT, Sigma, USA) to measure the viability of the cells. One hundred microliters of culture media containing 20 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for an additional 4 h. The MTT-containing media were discarded, and the formazan crystals in the living cells were dissolved with DMSO (100 μL/well). The absorbance of the formazan crystals was measured at 570 nm.

3. Results and discussion

Pullulan, a water-soluble, neutral, linear polysaccharide, has been extensively used as a drug delivery carrier due to its biocompatibility and biodegradability (Akiyoshi et al., 1998). However, hydrophilic pullulan itself cannot load drugs, and is difficult to have the thermal property. For this reason, several attempts for introduction of hydrophobic segments into pullulan have been made (Jeong et al., 2006; Jung, Jeong, Kim, & Kim, 2004; Na, Lee, & Bae, 2004; Na, Lee, & Bae, 2003). Of these, PLLA which has excellent biodegradability and biocompatibility is appropriate as a hydrophobic segment for hydrophobized pullulan. In this study, therefore, amphiphilic PLP copolymers composed of water-soluble pullulan and hydrophobic PLLA were obtained as reported in our previous study (Cho et al., 2009). The PLP copolymers in an aqueous environment are expected to have a core–shell type of structure due to their amphiphilic characteristics. The pullulan skeleton may act as a hydrophilic outer shell, and PLLA may form a hydrophobic inner core. PLLA as hydrophobic segments comprising the micelle inner core may play an important role in determining the release and activity of drugs loaded in the thermoresponsive polymeric micelles (Chung, Yokoyama, & Okano, 2000). The structured scheme is shown in Fig. 1.

3.1. Synthesis and characterization of PLP copolymer

The successful synthesis of PLP copolymers was verified by characterization with ^{1}H NMR spectroscopy as shown in Supplementary Data. Typically, the methylene proton signals of the pullulan were observed at δ = 3.5–5.5 ppm. Upon grafting PLLA onto pullulan, new peaks corresponding to PLLA appeared at δ = 1.5 and 5.1 ppm. The characteristic peaks observed at δ = 1.66 and 5.09 of the monomer (L-lactide) were not observed in Supplementary Data, indicating that the unreacted monomer in the product did not remain. By comparing the intensities of the peaks from δ = 5.1 to 5.6 ppm, the degree of substitution (DS) can be estimated and is shown in Table 1. DS refers to the average number of –OH groups on the anhydroglucose ring that reacted with L-lactide (Donabedian & McCarthy, 1998). According to our results, PLP with DS ranging from 0.5 to 2.0 can have thermal property in an aqueous environment. In the outer range, the resultant was insoluble in water or became a flaky substance (Cho et al., 2009). The DS range corresponding to thermoresponsiveness was maintained during synthesis by controlling the lactide contents. PLP copolymers with lower DS of the lactide moiety are expected to respond in higher temperature. As a result, two series of PLP copolymers with the higher (1.42) and lower (0.81) DS of the lactide moiety were obtained.

Table 2
Sizes, drug contents and loading efficiencies of the PLP nanogels.

Sample	Size (nm)	Drug contents (%, w/w)	Loading efficiency (%, w/w)
PLP 1	121.9 ± 16.60	4.276 ± 0.05	39
PLP 2	163.7 ± 14.48	4.966 ± 0.07	41

3.2. Hydrodynamic diameter of thermosensitive PLP nanogels in aqueous medium

The nanogels were prepared via a dialysis method, which prevented their uncontrolled rapid precipitation during the self-assembly process. Their hydrodynamic diameter in an aqueous environment was measured by dynamic light scattering (DLS) as shown in Table 2. The mean sizes of the PLP 1 and 2 nanogels were 121 and 163 nm at 25 °C, respectively. Polymeric micelles or nanospheres with a size less than 200 nm were reported as suitable drug-targeting carriers to solid tumors and specific disease sites (Gref et al., 1994; Yokoyama et al., 1990). The sizes and drug contents depending on the amount of lactide grafted to the pullulan increased as the ratio of the lactide contents increased. This result can be explained in that the higher lactide contents provide a better chance for hydrophobic interactions between the lactide portions within the interior structure of the PLP copolymers, resulting in secondary aggregation.

