Poly(benzyl-L-histidine)-*b*-Poly(ethylene glycol) Micelle Engineered for Tumor Acidic pH-Targeting, *in vitro* Evaluation

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A polymeric micelle, based on the poly(benzyl-L-histidine)-*b*-poly(ethylene glycol) (polyBz-His-*b*-PEG) diblock copolymer, was designed as a tumor-specific targeting carrier. The micelles (particle size: 67-80 nm, critical micelle concentration (CMC); 2-3 μg/mL) were formed from the diafilteration method at pH 7.4, as a result of self-assembly of the polyBz-His block at the core and PEG block on the shell. Removing benzyl (Bz) group from polyBz-His block provided pH-sensitivity of the micellar core; the micelles were physically destabilized in the pH range of pH 7.4-5.5, depending on the content of the His group free from Bz group. The ionization of His group at a slightly acidic pH promoted the deformation of the interior core. These pH-dependent physical changes of the micelles provide the mechanism for pH-triggering anticancer drug (*e.g.*, doxorubicin: DOX) release from the micelle in response to the tumor's extracellular pH range (pH 7.2-6.5).

Key Words : Poly(benzyl-L-histidine), Acidic pH targeting, Triggering drug release

Introduction

Recently medical application of a polymeric micelle system[1-4] has burst onto the scene with challenging innovations, designed to enable spatial site-specific drug delivery.[1,2,5,6] Especially, in tumor treatment, effectively regressing tumor while minimizing damage to healthy tissues is undeniably linked to this drug delivery system.[7] For example, delivery of high doses of therapeutic agents to target tumor sites and minimal accumulation of therapeutic agents in healthy tissue has been achieved using nano-sized micellar carrier systems.[8] This is primarily attributed to passive accumulation in solid tumors by the enhanced permeability retention (EPR) effect[9] of the micellar carriers with nano-size (20-200 nm),[7] followed by passive diffusional release of the drug in the extracellular space and/or active internalization into the tumor cells *via* various entry mechanisms.[7,10] Herein, to further improve local high-dose tumor therapy, drug-carrying polymeric micelles need to be inert, meaning minimal interactions with biological components and negligible drug release while circulating in the blood stream.[7] However, upon reaching their target sites, the micelles may need to switch their nature to induce enhanced drug release kinetics.[7,11] The switching property could be endowed by employing stimuli-sensitive components when constructing nanocarriers. Hyperthermia,[12,13] ultrasound,[14] specific enzyme-induced cleavable bonds,[15-17] and specific ligands or antibodies[18-21] have been intensively investigated for this purpose using micelle systems.

Recently, a pH-sensitive polymeric micelle system has been potentially used for targeted antitumor drug delivery,[5,6,22-31] based on the observation that one of the consistent differences between various solid tumors and the surrounding normal tissue is the acidity of the surrounding tissue.[32] The extracellular pH (pH_e) in most tumors is more acidic (pH, 6.5-7.2) than in normal tissues.[33,34] In all measurements (involving either invasive or noninvasive methods) of the pH_e of human and animal solid tumors, more than 80% of all measured values consistently fell below a pH of 7.2.[33,34] It is interesting to note that this physical difference in pH is intrinsic in distinguishing diseased tissue (*i.e.*, tumor) from normal tissue. In this respect, the development of pH-sensitive drug-carriers is unique for tumor targeting compared to the cultivation of drug carriers depending external stimuli (such as hyperthermia and ultrasound).[7] In particular, the polyHis[5,6,29] is a promising component for constructing a pH-sensitive polymeric micellar carrier capable of targeting tumor cells. The imidazole ring of a polyHis-block (pK_b ~ 7.0) has lone pairs of electrons on the unsaturated nitrogen that endow it with pH-dependent amphoteric properties[5]; polyHis is lipophilic above pK_b but lipophobic (resulting from ionization of polyHis) below pK_b.[5] These properties of polyHis have been utilized for the development of tumor acidic pH-sensitive polymeric micelle system.[5,6,29] Considering that the micelle system with pH-labile chemical bonds (such as hydrazone and acetal) between the drug and the micelle, responds only to pH 5.0-5.5,[2,26-28] a polyHis-based mixed micelle system may be more useful for tumor acidic pH (pH 7.2-6.5) targeting. In previous studies, pH-sensitive mixed micelles based on polyHis-*b*-PEG and poly(L-lactic acid)-*b*-PEG (PLA-*b*-PEG) block copolymers contributed improved anticancer activity and enhanced drug release when they were triggered to release their cargo by tumor-associated acidic pH.[5,6,29] However, despite its significant potential, this system depended on complicated interactions between the polymers in the mixed micellar core. Additionally, homogenous mixing of the two block copolymers was required for the micelles to exhibit reproducible behavior.[5,6,29]

