International Journal of Pharmaceutics 375 (2009) 163–169

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

A smart flower-like polymeric micelle for pH-triggered anticancer drug release

Kyung Taek Oh[a,1], Young Taik Oh[b,1], Nam-Muk Oh[c], Kwangmyung Kim[d], Don Haeng Lee[e], Eun Seong Lee[c,*]

[a] College of Pharmacy, Chung-Ang University, 221 Heukseok dong, Dongjak-gu, Seoul 155-756, South Korea
[b] Department of Diagnostic Radiology, Yonsei University College of Medicine, Seodaemun-ku Shinchon-dong 134, Seoul 120-752, South Korea
[c] Division of Biotechnology, The Catholic University of Korea, 43-1 Yeokgok 2-dong, Wonmi-gu, Bucheon-si, Gyeonggi-do 420-743, South Korea
[d] Biomedical Research Center, Korea Institute of Science and Technology, 39-1 Hawolgok-dong, Seongbuk-gu, Seoul 136-791, South Korea
[e] Department of Internal Medicine, Inha University, 253 Yonghyun-dong Nam-gu, Incheon, Gyeonggi-do 402-751, South Korea

ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 31 March 2009
Accepted 5 April 2009
Available online 11 April 2009

Keywords:
pH-sensitive flower-like micelle
3-Diethylamino propyl
isothiocyanato-L-lysine
pH-dependent drug release
Tumor targeting

ABSTRACT

Novel pH-responsive flower-like micelles were developed to provide the mechanism for pH-triggered drug release from drug carriers. The micelles (particle size: ~165 nm; critical micelle concentration (CMC): ~4 μg/ml), constructed from poly(N$^{\varepsilon}$-(3-diethylamino)propyl isothiocyanato-L-lysine)-*b*-poly(ethylene glycol)-*b*-poly(L-lactide) [poly(DEAP-Lys)-*b*-PEG-*b*-PLLA], were designed to have a self-assembled flower-like arrangement consisting of two hydrophobic blocks [deprotonated poly(DEAP-Lys) block and PLLA block] and a petal-like hydrophilic PEG block at physiological pH. As the pH decreases to slightly acidic pH (<pH 7.0), as in tumor extracellular pH (pH_e), the flower-like micelles undergo a change in the hydrophobicity of the micellar core. The protonation of poly(DEAP-Lys) changed the physical property of the polymer from hydrophobic to hydrophilic, resulting in disintegration of the micellar core. The co-presence of a pH-insensitive PLLA block in the micellar core affected the protonation of poly(DEAP-Lys), allowing the micelle to be stable at pH 7.0–7.4. In this study using doxorubicin (DOX) as the model drug, DOX release from the micelles accelerated in response to tumor pH_e.

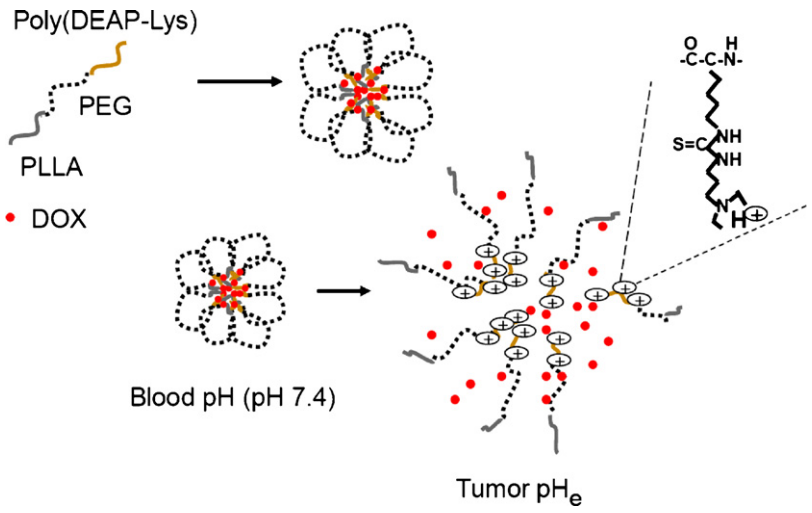
1. Introduction

Cancer is a leading cause of death in the world due to the heterogeneous nature of the disease (Ferrari et al., 2005; Oh et al., 2007), despite the advancement of tremendous technology in cancer prevention, diagnosis and treatment (Rothenberg et al., 2003; Ehdaie, 2007). Nanotechnology has been targeted as a promising system for the prevention and overcoming of cancer with early detection and chemotherapy (Ferrari, 2005; Ferrari et al., 2005; Oh et al., 2007; Heath and Davis, 2008). In chemotherapy, the goal is to develop therapeutic agents with high efficacy in the regression of tumor size while minimizing toxicity to the healthy tissues. Recently, nano-sized anticancer carriers using polymers have been frequently utilized due to their favorable properties, such as high drug loading capacity, controlled drug release and simple fabrication of targeted carriers (Kataoka et al., 2001; Lavasanifar et al., 2002). These polymeric nanovehicles can accumulate in tumor tissue much more than in normal tissue by the enhanced permeability and retention (EPR) effect (Maeda et al., 2000; Maeda, 2001). Additionally, nanovehicles that respond to environmental stimuli, i.e., pH, temperature and ultrasound, are being actively investigated and developed (Oh et al., 2007; Campbell, 2006; Park et al., 2007; Bossard et al., 2006).