Owing to these hydrophobic lactide portions, the size of the PLP copolymers in an aqueous environment can be changed with increasing temperature. As expected, the size of PLP 2 nanogels, having relatively higher lactide contents, rapidly increased over 32 °C. The size of the PLP 1 nanogels, having relatively lower lactide contents, did not significantly change and remained under 100 nm until near 40 °C as shown in Fig. 2a. Supporting the result in Fig. 2a and b shows strong evidence for their phase-transition temperature. PLP 2 solution was noticeably turbid at 37 °C as compared with that at 25 °C. No changes in terms of turbidity were observed for pullulan between the two temperatures. The PLP 2 solution became more insoluble in an aqueous environment as the temperature increased, resulting in secondary aggregation between the nanogels. While secondary aggregation between the nanogels occur, each particle size of PLP nanogels is thought to decrease with increasing temperature due to squeezing effects of each particle caused by increasing hydrophobicity (Cho et al., 2009; Gref et al., 1994). The results indicate that the water solubility of the nanogels decreased at high temperatures as a result of the hydrophobic interactions between the nanogels. Generally, micelles or nanospheres maintain a constant size and an approximately spherical shape as the temperature is raised. However, critical concentration fluctuations arise due to increasingly attractive self-aggregation interactions as a lower consolution temperature is approached, which gives rise to a cloud point (Hayter & Zulauf, 1982).

3.3. Self-assembled formation behaviors of thermosensitive PLP nanogels

Fig. 3 shows the phase-transition temperature of PLP nanogels as determined by UV spectrophotometric analysis to further understand their temperature-dependent properties. As the temperature increased, the transmittance of PLP 2 rapidly decreased over 32 °C, whereas the transmittance of PLP 1 slowly decreased (Fig. 3a). Their transmittance decreased as the temperature increased due to the secondary aggregation between the nanogels. The water

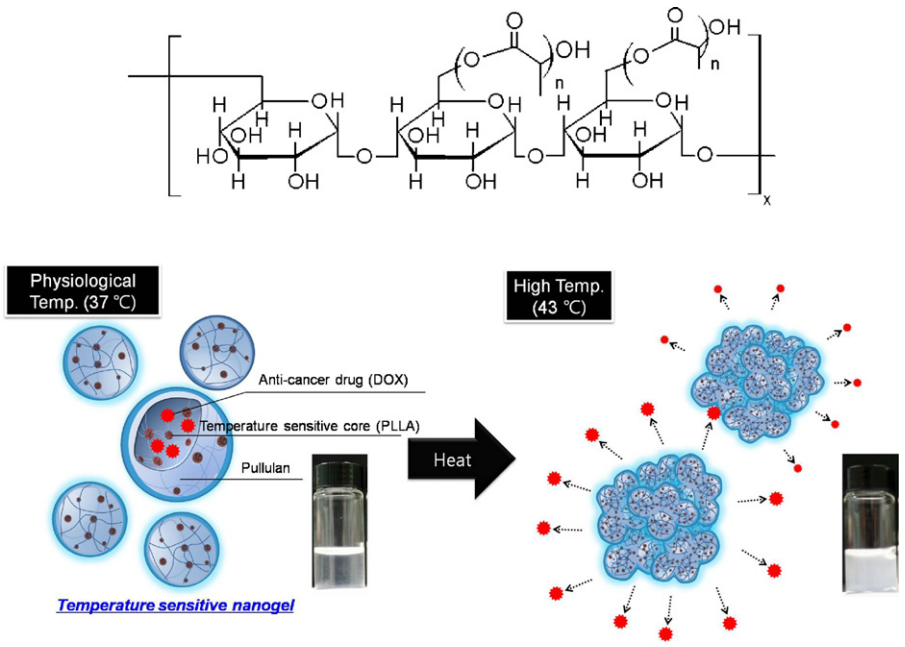

Fig. 1. (a) Molecular structure of the PLP copolymer and (b) the schematic diagram for anticancer drug release from thermosensitive PLP nanogels by triggering temperature.