To improve upon this design, therefore, we prepared pH-sensitive polymeric micelles from one block copolymer,

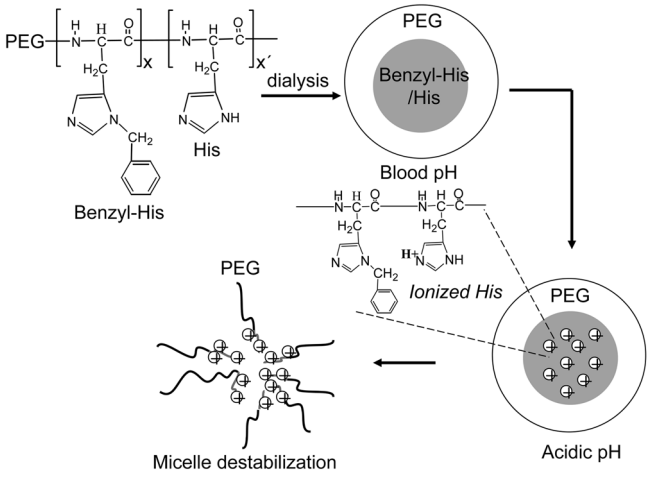

Figure 1. Schematic diagram depicting the central concept.

polyBz-His-*b*-PEG. The benzyl (Bz) group (used as a protecting group for polyHis synthesis)[5,6,29] remaining in polyHis, as shown in Figure 1, may be useful in tuning drug release behaviors of the polymer micelle with pH.[5,6,22-31] For proof of this concept, we examined the pH-sensitive properties of the polyBz-His-*b*-PEG micelles and monitored pH-dependent cell cytotoxicity against human breast carcinoma cells (MCF-7).

Experimental

Materials. Triethylamine (TEA), dimethylsulfoxide (DMSO), dimethylformamide (DMF), anhydrous liquid ammonia, metallic sodium, HCl, NaOH, ammonium chloride, diethyl ether, tetrazolium salt MTT, L-glutamine, *n*-propyl galate, glycerol, and DOX·HCl were purchased from Sigma-Aldrich (St.Louis, MO, USA). Penicillin-streptomycin, Tris-HCl (pH 8.4), fetal bovine serum (FBS), 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution, and RPMI1640 medium were purchased from Gibco (Uxbridge, UK). PolyBz-His-*b*-PEG (repeating unit of Bz-His = 28, M_n of PEG = 2000) was synthesized by ring-opening co-polymerization of His *N*-carboxyanhydride (NCA)·HCl, followed by PEG conjugation, as described in detail in our previous reports.[5,6,29]

Controlling deprotection of Bz group. The protecting Bz group of His was deprotected using metallic sodium and anhydrous liquid ammonia.[29] Briefly, the suspension of polyBz-His-*b*-PEG (1 g) in anhydrous liquid ammonia (20 ml) was mixed with metallic sodium (0 (His0), 0.03 (His1), 0.05 (His2), 0.10 (His3), 0.20 (His4) g, Table 1) over a period of 15 min. After evaporation of liquid ammonia, the residue was dissolved in 1 M HCl (10 mL). The 1 M HCl solution was extracted three times with diethyl ether (10 mL). The 1 M HCl solution was neutralized with 3 M NaOH solution and the precipitate was filtered. The precipitate was three times washed with diethyl ether and was dried *in vacuo* for 2 days. His or Bz-His mol% in polymer was estimated from the ^{1}H-NMR spectrum (DMSO-d_6 with TMS) using the integration ratio of the peaks from the imidazole ring unit (δ7.6, -N-C**H**=) and the Bz unit (δ7.21, -C=C**H**-).

pK_b determination from acid-base titration. The polymer and NaCl (control) were dissolved in 30 mL of deionized water (30 mmol/L) and the solution was adjusted to pH 12 with 1 N NaOH. The diluted solution was titrated by stepwise addition of 0.1 N HCl solution to obtain the titration profile.[29]