Among them, pH-sensitive polymeric carriers have been used in targeted antitumor drug delivery (Lee et al., 2003, 2005a,b, 2007; Bae et al., 2005; Gillies and Frechet, 2005; Kim et al., 2005; Sawant et al., 2006; Oh et al., 2007; Kim et al., 2008; Oh and Lee, 2008; Lee and Youn, 2008) based on the intrinsic differences between various solid tumors and the surrounding normal tissues in terms of their relative acidity (Leeper et al., 1994). Typical tumor pH_e ranges from 7.0 to 6.5 in a xenograft animal model and appears to be more diffuse in clinical tumors. This acidic pH is thought to be caused by anaerobic respiration, subsequent glycolysis and/or a tumor phenotype (Tannock and Rotin, 1989; Stubbs et al., 2000; Gillies et al., 2004). In particular, tumor pH_e (but not in normal tissues) can be lowered 0.2–0.4 pH units by glucose given orally or intravenously to animals as well as patients (Leeper et al., 1994). Thus, pH 7.0–6.8 can be used as a safe and natural target for most solid tumors in animal models and also in clinical patients with a glucose challenge if necessary. In this respect, the development of drug-carriers that respond to the pH in tumors may be a favorable strategy to increase anticancer efficacy and to decrease the side effect in normal tissues.

* Corresponding author. Tel.: +82 2 2164 4921; fax: +82 2 2164 4865.
E-mail address: hejulu@hanmail.net (E.S. Lee).
[1] These authors equally contributed to this work as the first authors.

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.04.005



Scheme 1. Schematic diagram depicting the central concept.

This development would be an improvement from the drug carriers that depend on external stimuli such as hyperthermia and ultrasound and require to know the exact tumor sites in the body for tumor treatment (Oh et al., 2007).

Several anticancer carriers were developed using pH labile chemical bonds, such as hydrazone and acetal bonds (Bae et al., 2005; Gillies and Frechet, 2005; Sawant et al., 2006). These were shown to be sensitive to endosomal pH (~pH 6.0) but not to tumor pH_e. Meanwhile, polymeric micelles that underwent pH-induced micellar destabilization (Lee et al., 2003, 2005a,b, 2007, 2008a; Kim et al., 2005, 2008; Oh and Lee, 2008) were able to recognize small differences in pH such as with tumor pH_e (Oh et al., 2007).

In this study, we prepared flower-like pH-sensitive polymer micelles based on the poly(N^{ε}-(3-diethylamino)propyl isothiocyanato-L-lysine)-*b*-poly(ethylene glycol)-*b*-poly(L-lactide) [poly(DEAP-Lys)-*b*-PEG-*b*-PLLA] block copolymer. A 3-diethylaminopropyl group conjugated with a biodegradable polypeptide [poly(Lys)] (van Dijk-Wolthuis et al., 1997) is a promising component for constructing a pH-sensitive polymeric micellar carrier capable of targeting tumor pH_e. The pK_b of the 3-diethylaminopropyl group is 7.0–7.3 (Xue et al., 2007; Mundargi et al., 2008), similar to tumor pH_e.

As shown in Scheme 1, the micelle had a flower-like arrangement, as a result of the self-assembly of poly(DEAP-Lys) and biodegradable PLLA (Athanasiou et al., 1996) at the core and PEG on the shell. This flower-like ABC (A block: hydrophobic or hydrophilic with pH, B block: hydrophilic, C block: hydrophobic) micellar structure is predicted to be highly responsive to environment change (Lee et al., 2007). The poly(L-histidine) (M_n 5K)-*b*-PEG (M_n 2K)-*b*-PLLA (M_n 3K) flower-like micelles (Lee et al., 2007) showed triggered release of the anticancer drug in decreasing pH from 7.4 to 6.0, thus presenting physical transitions such as micellar swelling, micellar bridging, and micellar destabilization due to the protonation of poly(L-histidine) and the effect of the pH-insensitive PLLA block co-located in the micellar core. Similarly, the poly(DEAP-Lys)-*b*-PEG-*b*-PLLA micelle is also expected to be sensitive to external pH stimuli depending on the 3-diethylaminopropyl group. Of course, unlike poly(L-histidine)-*b*-PEG-*b*-PLLA micelles coupled with costly poly(L-histidine) preparation (Lee et al., 2003; Na et al., 2007), simple conjugation of the 3-diethylaminopropyl group with poly(Lys) may be more practical for the development of a pH-sensitive drug carrier for tumor treatment. We preferentially examined the pH-sensitive properties of the flower-like micelles with a fluorescence probe technique, particle size measurement, and a transmittance study. In addition, DOX-loaded flower-like micelles were evaluated by monitoring the pH-dependent drug release rate.

2. Materials and experimental design

2.1. Materials

3-Diethylaminopropyl isothiocyanate (DEAP), N^{ε}-benzyloxycarbonyl-L-lysine, triphosgene, N-(2-aminoethyl) maleimide (AEM), dimethylsulfoxide (DMSO), pyrene, doxorubicin·HCl (DOX·HCl), triethylamine (TEA), trifluoroacetic acid (TFA), $Na_2B_4O_7$, NaOH, HCl, 33% HBr in acetic acid, anhydrous dioxane, dichloromethane (DCM), anhydrous dimethylformamide (DMF), cystamine, triscarboxyethylphosphine (TCEP), N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), diethyl ether, ethanol, tetramethylsilane (TMS), and NaCl were purchased from Sigma–Aldrich (St. Louis, MO, USA). PLLA (M_n 1K)-*b*-PEG-COOH (M_n 2K) was prepared by conventional method. PLLA (M_n 1K)-*b*-PEG-COOH (M_n 2K) preactivated with NHS and DCC was reacted with cystamine and transferred to PLLA (M_n 1K)-*b*-PEG (M_n 2K)-SH by TCEP, as described in detail in our previous report (Lee et al., 2007).