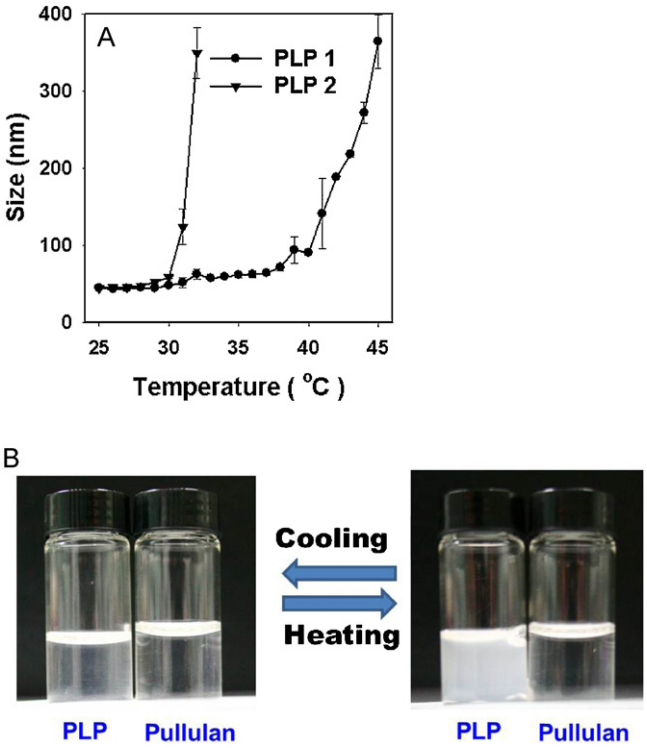

Fig. 2. (a) Sizes of PLP 1 and PLP 2 nanogels as a function of temperature. (b) Pictures representing the thermosensitive PLP nanogel solution obtained at 25 and 37 °C by comparing two solutions of PLP and pullulan without the lactide contents.

density is depleted near the hydrophobic surface, especially when water molecules became more mobile at the higher temperatures. Because of water depletion, a large energetic contribution is added to the free energy of hydration of extended surfaces, as manifested in the surface tension of a water interface (Huang & Chandler, 2000). Fig. 3b shows the phase-transition temperature curves of PLP 1 with increasing NaCl concentrations. Upon adding 0.15 and 0.2 M NaCl to PLP 1, the transmittances rapidly decreased over 32 °C but did not in the absence of NaCl. A simple plot of the