Preparation of polymeric micelle. The polymer (20 mg) dissolved in DMSO (20 mL) was transferred to a pre-swollen dialysis membrane tube (Spectra/Por; MWCO 15 K) and dialyzed against HCl (or NaOH)–$Na_2B_4O_7$ buffer solution (pH 7.4, 20 mM) for 24 hours. The outer phase was three times replaced with fresh $Na_2B_4O_7$ buffer solution. The solution was then mixed with phosphate buffer saline (PBS) solution (pH 7.4). The solution (ion strength: 0.15) was subsequently lyophilized after filtering through a 0.8 μm syringe filter. The yield (wt.%) of micelles was 91 ± 7 wt.%.

Fluorescence spectroscopy. For the measurement of steady-state fluorescence spectra, the pyrene solution (6.0×10^{-2} M) in acetone was added to deionized water to give a pyrene concentration of 12×10^{-7} M. The solution was then distilled under vacuum at 60 °C for 1 hour to remove acetone from the solution. The acetone-free pyrene solution was mixed with the polymeric micelles ($1 \times 10^{-4} \sim 1 \times 10^{-1}$ g/L), yielding final pyrene concentration of 6.0×10^{-7} M. The change of the intensity ratio (I_{337}/I_{333}) of the pyrene with polymer concentration was plotted from excitation spectra from 300 to 360 nm at emission wavelength 390 nm.[35] The CMC (μg/mL) at pH 7.4 was determined from crossover point at low polymer concentration on this plot.[35] In order to quantify the polarity around the pyrene molecules retained in the polymeric micelle (0.1 g/L) with pH, the intensity ratio (I_{337}/I_{333}, higher ratios means a less polar environment) of the pyrene at different pHs (pH 7.4-5.5) was measured.

Particle size analysis. Photon correlation spectroscopy (PCS) was conducted using a Zetasizer 2000 (Malvern Instruments, USA) with a He-Ne Laser beam at a wavelength of 633 nm, and a fixed scattering angle of 90°. The

Table 1. Characterization of polymer and micelle system

	His content (mol%) in polyBz-His	BZ-His content (mol%) in polyBz-His	pK_b	CMC (μg/mL) of micelle at pH 7.4	Particle size of micelle at pH 7.4
His0	0	100	–	2.1 ± 0.6	67 nm
His1	48	52	6.10	2.6 ± 0.4	70 nm
His2	71	29	6.54	2.5 ± 0.3	78 nm
His3	86	14	6.83	2.6 ± 0.5	76 nm
His4	100	0	7.02	2.8 ± 0.4	80 nm

polymeric micelles (0.1 g/L, ion strength: 0.15) were then exposed to different pH values (pH 7.4-5.5) at 37 °C for 24 hours before measurement of the particle size and particle size distribution.[5,36]

DOX loading. DOX·HCl (1 mole) was stirred with TEA (2 mole) in DMSO for overnight, in order to detach HCl salt and make DOX lipophilic.[5,6] And then DOX (2 mg) was blended with polymer (10 mg) in DMSO (20 mL). The mixture was transferred to a preswollen dialysis membrane (Spectra/Por molecular weight cut-off 15 K) and dialyzed against HCl (or NaOH)-$Na_2B_4O_7$ buffer solution (pH 7.4, 20 mM) for 24 hours. The medium was exchanged several times and the content inside the dialysis tube was subsequently lyophilized. The amount of entrapped DOX was determined by measuring the UV absorbance at 481 nm of the drug-loaded polymeric micelles dissolved in DMSO. The DOX loading efficiency was 80-90 wt.% calculated by dividing the loaded DOX concentration with the initial DOX concentration.