2.2. Polymer synthesis

Poly(N^{ε}-benzyloxycarbonyl-L-lysine) was synthesized by ring-opening polymerization of N-carboxy-(N^{ε}-benzyloxycarbonyl)-L-lysine anhydride (L-lysine NCA) using AEM as an initiator. L-lysine NCA (30 mmol), prepared under anhydrous dioxane at 65 °C using triphosgene as described by van Dijk-Wolthuis et al. (1997), was dissolved in anhydrous DMF (20 ml) in the presence of AEM (1 or 2 mmol: corresponding with a molar NCA/initiator ratio of 30 or 15) (Lee et al., 2008b) and reacted for 3 days at room temperature. Poly(N^{ε}-benzyloxycarbonyl-L-lysine) was obtained after reprecipitation from excess diethyl ether. In order to remove the benzyloxylcarbonyl group from the polymer, poly(N^{ε}-benzyloxycarbonyl-L-lysine) (1 g) was dissolved in TFA (5 ml) and then was mixed with 33% HBr in acetic acid (5 ml) at room temperature for 30 min. Poly(L-lysine)·HBr [poly(Lys)·HBr] was recrystallized from excess diethyl ether/ethanol (50/50 vol.%). The removal of the benzyloxylcarbonyl group was confirmed by ^{1}H NMR (DMSO-d_6 with TMS) peaks: no peak at δ 7.40–7.21 (benzyl group) (data not shown). The molecular weight of poly(Lys) was estimated from ^{1}H NMR (DMSO-d_6 with TMS) peaks using the integration ratio of the peaks from the repeating unit

(—CH—, δ 4.72) and the initiator (—CH=CH—, δ 6.92) (data not shown). The molecular weight (M_n) of poly(Lys) was 2105 (2K) and 4069 (4K) Daltons. Subsequently, poly(Lys) (2K or 4K) (0.5 mmol) was reacted with DEAP (15 mmol or 30 mmol) in DMSO (20 ml) at room temperature for 2 days. After the reaction, the solution was transferred to a pre-swollen dialysis membrane tube (Spectra/Por; MWCO 2K) and was dialyzed against deionized water to remove non-reacted DEAP. The resulting solution was freeze-dried. The DEAP conjugation was confirmed by the presence of a ^{1}H NMR (DMSO-d_6 with TMS) peak at δ 1.2 (—CH_3, DEAP) (data not shown). The percent conjugation was 97 ± 2%, and the molecular weight of poly(N^{ε}-(3-diethylamino)propyl isothiocyanato-L-lysine) [poly(DEAP-lysine)] was 4890 (5K) and 9862 (10K) Daltons, as calculated by the comparison of the two ^{1}H NMR peaks [δ 4.72 (—CH—, repeating unit) and δ 1.2 (—CH_3, DEAP)] (data not shown). The coupling of poly(DEAP-lysine) and PLLA-*b*-PEG-SH (Lee et al., 2007; Lee et al., 2008b) was carried out in DCM overnight. Excess diethyl ether was added to form precipitate poly(DEAP-lysine)-*b*-PEG-*b*-PLLA. The complete coupling of poly(DEAP-lysine) and PLLA-*b*-PEG-SH was confirmed by the transfer of ^{1}H NMR at δ 6.92 (—CH=CH—, at terminal maleimide of poly(DEAP-lysine)) to δ 3.32 (—CH—CH_2—, at terminal maleimide of poly(DEAP-lysine)) (data not shown). The synthesis of poly(DEAP-Lys)-*b*-PEG-*b*-PLLA is outlined in Scheme 2.

2.3. Acid–base titration

The polymer or NaCl (as a control) in deionized water (30 mmol/l) was adjusted to pH 12 with 1N NaOH. The solution including block copolymers was titrated by stepwise addition of 0.1N HCl solution to obtain the pH profile.

2.4. Preparation of polymeric micelle

The polymer (50 mg) dissolved in DMSO (5 ml) was transferred to a pre-swollen dialysis membrane tube (Spectra/Por; MWCO 8K) and dialyzed against HCl (or NaOH)–$Na_2B_4O_7$ buffer solution (pH 7.4–6.0, 10 mM) for 24 h. The outer phase was replaced three times with fresh $Na_2B_4O_7$ buffer solution. The solution was then mixed with phosphate buffer saline (PBS) solution. The solution was subsequently lyophilized after filtering through a 0.8 μm syringe filter. After freeze-drying of the solution for 2 days, the micelle powder was obtained. The yield (wt.%) of the micelles was calculated by weighing the freeze-dried micelle powder. The yield (wt.%) of the micelles was 92 ± 3 wt.%.