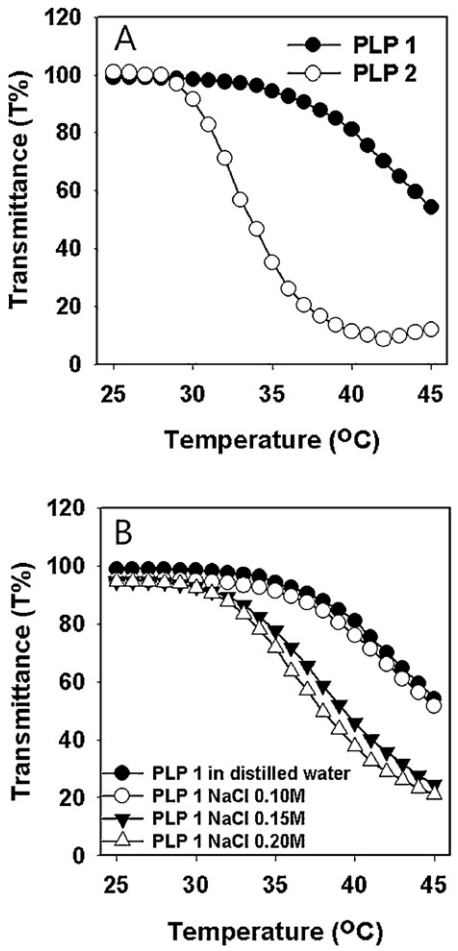

Fig. 3. (a) The hydrophobic effect of lactide contents on the phase-transition temperature of the PLP nanogels by monitoring their transmittance change with different lactide contents as a function of temperature. (b) The salt effect on the phase-transition temperature of the PLP 1 nanogels by monitoring their transmittance change with different NaCl concentrations as a function of temperature ($n = 5$).

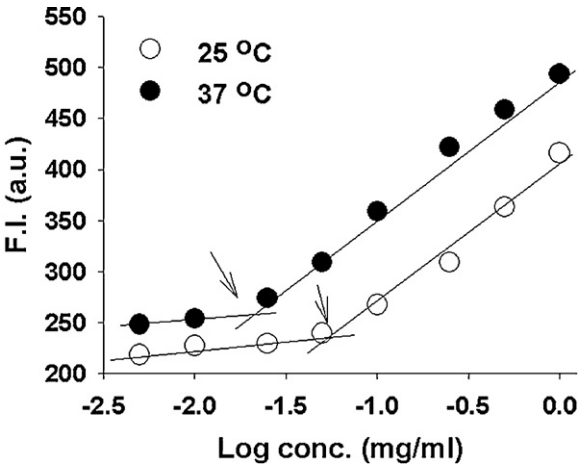

Fig. 4. The excitation spectra of Hoechst 33342 (7 mM) in distilled water in the presence of PLP 2 nanogels as a function of polymer concentration at 25 and 37 °C. The plots of intensity ratio were obtained from the excitation spectra vs. log concentration of PLP 2 nanogels at 25 and 37 °C ($n = 3$).

transmittance change as a function of salt concentration and lactide contents with increasing temperature determines the phase transition of the PLP nanogels. The thermo-responsive behavior of the polymers is due to the breakdown of polymer–water hydrogen bonding in controlling the macromolecular contraction, chain collapse as a result of changes in the hydrophobic effects or both (Inomata, Goto, & Saito, 1990; Otake, Inomata, Konno, & Saito, 1989; Otake, Inomata, Konno, & Saito, 1990). Some salts increase the LCST, in which case it is called the 'salting-in effect', whereas other salts decrease the LCST, in which case it is called the 'salting out effect' (Lee, Song, Jin, & Sohn, 1999). The NaCl effects on the present polymers are clearly due to the 'salting out effect'. The addition of NaCl increases the hydrogen bonding among the water molecules and therefore decreases the hydrogen bonding between the water molecules and the hydrophilic chains. Subsequently, the hydrogen bonding among the hydrophilic chains becomes dominant, which results in a stronger tendency for the polymers to associate and decrease their LCSTs (Liu, Yang, & Leong, 2003). The presence of NaCl increases the polarity of aqueous media and enhances hydrophobic–hydrophobic interactions. The stronger hydrophobic–hydrophobic interaction indicates the stronger tendency for the polymers to self-aggregate. Self-aggregation will result in decreased solubility for the polymer in water, decreasing its LCST (Garret Flaudy & Freitag, 2000; Liu, Wang, Wang, Huang, & He, 2004).