DOX release. DOX-loaded micelles in PBS pH 7.4 solution (1 mL, ionic strength: 0.15) in a dialysis membrane tube (Spectra/Por MWCO 15 K) were immersed in a vial containing fresh PBS (20 mL) with different pHs (pH 7.4-5.5) at 37 °C, including mechanical shaking (100 rev./min). DOX concentration was determined with a UV/Visible spectrophotometer.[5,6]

Cell cytotoxicity. Human breast carcinoma MCF-7 cells (from Korean Cell Line Bank) were maintained in RPMI-1640/PBS medium (Gibco) with 0.5 M PBS, 2 mM L-glutamine (Sigma), 5% penicillin-streptomycin (Gibco), 10 % fetal bovine serum (Gibco) in a humidified incubator at 37 °C and 5% CO_2 atmosphere. Before testing, cultured cell (1×10^5 cells/mL) monolayers were harvested by 0.25% (w/v) trypsin-0.03% (w/v) EDTA solution (Gibco). For cytotoxicity test, free DOX (5 μg/mL), equivalent DOX-loaded micelles (DOX 5 μg/mL, polymer 33 μg/mL), blank micelles (polymer 0-200 μg/mL, no DOX) in RPMI-1640/ PBS medium were added to the medium-removed 96-well palate. The pH of culture medium was readjusted with 0.1 N HCl or 0.1 N NaCl.[5,23] During 24 hours incubation, no considerable pH shift in the culture medium was observed. After the incubation, chemosensitivity was measured using tetrazolium salt MTT assay.[5,6,23]

Results and Discussion

pH-sensitive polymer. PolyBz-His was prepared by the ring-opening polymerization of His *N*-carboxyanhydride.[29] PolyBz-His was coupled with PEG by simple 1,3-dicyclohexylcarbodiimide (DCC) chemistry.[5,29] The deprotection of the Bz group was managed to evaluate the effect of Bz on the pH-sensitivity of polyHis. The pH-dependent amphoteric properties of the His component may be affected by the pH-insensitive lipophilic Bz component, similar to our previous polyHis-based mixed micelle application.[5] Physical blending of the pH-insensitive block (poly(L-lactic acid)) to polyHis micelle shifted the micelle destabilizing pH to below pH 7.0, depending on the amount of pH-insensitive block.[5] Table 1 shows that increasing the Bz content reduced the pK_b value of the polymer. The pK_b of His2 and His3 were found to be 6.83 and 6.54, respectively (Table 1).

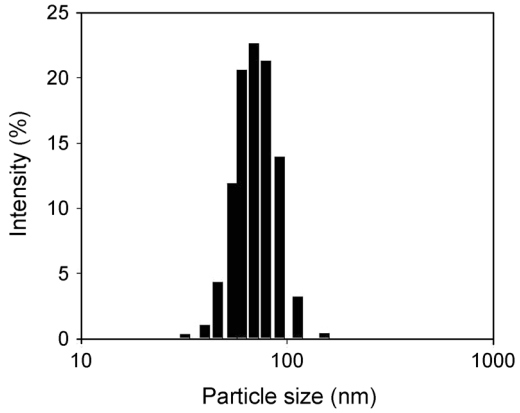

Figure 2. The particle size distribution of His1.

pH-sensitivity of micelles. The micelles constructed by the self-assembling process of these polymers presents relatively low CMCs (2-3 μg/mL) and average 67-80 nm sizes at pH 7.4. All micelles showed a narrow particle size distribution as shown in Figure 2.

The CMC and particle size of the micelles decreased as the Bz-His content increased, which may be due to the enhanced lipophilicity[35] of the micellar core by the presence of Bz. In addition, 67-80 nm sizes of micelles will be useful to the extravasation of micelles due to the EPR effect.[4]

The ionization of His in the micellar core will facilitate micelle destabilization (Figure 1). Non-ionized His (*i.e.*, Bz-His) is expected to shift the micelle destabilizing pH to a lower value, reducing ionization of the lipophilic core. Figure 3 shows that the particle size of the His1, His2, His3, His4 micelles decreased with a pH drop. The particle size of the His3 micelles decreased from 80 nm at pH 7.0 to less than 10 nm at pH 6.5, which exhibits disintegration of the polymeric micelles at a low pH. While, the His0 micelles (without ionizable His groups) present a negligible particle

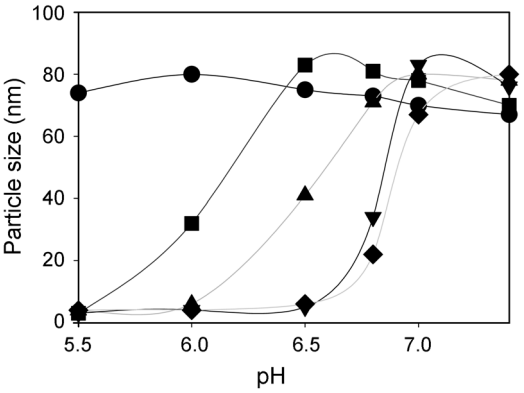

Figure 3. The particle size change (n = 3) of His0 (●), His1 (■), His2 (▲), His3 (▼), His4 (◆) with pH of the solution. The micellar solution (ionic strength: 0.15) was kept to 0.1 g/L.