2.5. DOX loading

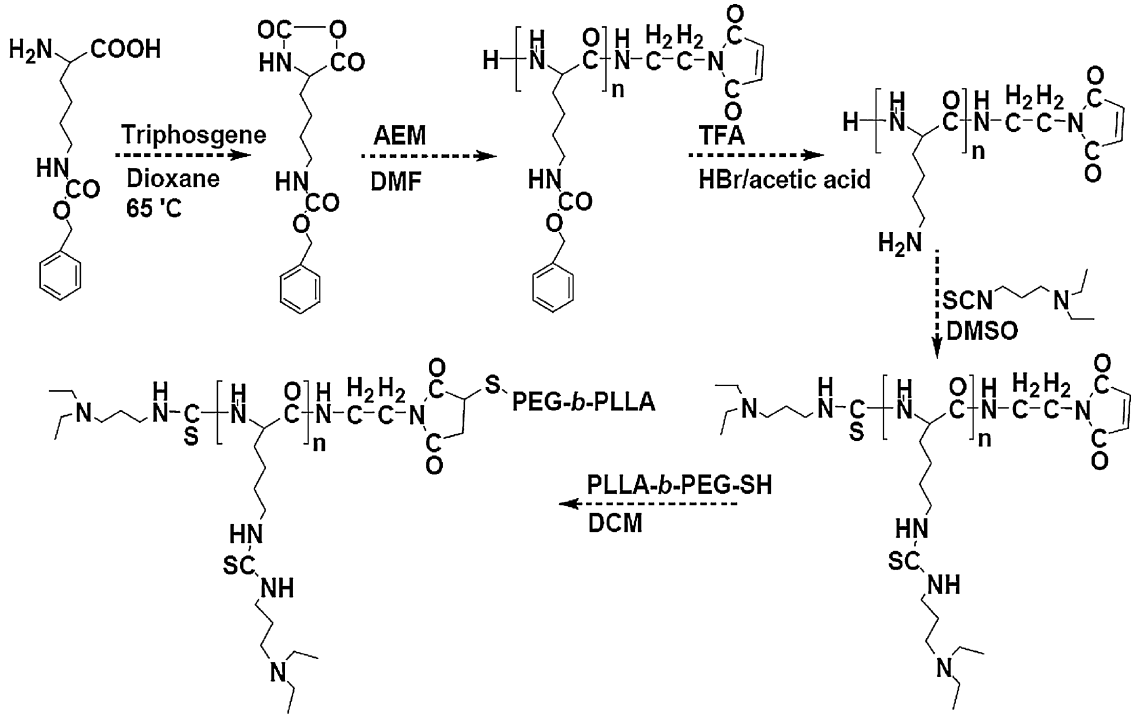
DOX·HCl was stirred with a 2 mole ratio of TEA in DMSO overnight to detach the HCl salt from DOX (Lee et al., 2005a). A total of 20 mg of polymer with DOX (4 mg) in DMSO (2 ml) was dialyzed against HCl (or NaOH)–$Na_2B_4O_7$ buffer solution (pH 8.0, 10 mM) for 24 h to fabricate DOX-loaded polymeric micelles. The outer phase of the dialysis bag containing DOX and polymer was replaced three times, and non-encapsulated free DOX was removed during this procedure. The measurement of the DOX concentration in micelles was performed with a UV–visible spectrophotometer by measuring the UV absorbance at 481 nm of the DOX-loaded polymeric micelles dissolved in DMSO/HCl (or NaOH)–$Na_2B_4O_7$ buffer mixture solution (95: 5 vol.%). DOX loading efficiency was 80 ± 3 wt.%, calculated by dividing the loading DOX content by the feeding DOX content (Lee et al., 2005a,b).

2.6. Photon correlation spectroscopy (PCS) by Zetasizer

Dynamic light scattering (Malvern Instrument) was used to determine the particle size distribution of the polymeric micelles (0.1 g/l) in PBS solution (pH 7.4–6.0, ion strength: 0.15).

2.7. Transmittance of micellar solution

Transmittance was measured using a Varian CARY 1E UV–vis spectrophotometer. Before the test, all micellar solutions (0.1 g/l, ionic strength: 0.15) with different pHs (pH 7.4–6.0) were stabilized



Scheme 2. Overall scheme for the synthesis of poly(DEAP-Lys)-*b*-PEG-*b*-PLLA.

at 37 °C for 4 h. Relative transmittance of the micellar solution was measured in the selected pH range with respect to transmittance at pH 7.4.

2.8. CMC analysis

All fluorescence measurements for CMC determination were performed using a Shimadzu RF-5301PC Spectrofluorometer. Pyrene was used as the fluorescence probe to obtain the steady-state fluorescence spectra of the micelles (Lee et al., 2003). For the preparation of each micellar solution, a freeze-dried micelle sample was dispersed in HCl (or NaOH)–$Na_2B_4O_7$ buffer solution (pH 7.4–6.0, ionic strength: 0.15). The initial pH of each micelle solution used for this study was tuned to the diafilteration pH used in micelle fabrication. The micellar solution (1×10^{-4} to 1×10^{-1} g/l) was mixed with pyrene at 50 °C for 6 h, yielding a final pyrene concentration of 6.0×10^{-7} M. The pyrene emission at λ_{ex} 339 nm was recorded. The CMC was estimated by plotting I_1 (intensity of first peak) of the emission spectra profile against the log of the micelle concentration. The CMC at each pH was determined by the crossover point of low polymer concentrations on this plot (Lee et al., 2003).

2.9. DOX release behavior

For the drug release test, DOX-loaded micelle solution (0.5 ml) was added into a dialysis membrane tube (Spectra/Por MWCO 5 kDa), immersed in a vial containing fresh PBS (10 ml, ionic strength: 0.15) with different pHs (pH 7.4–6.0), and put through mechanical shaking (100 rev./min) at 37 °C. The outer phase of the dialysis bag was withdrawn and replaced with fresh buffer solution at predetermined time intervals in order to keep the sink condition of DOX. The measurement of the DOX concentration was performed with a UV–visible spectrophotometer as described above.

3. Results and discussion

3.1. Characterization of poly(DEAP-Lys)-b-PEG-b-PLLA

A new ABC type of polyelectrolyte block copolymer was composed of poly(DEAP-Lys)-*b*-PEG-*b*-PLLA. Herein, the poly(DEAP-Lys) block was prepared by conjugating a pH-sensitive DEAP to a biocompatible polypeptide [poly(Lys), M_n 2K, 4K]. Coupling of the thiol groups at the end of PLLA (1K)-*b*-PEG (2K) to the maleimide of the poly(DEAP-Lys) block was employed for the preparation of poly(DEAP-Lys)-*b*-PEG-*b*-PLLA (Scheme 2). To determine the pK_b of block copolymers, acid–base titration of poly(DEAP-Lys)-*b*-PEG-*b*-PLLA was performed (Fig. 1). The apparent pK_b of both polymers was around 6.5. The pH-buffering region of the polymers was at pH 6–7. However, the buffering capacity of the three block copolymers was greater in the case of high molecular weight poly(DEAP-Lys) (10K). These results suggest that the poly(DEAP-Lys) block exhibited hydrophobicity due to deprotonation of DEAP attached to poly(Lys) at physiological pH and changed to a hydrophilic block below pH 7.0 through protonation of the tertiary amine in DEAP. Therefore, the ABC type of three block copolymer may show a flower-like micelle with a hydrophobic core composed of deprotonated poly(DEAP-Lys) blocks, a hydrophobic PLLA block and a hydrophilic PEG shell block at physiological pH. However, as pH decreased, protonation of poly(DEAP-Lys) resulted in destabilization of the micelles due to the change of hydrophobicity of the polymer (Scheme 1).