3.4. Critical association concentration of thermosensitive PLP nanogels

The CAC is a fundamental parameter describing the physicochemical characteristics of polymeric self-organized nanogels. In general, the CAC is determined by a fluorescence method (Cao, Munk, Ramireddy, Tuzar, & Webber, 1991). At the CAC, micelles are formed, and fluorescence probes partition between the aqueous and self-assembled micelle domains (Wilhelm et al., 1991). Also, single block copolymers that do not have CAC properties are considered to be very different from self-organized nanogels in terms of delivery behavior in the bloodstream. We used Hoechst 33342 as a fluorescence probe to monitor the organizing behavior of PLP 2 nanogels in aqueous media (Fig. 4). As the temperature increased, a change in the fluorescence intensity and a shift in the critical aggregation point could be clearly observed. This finding indicates that Hoechst 33342 was transferred from the aqueous polar environment to the less polar micellar domain (Wilhelm et al., 1991). The total fluorescence intensity increased at 37 °C, compared with that at 25 °C. The CACs of PLP 2 determined by the crossover point were 0.02 and 0.06 mg/mL at 37 and 25 °C, respectively. The CAC at 25 °C was approximately twofold higher than that at 37 °C. The results indicate that the number of stable micelles increases with the decrease in the CAC. This stability can be explained by the increased hydrophobic interactions and the rigidity of the lactide chains in the copolymers as a function of the hydrophobic segments as the temperature increased (Cho et al., 2009).

3.5. In vitro drug release test of thermosensitive PLP nanogels

The DOX loading efficiencies of the PLP 1 and 2 nanogels were found to be 39 and 41%, respectively, and the maximum loading content was near 5% (Table 2). Fig. 5 shows that the DOX release profiles from the PLP 1 and 2 nanogels were monitored at 25, 37 and 42 °C. The total amount of released DOX after 50 h reached 40, 60 and 100% from the PLP 1 nanogels (Fig. 5a) and 40, 70 and 80% from the PLP 2 nanogels (Fig. 5b) at 25, 37 and 42 °C, respectively. A larger difference in DOX release between 37 and 42 °C was found for the PLP 1 nanogels than for the PLP 2 nanogels. Similar results are shown in Fig. 3a, in which significant differences in the transmittances at 37 and 42 °C were found for PLP 1 only. Fig. 5c shows the cumulative DOX release from the PLP 1 and 2 nanogels after 24 h. As the temperature increased, the cumulative DOX release from the nanogels increased. However, the release of DOX from the PLP 1 and PLP 2 nanogels after 24 h was similar. Therefore, the main effect determining DOX release seemed to depend on the temperature rather than on the lactide contents. To further investigate the relationship between hydrophobicity and temperature, the initial release profiles of DOX were plotted for the PLP 1 and 2 nanogels for 7 h (Fig. 5d and e). The initial release behaviors were analyzed by plotting the release data. The calculated regression slopes indicating the release speeds were 2.8, 3.5 and 6 for PLP 1 and 3, 2.7 and 4.3 for PLP 2 at 25, 37 and 42 °C, respectively. Fig. 5f shows a comparison of the initial slopes by linear regression for the PLP 1 and PLP 2 nanogels for the initial 7 h. The drug release was fastest at 42 °C, and further faster in PLP 1 than in PLP 2 at 42 °C. The initial drug release between 37 and 42 °C from the PLP 1 nanogels was noticeably different from the PLP 2 nanogels, as above explained in Fig. 5a and b. Higher lactide contents in PLP nanogels resulted in sharper critical concentration point of thermosensitive PLP nanogels with increasing temperature. PLP 2 nanogels, which had higher lactide contents, became almost hydrophobic over 32 °C as described in Figs. 2 and 3a. Thus, PLP 1 nanogels, which had lower lactide contents, showed a larger difference in initial release between 37 and 42 °C than PLP 2 nanogels, even if release conditions of the two nanogel samples are the same under PBS. The results suggest that greater and faster drug release occurs at higher temperatures because the stronger hydrophobic interactions between the lactide portions of the PLP nanogels in the interior structures render a squeezing effect on the particles at higher temperature. This means that each particle became more compact at higher temperature, thus resulting in faster and easier release of drug amount. The results are similar to previous reports (Chung et al., 2000; Gref et al., 1994; Jeong et al., 1998; Na, Lee, Lee, & Bae, 2006). Drug-loaded PLP nanogels may be applicable as a long-term drug delivery system because the hydrolysis of PLLA grafted to pullulan may result in the disintegration of the nanogels.