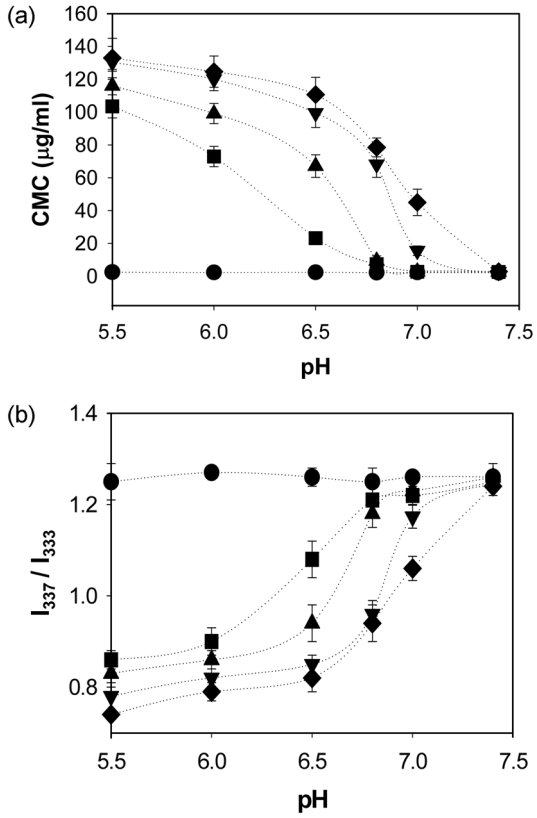

Figure 4. (a) CMC change (n = 3) of His0 (●), His1 (■), His2 (▲), His3 (▼), His4 (◆) and (b) a plot of the intensity ratio I_{337}/I_{333} (n = 3) of each micelle (was kept to 0.1 g/L) with pH of the solution (ionic strength: 0.15).

size change with pH.

The micelle formation by self-assembly of polyBz-His-*b*-PEG was monitored by fluorometry in the presence of pyrene as a fluorescent probe.[35] Pyrene is highly hydrophobic and therefore preferentially migrates into the micellar core in an aqueous solution.[35] When pyrene is located in a non-polar environment (*i.e.*, micellar core), it shows strong fluorescence. However, in a polar environment (*i.e.*, outside of micellar core), it shows a weak fluorescent intensity and a shift of the excitation peak. The change in the intensity ratio (I_{337}/I_{333}) of the pyrene extracted from the excitation graph indicates whether the polymer forms the micelle or exists as the unimer.[5,25,35] The CMCs of polyBz-His-*b*-PEG micelles ranged to 2-3 mg/mL (Table 1), as estimated from the crossover point of the intensity ratio (I_{337}/I_{333}) at low polymer concentration.[35] These CMCs of the micelles were changed with pH (Figure 4(a)). The CMC of His2 micelles were remarkably elevated below pH 6.8. The CMC of His3 micelles significantly increased below pH 7.0. Similarly, the CMC of His4 micelles with 100 mol% His group, increased below pH 7.4, while the His0 micelles showed a negligible CMC change. It is evident that the ionization of the His groups in the polymer at a lower pH level causes a reduction in lipophilicity, leading to an increase in CMC. In addition, higher His amount in the polymer induced CMC change at a higher pH. These behaviors of the micelles were also supported by the monitoring of micropolarity in the micellar core with pH (Figure 4(b)). The decrease of the pyrene intensity ratio (I_{337}/I_{333}) under the same polymer concentration (0.1 g/L) (Figure 4(b)) demonstrated that the His2 micelles or His3 micelles experienced a sharp increase in polarity as the pH decreased from 6.8 to 6.5, or from 7.0 to 6.8, respectively. In addition, the His1 or His4 micelles present a constant increase as the pH decreased from 6.8 to 6.0, or from 7.4 to 6.5, respectively. The amount of His in the micellar core affected micelle disintegration. A decrease in His (or an increase in Bz-His) content decreased micelle disintegrating pH, reducing the ionization of the micellar core.