3.2. pH-dependent micelle formation

The micelles of poly(DEAP-Lys)-*b*-PEG-*b*-PLLA were prepared in PBS buffers (pH 7.4–6.0) by the diafiltration method. The particle size of the formed micelle was measured using dynamic light scattering technique and was found to be about 165 nm with a unimodal size distribution at the pH range of 7.4–7.0 (Fig. 2). However, at less than pH 7.0, the size of the polymer particles could not be determined due to low intensity in the nano-size range (data not shown), indicating micelle disintegration. This is comparable with poly(L-histidine) (M_n 5K)-*b*-PEG (M_n 2K)-*b*-PLLA (M_n 3K) flower-like micelles (Lee et al., 2007) that increased the particle size as the pH decreased, resultant from micellar swelling and bridging. It was assumed that the relative high molecular weight of PLLA (M_n 3K) resisted micellar disintegration upon protonation of poly(L-histidine) co-present in the micellar core rather than the induction of micelle swelling and random aggregation. It appears that the PLLA (M_n 1K) block used in this study had no effect on micelle aggregation.

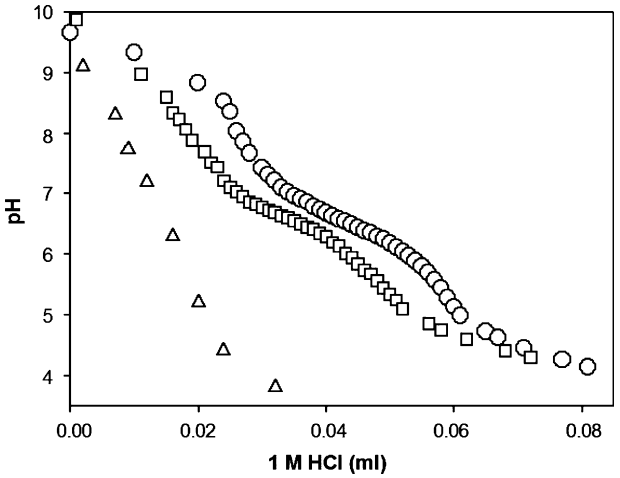


Fig. 1. The pH-profile of poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) (○), poly(DEAP-Lys) (5K)-*b*-PEG (2K)-*b*-PLLA (1K) (□), and NaCl (□) by acid–base titration. The average value from the triplicate titrations was plotted.

In Fig. 3, the poly(DEAP-Lys)-*b*-PEG-*b*-PLLA micelles showed a sharp increase in relative transmittance at pH 7.0–6.0, which was consistent with the results of size measurement. Conversely, the PEG-*b*-PLLA micelle had no significant difference in relative transmittance at all pHs tested. This result may be due to the protonation of poly(DEAP-Lys) in the micellar core, leading to micelle disintegration. In particular, the poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA micelle increased micelle destabilization at less than pH 7.0 compared to the poly(DEAP-Lys) (5K)-*b*-PEG-*b*-PLLA micelles. This elucidated that block copolymers with a long poly(DEAP-Lys) chain

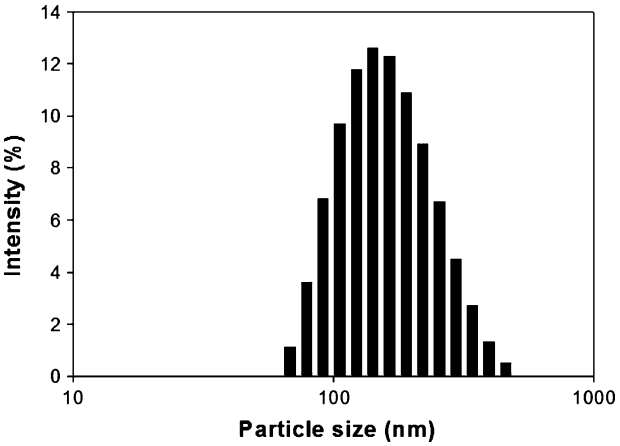


Fig. 2. The particle size distributions of poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micelles (pH 7.4).

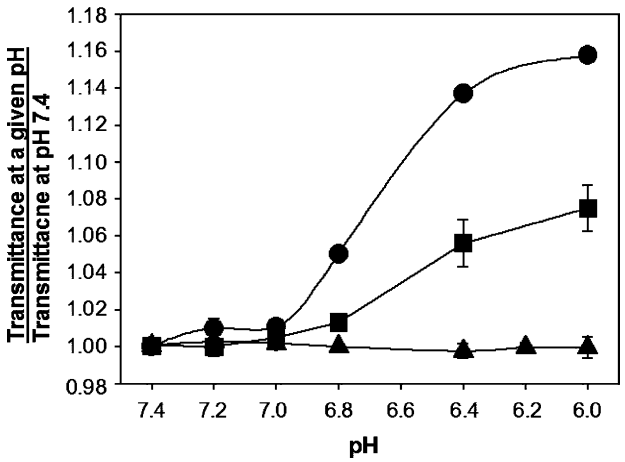


Fig. 3. The relative transmittance change according to the pH of each micellar solution [poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micelle (●), poly(DEAP-Lys) (5K)-*b*-PEG (2K)-*b*-PLLA (1K) micelle (■), and PEG(2K)-*b*-PLLA(1K) micelle (▲)] after 4 h. Each micellar solution (ionic strength: 0.15) was kept to 0.1 g/l ($n = 3$).