3.6. In vitro cytotoxicity of DOX-loaded thermosensitive PLP 1 nanogels

We conducted an MTT assay to understand the thermo-responsive effects of DOX-loaded PLP 1 nanogels on killing cancer cells at 42 °C. The *in vitro* cytotoxicity to HeLa cells by DOX-loaded

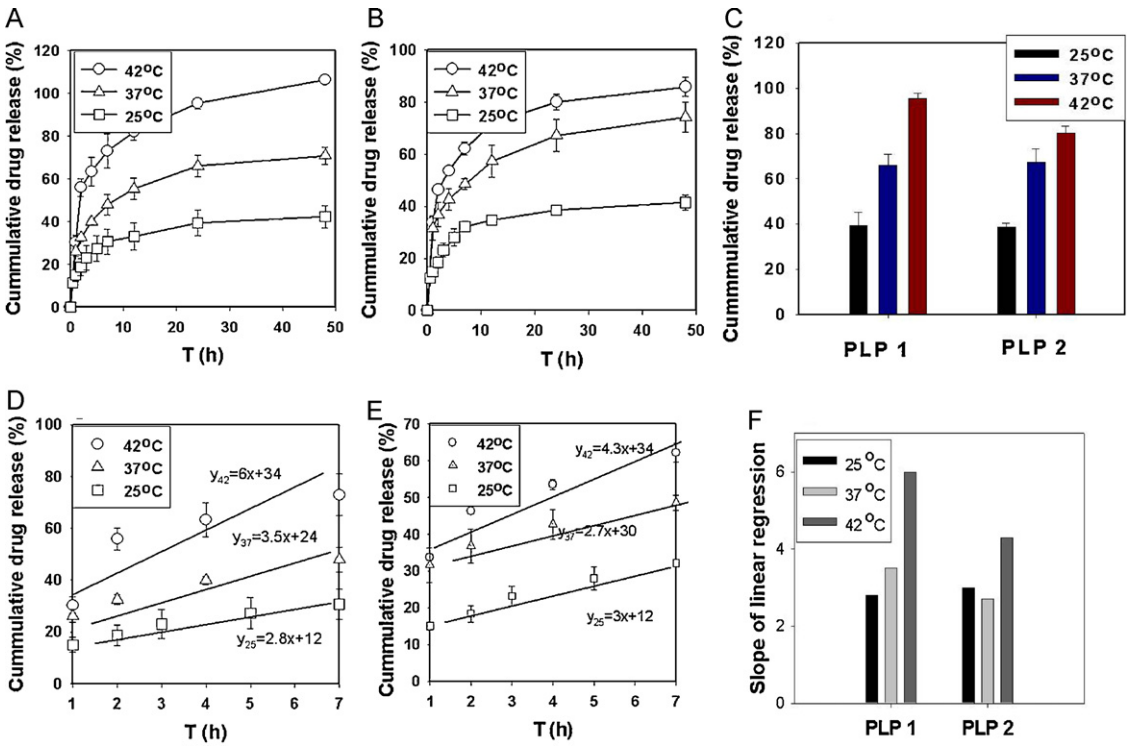

Fig. 5. DOX release kinetics from (a) PLP 1 and (b) PLP 2 nanogels at 25, 37 and 42 °C for 50 h ($n = 3$). (c) The cumulative DOX release amounts from PLP 1 and PLP 2 nanogels at 25, 37 and 42 °C for 24 h ($n = 3$). The initial DOX release profiles for the initial 7 h by a linear regression analysis from (d) PLP 1 and (e) PLP 2 nanogels at 25, 37 and 42 °C. (f) The slopes of the initial DOX release as determined by linear regression analysis from the PLP 1 and PLP 2 nanogels at 25, 37 and 42 °C. The results are reported as mean ± SD ($n = 5$).