Figure 5(a) shows the cumulative DOX release from the DOX-loaded polymeric micelles with pH. The pH-depen-

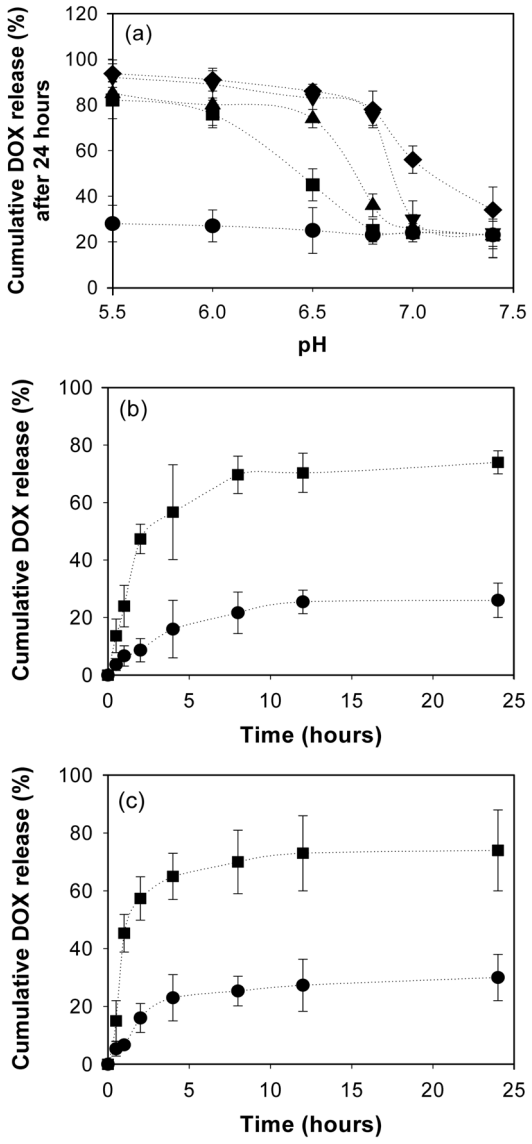

Figure 5. (a) The pH-dependent cumulative DOX release from the His0 (●), His1 (■), His2 (▲), His3 (▼), and His4 (◆) with pH (n = 3) after 24 hours. The time-dependent cumulative DOX release from (b) His2 (pH 7.0: ●, pH 6.5: ■) and (c) His3 (pH 7.0: ●, pH 6.8: ■) with pH (n = 3).

dent micelle properties facilitated pH-induced DOX release from the micelles. The His0 micelles showed no pH-dependent DOX release. However, the His1 micelles released 30 wt.% of DOX at pH 6.8 and 45 wt.% of DOX at pH 6.5. The His2 micelles released 20-36 wt.% of DOX at pH 7.4-6.8 and 76 wt.% of DOX at pH 6.5 (*e.g.*, endosomal pH targeted for cytosolic drug delivery[5-7]). The His3 micelles showed a desirable tumor extracellular pH-dependency such that 20-30 wt.% of DOX was released at pH 7.4-7.0 and 75 wt.% of DOX at pH 6.8 (*e.g.*, tumor extracellular pH), as well as 80 wt.% of DOX at pH 6.5. Unlike His0, His1, His2, and His3 micelles that released approximately 20 wt.% of DOX at pH 7.4, the His4 micelles released 35 wt.% of DOX at pH 7.4, indicating relative instability of His4 micelles at blood pH.[5] In addition, the DOX release pattern with the time followed nearly first-order kinetics and reached a plateau in 8 hours (His2) or 4 hours (His3) (Figure 5(b)-5(c)).

These results mean that the micelles responded a small pH difference (between pH 7.0 and 6.5); accelerated DOX release from the micelles destabilized with the drop in pH.