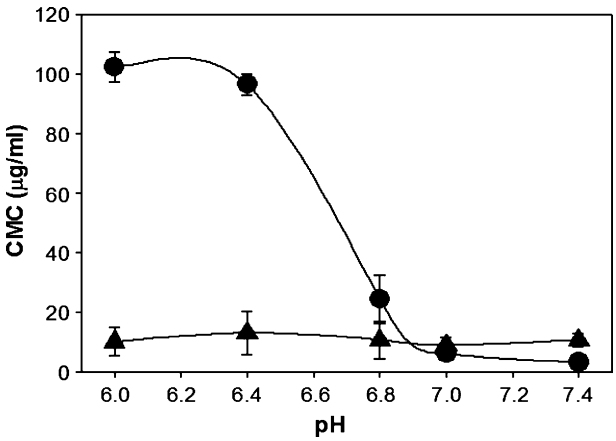


Fig. 4. The CMC change of each micelle [poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micelle (●) and PEG(2K)-*b*-PLLA(1K) micelle (▲)] ($n = 3$).

can have increased hydrophobic blocks via deprotonation, providing a stable structure at physiological pH and can be hydrophilic in a hydrophilic–lipophilic balance (HLB) as the number of charged DEAP units increase in the copolymer. This result suggested that the poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA micelle can be effectively used as an anticancer drug carrier by forming a stable structure in blood circulation and normal tissues (pH 7.4). The destabilization of carriers that sensitively respond to tumor pH_e resulted in anticancer drug accumulation around tumor tissues. Therefore, based on the above results, the poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA micelle was selected for further study.

3.3. The CMC of poly(DEAP-Lys) (10K)-b-PEG-b-PLLA dependent on pH

Micelle formation by the self-assembly of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA was studied using CMC measurement determined by fluorometry in the presence of pyrene as a fluorescent probe. Fig. 4 presented the CMC of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA and PEG-*b*-PLLA (as a control) as a function of pH. The CMC of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA at pH 7.4 showed a relatively low value of 4 μg/ml compared to those of PEG-*b*-PLLA (12 μg/ml) and other polymeric amphilphiles (5–1000 μg/ml) (Kabanov et al., 2002). This low CMC may be due to increased hydrophobicity caused by the two hydrophobic blocks of deprotonated poly(DEAP-Lys) (10K) and PLLA (1K). In contrast, decreased pH induced a micropolarity change of the micellar core and resulted in an increase of the CMC of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA. Interestingly, at pH 6.8 (>pK_b), the CMC of partially charged poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA was represented by a low value (~23 μg/ml), and at pH 6.4 (<pK_b), the CMC of fully charged poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA drastically increased (~95 μg/ml). However, the CMC of PEG-*b*-PLLA did not change at any of the pHs. These results suggested that the pH-sensitivity of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA is attributable to the pH-dependent change in amphiphatic properties. Here, control of the molecular weight of each ABC block may open the way to the development of other controlled drug release carriers.

3.4. pH-dependent DOX release

To study the correlation between the release rate of encapsulated drugs from micelles and pH-sensitive micelle destabilization, DOX was selected as a model anticancer agent and loaded into the micelles of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA and PEG-*b*-PLLA (as a control) by the diafiltration method (Lee et al., 2003; Lee et al., 2007). Fig. 5(a) showed the relative transmittances of the two-micelle solutions as a function of pH. The micelle solution

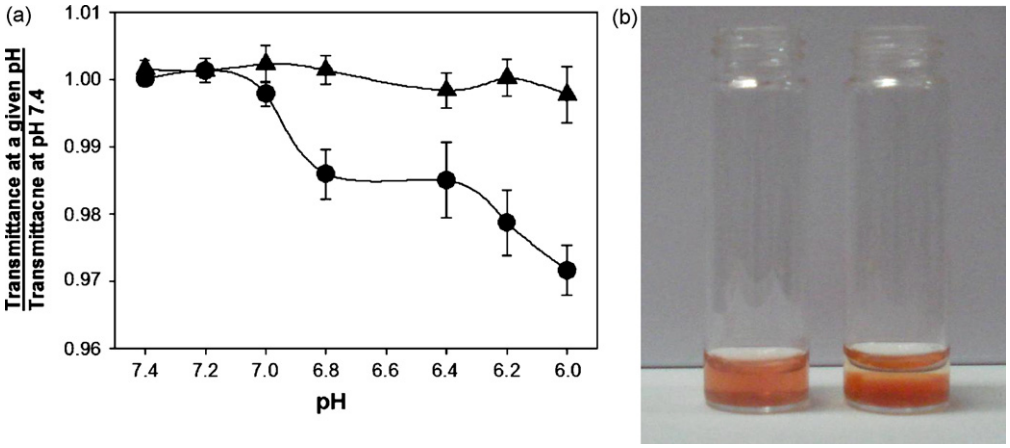


Fig. 5. (a) The relative transmittance change of DOX-loaded polymeric micelles [poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micelle (●) and PEG(2K)-*b*-PLLA(1K) micelle (▲)] after 2 h. ($n = 3$) (b) Photographic image of DOX-loaded poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micellar solution (pH 6.8, right) and PEG(2K)-*b*-PLLA(1K) micelle (pH 6.8, left) at room temperature after 4 h. Each micellar solution (ionic strength: 0.15) was kept to 0.1 g/l. DOX content in each micelle was fixed to 16 wt.%.