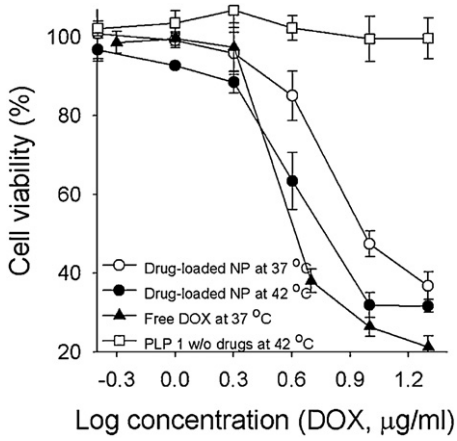

Fig. 6. The *in vitro* cytotoxicity of HeLa cells treated with free DOX, PLP 1 and DOX-loaded PLP 1 nanogels at 37 and 42 °C as a function of log concentration of DOX. The results are reported as mean ± SD ($n = 5$).

nanogels was investigated at 37 and 42 °C. The cells were exposed to DOX-loaded nanogels for 6 h at the indicated temperature and were additionally incubated for 24 h at 37 °C after removing the samples. Fig. 6 shows the cell viability of the HeLa cells treated with the DOX-loaded nanogels at 37 and 42 °C. Almost all of the cells treated with PLP 1 at 37 (data not shown) and 42 °C were alive, indicating that PLP is biocompatible. However, most of the cells treated with free DOX (1–2 μg/mL) were killed at 37 and 42 °C (data not shown) due to DOX cytotoxicity. In our previous report, the cytotoxicity over the DOX concentration range of 1 ng/mL to 10 μg/mL at the two temperatures was unchanged (Na et al., 2006). Different shapes were shown for the viabilities of the cells treated with DOX-loaded nanogels at the two temperatures. Much stronger cytotoxic effects due to DOX released from the nanogels were observed at 42 °C than at 37 °C, based on cell death. The 50% inhibitory concentration (IC_{50}) of DOX released from the PLP 1 nanogels was analyzed. The IC_{50} values were approximately 5.9 and 9.3 μg/mL at 37 and 42 °C, respectively. This finding indicates that the cytotoxicity at 42 °C was approximately 1.6 times higher than at 37 °C. The effect of the triggering temperature on the killing of the cancer cells by the DOX-loaded PLP 1 nanogels was expected because the amount of DOX released at 42 °C after 6 h was more than the amount at 37 °C. The more hydrophobic interior structure of the PLP 1 nanogels induced by the higher temperature facilitates the internalization of the DOX-loaded PLP 1 nanogels in the cells as observed with the pH-sensitive micelles (Na et al., 2003) and other temperature-sensitive micelles (Chung et al., 1999; Na et al., 2006).

4. Conclusions

In conclusion, we successfully prepared thermo-sensitive and self-assembled nanogels composed of hydrophilic pullulan grafted to hydrophobic PLLA as an anticancer drug delivery system. The DOX-loaded PLP nanogels showed more efficient long-term drug release and were effective for killing cancer cells at higher temperatures, suggesting facilitating their internalization in the cells. Thus, self-assembled PLP nanogels that can be triggered by temperature may be promising nanocarriers for a long-term drug delivery system in cancer treatments.

Acknowledgements

This research was financially supported by the Fundamental R&D Program for Core Technology of Materials, Ministry of Knowledge Economy, Republic of Korea, the Gyeonggi Regional Research Center (GRRC), the Ministry of Knowledge Economy (MKE), and the Korea Industrial Technology Foundation (KOTEF)

through the Human Resource Training Project for Strategic Technology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.08.061.

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