***In vitro* cell viability.** Figure 6(a) shows pH-dependent cytotoxicity of the DOX-loaded micelles against MCF-7 cells. The acidic pH enhances the DOX release rate of the His2 or His3 micelles and makes the DOX-loaded His2 or His3 micelle effective for killing MCF-7 cells. In an *in vitro* cytotoxicity test, these micelle systems demonstrated limited cell killing effect at pH 7.4-7.0, but cell viability was greatly reduced as the pH decreased from 6.8 to 6.5 (His2 micelles for endosomal pH targeting) or from 7.0 to 6.8 (His3 micelles for tumor extracellular pH targeting). The viability of MCF-7 cells treated with the DOX-loaded His2 micelle was 89% at pH 7.4, 75% at pH 6.8, and 23% at pH 6.5. On the other hand, the viability of MCF-7 cells treated with the DOX-loaded His3 micelle was 88% at pH 7.4, 78% at pH 7.0, and 24% at pH 6.8. No cytotoxicity was observed up to 200 mg/ml of the blank micelles for MCF-7 cells after 24 hours incubation, regardless of culture medium pH (data not shown). In addition, the change in pH from 7.4 to 6.5 may influence cellular physiology and cell viability. However, acidic pH is a favorable environment for tumor cells, although acidic pH has opposite effect on normal cells.[37] The cell viability expressed in this study is relative to those at each pH in the culture medium without DOX. Furthermore, noticeable difference in cell viability by pH was not observed when MCF-7 cells were treated with free DOX (data not shown). Consequently, the DOX-loaded His2 or His3 micelles presented tumor cell killing effectiveness in a pH-dependent manner. These results confirmed the idea that the simply prepared polyBz-His-*b*-PEG micelles respond to minute differences in pH and discriminates the tumor extracellular pH and endosomal pH by drug release rate. In particular, this micelle system may be useful to replace both the polyHis-based mixed micelle approach[5] presenting complicated behaviors of polymers in the mixed micellar core[30] and the polyHis-based flower-like micelle system[22] with the ability to only target tumor extracellular pH. Of course, the proof of this hypothesis will require further *in vivo* investigations including tumor regression and toxicological studies.

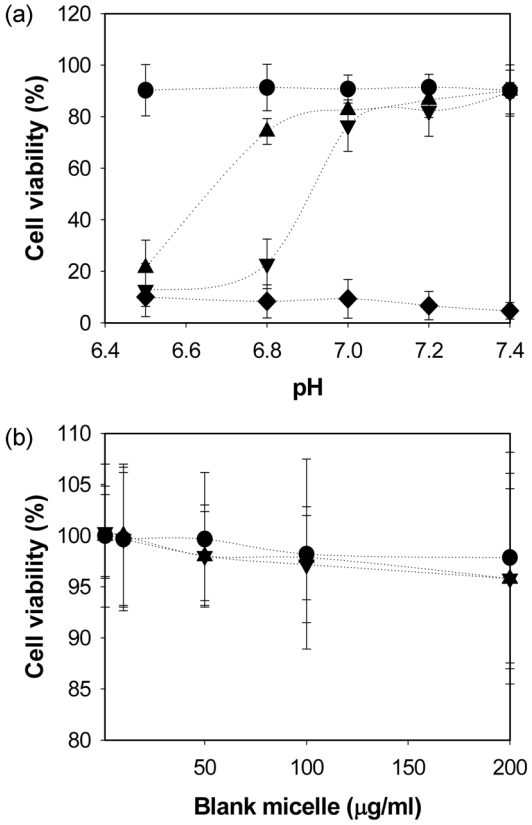

Figure 6. (a) pH-dependent MCF-7 cell viability of DOX-loaded (a) His0 (●), His2 (▲), His3 (▼) (equivalent DOX: 5 μg/mL), and free DOX (◆) (DOX: 5 μg/mL) with pH (n = 7) (b) *in vitro* cell toxicity of blank micelles (His0 (●), His2 (▲), and His3 (▼)) (n = 7).

Conclusions

PolyBz-His-*b*-PEG micelles with different His content was optimized to have pH-responsive properties for slightly acidic solid tumors. The micelles showed DOX triggering release as decreasing pH from 7.4 to 5.5. The micelles with 86 mol% of His and 14 mol% of Bz-His triggered DOX release at pH 6.8 that is similar to tumor extracellular pH. The micelles with 71 mol% of His and 29 mol% of Bz-His allowed triggering DOX release at pH 6.5 that is similar to endosomal pH. This pH effect was demonstrated by the pH-dependent cell cytotoxicity results of the DOX-loaded micelles. This approach may provide maximal therapeutic efficacy at the tumor site, resulting in tumor pH-specific drug accumulation, while preserving the lowest probability of drug accumulation in normal tissues for reduced side effects. Of course, this hypothesis requires further investigation.

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