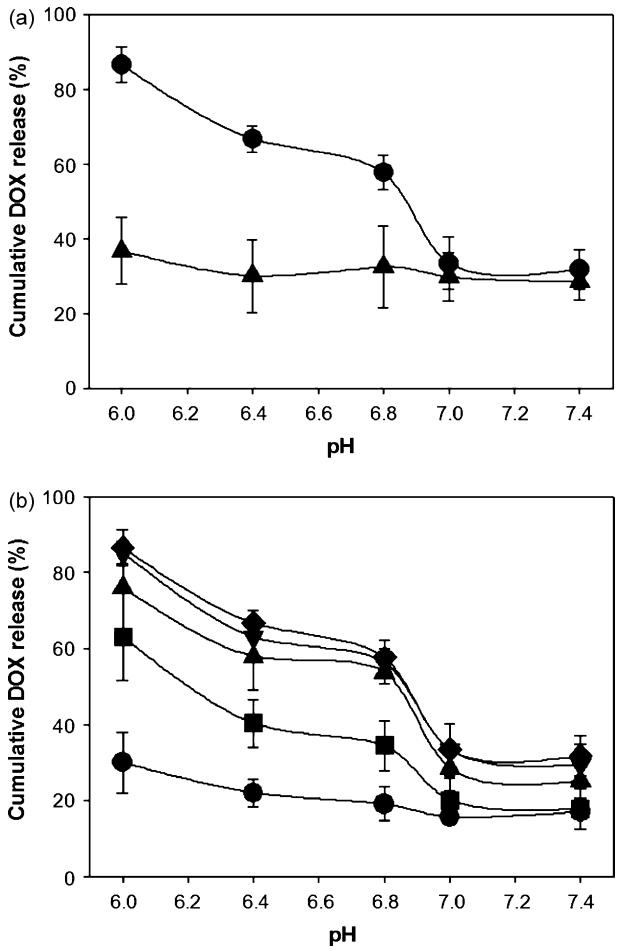


Fig. 6. (a) The pH-dependent DOX release from poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micelle (●) and PEG(2K)-*b*-PLLA(1K) micelle (▲) ($n = 3$) after 24 h (b) Time-dependent DOX release from poly(DEAP-Lys) (10K)-*b*-PEG (2K)-*b*-PLLA (1K) micelle at each pH (after 1 h (●), 2 h (■), 4 h (▲), 6 h (▼), and 24 h (♦) incubation) ($n = 3$).

of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA represented the decreased transmittance; the micelle led to the release of DOX from the micelles as pH decreased, resulting in drug precipitation (Fig. 5(b)). Fig. 6 showed the pH-dependent cumulative DOX release from the poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA and PEG-*b*-PLLA (as a control) micelles over the course of 24 h. Both DOX-loaded micelles exhibited a ca. 35% drug release at pH 7.4. The pH-sensitive poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA micelles gradually increased DOX release from the micelles as the pH decreased and showed a 90% drug release at pH 6.0, while pH-insensitive PEG-*b*-PLLA micelles did not change the release rate of DOX from the micelles in any of the pHs. The kinetic profiles of DOX release from poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA micelles indicated that the differences in DOX release in regard to pH were remarkable (Fig. 6(b)). For 1 h, a small initial burst release of DOX (20–30 wt.%) from the micelles was observed at all pHs. However, at pH 7.4, drug release from the micelles demonstrated a small increase to 35% for 24 h, whereas, the release of DOX apparently accelerated at pHs lower than pH 7.0 and only within 4 h, appeared to reach a ca. 80% of plateau at 24 h. In particular, these micelles responded to small pH differences between 7.0 and 6.8, which indicated that the micelles constructed from poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA were more sensitive to a little change in tumor pH_e compared to physiological pH. This pH-sensitivity of poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA micelles may be due to the destabilization of the micellar core resulting from the ioniziation of poly(DEAP-Lys) below pH 7.0. These results confirmed that the PLLA-*b*-PEG-*b*-polyHis micelles distinguish tumor extracellular pH from physiological pH by accelerating drug release rate.

4. Conclusion

Poly(DEAP-Lys) (10K)-*b*-PEG-*b*-PLLA was constituted into a novel pH-sensitive flower-like polymeric micelle. The micelles (~165 nm in diameter) showed triggered release of DOX in decreasing pH from 7.0 to 6.8. This flower-like micelle system may provide maximal therapeutic efficacy in the tumor site due to increased drug accumulation while having low probability of drug accumulation in normal tissues resulting in reduced side effects. The results of this study justify the need of further investigation.

Acknowledgements

This work was supported by the Research Fund, 2009 of the Catholic University of Korea, by the Research Grant funded by the Gyeonggi Regional Research Center (GRRC), by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean government (MEST) (NO. R01-2008-000-20197-0), and by a grant to the Intramural Research Program of the KIST.

References

Athanasiou, K.A., Niederauer, G.G., Agrawal, C.M., 1996. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. Biomaterials 17, 93–102.
Bae, Y., Nishiyama, N., Fukushima, S., Koyama, H., Yasuhiro, M., Kataoka, K., 2005. Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced in vivo antitumor efficacy. Bioconjug. Chem. 16, 122–130.
Bossard, F., Aubry, T., Gotzamanis, G., Tsitsilianis, C., 2006. pH-tunable rheological properties of a telechelic cationic polyelectrolyte reversible hydrogel. Soft Matter 2, 510–516.
Campbell, R.B., 2006. Tumor Physiology and delivery of nanopharmaceuticals. Anti-Canc. Agents Med. Chem. 6, 503–512.
Ehdaie, B., 2007. Application of nanotechnology in cancer research: review of progress in the national cancer institute's alliance for nanotechnology. Int. J. Biol. Sci. 3, 108–110.
Ferrari, M., 2005. Cancer nanotechnology: opportunities and challenges. Nat. Rev. Cancer 5, 161–171.
Ferrari, M., Barker, A., Downing, G., 2005. A cancer nanotechnology strategy. NanoBio Technol. 1, 129–131.
Gillies, E.R., Frechet, J.M., 2005. pH-responsive copolymer assemblies for controlled release of doxorubicin. Bioconjug. Chem. 16, 361–368.
Gillies, R.J., Raghunand, N., Garcia-Martin, M.L., Gatenby, R.A., 2004. pH imaging. A review of pH measurement methods and applications in cancers. IEEE Eng. Med. Biol. Mag. 23, 57–64.
Heath, J.R., Davis, M.E., 2008. Nanotechnology and Cancer. Annu. Rev. Med. 59, 251–265.
Kabanov, A.V., Batrakova, E.V., Alakhov, V.Y., 2002. Pluronic block copolymers for overcoming drug resistance in cancer. Adv. Drug Deliv. Rev. 54, 759–779.
Kataoka, K., Harada, A., Nagasaki, Y., 2001. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv. Drug Deliv. Rev. 47, 113–131.
Kim, D., Lee, E.S., Oh, K.T., Gao, Z.G., Bae, Y.H., 2008. Doxorubicin-loaded polymeric micelle overcomes multidrug resistance of cancer by double-targeting folate receptor and early endosomal pH. Small 4, 2043–2050.
Kim, G.M., Bae, Y.H., Jo, W.H., 2005. pH-induced micelle formation of poly(histidine-co-phenylalanine)-block-poly(ethylene glycol) in aqueous media. Macromol. Biosci. 5, 1118–1124.
Lavasanifar, A., Samuel, J., Kwon, G.S., 2002. Poly(ethylene oxide)-block-poly(L-amino acid) micelles for drug delivery. Adv. Drug Deliv. Rev. 54, 169–190.
Lee, E.S., Gao, Z., Kim, D., Park, K., Kwon, I.C., Bae, Y.H., 2008b. Super pH-sensitive multifunctional polymeric micelle for tumor pH(e) specific TAT exposure and multidrug resistance. J. Control. Release 129, 228–236.
Lee, E.S., Kim, D., Youn, Y.S., Oh, K.T., Bae, Y.H., 2008a. A virus-mimetic nanogel vehicle13. Angew. Chem. Int. Ed. 47, 2418–2421.
Lee, E.S., Na, K., Bae, Y.H., 2005a. Doxorubicin loaded pH-sensitive polymeric micelles for reversal of resistant MCF-7 tumor. J. Control. Release 103, 405–418.
Lee, E.S., Na, K., Bae, Y.H., 2005b. Super pH-sensitive multifunctional polymeric micelle. Nano Lett. 5, 325–329.
Lee, E.S., Na, K., Bae, Y.H., 2003. Polymeric micelle for tumor pH and folate-mediated targeting. J. Control. Release 91, 103–113.

Lee, E.S., Oh, K.T., Kim, D., Youn, Y.S., Bae, Y.H., 2007. Tumor pH-responsive flower-like micelles of poly(L-lactic acid)-*b*-poly(ethylene glycol)-*b*-poly(L-histidine). J. Control. Release 123, 19–26.

Lee, E.S., Youn, Y.S., 2008. Poly(benyzl-L-histidine)-*b*-poly(ethylene glycol) micelle engineered for tumor acidic pH-targeting, *in vitro* evaluation. Bull. Korean Chem. Soc. 29, 1539–1544.

Leeper, D.B., Engin, K., Thistlethwaite, A.J., Hitchon, H.D., Dover, J.D., Li, D.J., Tupchong, L., 1994. Human tumor extracellular pH as a function of blood glucose concentration. Int. J. Radiat. Oncol. Biol. Phys. 28, 935–943.

Maeda, H., 2001. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv. Enzyme Regul. 41, 189–207.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284.

Mundargi, R.C., Babu, V.R., Rangaswamy, V., Patel, P., Aminabhavi, T.M., 2008. Nano/micro technologies for delivering macromolecular therapeutics using poly(d,L-lactide-co-glycolide) and its derivatives. J. Control. Release 125, 193–209.

Na, K., Lee, E.S., Bae, Y.H., 2007. Self-organized nanogels responding to tumor extracellular pH: pH-dependent drug release and *in vitro* cytotoxicity against MCF-7 cells. Bioconjug. Chem. 18, 1568–1574.

Oh, K.T., Lee, E.S., 2008. Cancer-associated pH-responsive tetracopolymeric micelles composed of poly(ethylene glycol)-*b*-poly(L-histidine)-*b*-poly(L-lactic acid)-*b*-poly(ethylene glycol). Polym. Adv. Technol. 19, 1907–1913.

Oh, K.T., Yin, H., Lee, E.S., Bae, Y.H., 2007. Polymeric nanovehicles for anticancer drugs with triggering release mechanisms. J. Mater. Chem. 17, 3987–4001.

Park, K.H., Song, H.C., Na, K., Bom, H.S., Lee, K.H., Kim, S., Kang, D., Lee, D.H., 2007. Ionic strength-sensitive pullulan acetate nanoparticles (PAN) for intratumoral administration of radioisotope: ionic strength-dependent aggregation behavior and 99mTechnetium retention property. Colloids Surf. B. 59, 16–23.

Rothenberg, M.L., Carbone, D.P., Johnson, D.H., 2003. Improving the evaluation of new cancer treatments: challenges and opportunities. Nat. Rev. Cancer 3, 303–309.

Sawant, R.M., Hurley, J.P., Salmaso, S., Kale, A., Tolcheva, E., Levchenko, T.S., Torchilin, V.P., 2006. SMART drug delivery systems: double-targeted pH-responsive pharmaceutical nanocarriers. Bioconjug. Chem. 17, 943–949.

Stubbs, M., McSheehy, P.M., Griffiths, J.R., Bashford, C.L., 2000. Causes and consequences of tumour acidity and implications for treatment. Mol. Med. Today 6, 15–19.

Tannock, I.F., Rotin, D., 1989. Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res. 49, 4373–4384.

van Dijk-Wolthuis, W.N.E., van de Water, L., van de Wetering, P., van Steenbergen, M.J., van den Bosch, J.J.K., Schuyl, W.J.W., Hennink, W.E., 1997. Synthesis and characterization of poly-L-lysine with controlled low molecular weight. Macromol. Chem. Phys. 198, 3893–3906.

Xue, W., Diao, H., Chen, X., Wang, C., Chen, J., Junfeng, Z., 2007. An efficient vector for gene delivery: α, β-poly(3-dimethylaminopropyl-D, L-aspartamide). Eur. J. Pharm. Biopharm. 66, 327–333.