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## Doxorubicin-Loaded Polymeric Micelle Overcomes Multidrug Resistance of Cancer by Double-Targeting Folate Receptor and Early Endosomal pH

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### Abstract

An optimized, pH-sensitive mixed micelle system conjugated with folic acid was prepared to challenge multidrug resistance (MDR) in cancers. The micelles were composed of poly(histidine (His)-*co*-phenylalanine (Phe))-*b*-poly(ethylene glycol) (PEG) and poly(L-lactic acid) (PLLA)-*b*-PEG-folate. Core-forming, pH-sensitive hydrophobic blocks of poly(His-*co*-Phe) of varying composition were synthesized. The composition-dependent pK values of poly(His-*co*-Phe) (Mn=5,000–5,500 Da) were examined. The size and critical micelle concentration were evaluated as a function of pH. The pH sensitivity of the micelles was roughly controlled by the copolymer composition, and its fine tuning to early endosomal pH was achieved by blending PLLA(3K)-*b*-PEG(2K)-folate, especially in the presence of a basic anticancer drug, doxorubicin (DOX).

To prove the efficacy of the micellar system, *in vitro* tests including cell viability, folate receptor-mediated endocytosis, and endosomolytic activity were conducted against both wild-type (A2780) and DOX-resistant ovarian carcinoma cell lines (A2780/DOX<sup>R</sup>). From the physicochemical properties and *in vitro* results, a mixed micelle system composed of poly(His-*co*-Phe (16 mole%))-*b*-PEG (80 wt%) and PLLA-*b*-PEG-folate (20 wt%) was selected to target early endosomal pH. DOX-loaded (ca. 20 wt%) micelles effectively killed both wild-type sensitive and MDR cancer cell lines through an instantaneous high dose of DOX in the cytosol, resulting from active internalization, accelerated DOX release triggered by endosomal pH, and a disruption of endosomal pH.

### Keywords

histidine; pH-sensitive micelle; folate; early endosome; multidrug resistance

### Introduction

One of the major challenges in successful cancer chemotherapy is to overcome intrinsic or acquired multidrug resistance (MDR) in tumors.[1-5] MDR mechanisms are associated with a number of efflux pumps including P-glycoprotein (Pgp)[6-9] and multidrug-resistant protein (MRP),[10] altered expression of antiapoptotic protein Bcl-2[11] and tumor suppressor protein p53,[12] changes in topoisomerase II activity,[13] and modifications in glutathione S-

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transferase[14] and others. Sequestration[15] and subsequent exocytosis[16,17] are additional factors that limit the therapeutic efficacy of basic anticancer agents. In an effort to reverse MDR, Pgp modulators have been central in the pharmaceutical industry as well as in academic research.[18-20] Despite great promise in *in vitro* tests, their use in the clinical setting had extremely limited success due to the nonspecific nature of Pgp modulators, their binding properties, and pharmacokinetic interference of anticancer drugs.[21-24]

Other approaches using poly(ethylene oxide)-*b*-poly( $\epsilon$ -caprolactone) nanoparticles[25] and polymeric nonionic surfactants such as Pluronic[26] and polyalkylcyanoacrylate nanoparticles[27] have been studied to overcome MDR. However, their validity *in vivo* is not well identified.

Because the resistance of clinical tumors is not often explained by Pgp overexpression, various other mechanisms associated with multicellular events, soluble factors, and cellular adhesion have been proposed for clinically relevant MDR.[28-30] These imply that tackling a single mechanism may not be practical in overcoming MDR. Most living organisms, including bacteria and healthy organs in the body, share common MDR mechanisms expressed by cancer cells. This defense system has evolved for billions of years to survive toxic environmental chemicals.[31] Despite such survival mechanisms, resistant cancer cells eventually die upon exposure to 10- to 100-fold higher drug concentrations than would kill sensitive counterparts, [32-34] yielding to the hypothesis that there may be a limit for cells to defend themselves against cytotoxic chemicals by various MDR mechanisms.

This hypothesis has been partially proven through the design of pH-sensitive micelles carrying DOX.[35] The micelles were composed of poly(L-histidine)-*b*-PEG-folate[36] or polyHis-*b*-PEG blended with poly(lactic acid) (PLLA)-*b*-PEG-folate.[37] The former micelle was destabilized around pH 7.2, while the latter micelles were destabilized at a pH range of 6.5 to 7, depending on the amount of blended PLLA-*b*-PEG.[36,37] All the micelles tested were effective against sensitive cells; however, to kill MDR cells, the micelle was required to be equipped with an active cellular entry tool and to be destabilized at a pH lower than the extracellular conditions (pH 6.5–7.2) of most solid tumors. The endosomolytic function of polyHis is essential for quick drug delivery to the cytosol.[38-40] Although the blending approach of two polymer components made the destabilization pH as low as 6.6 by increasing PLLA-*b*-PEG up to 40 wt%, further increases led to a loss of pH sensitivity of the mixed micelles.[37]

The primary objective of this study was to create drug-loaded micelles that are destabilized at an early endosomal pH of 6 to avoid the tumor extracellular pH ( $pH_e$ ) as well as lysosomal pH ranges. Destabilization of the micelle before entry into cells causes drug loss due to premature release, and destabilization at lysosomal pH may induce unwanted toxicity due to leakage of digestive lysosomal enzymes.[41-43] For these reasons, we designed pH-sensitive polymeric micelles that precisely target the early endosomal pH ( $\sim 6$ ) between  $pH_e$  and lysosomal pH.

## Results and discussion

### 3.1. Characterization of poly(histidine-co-phenylalanine)

The characterization of synthesized poly(His-*co*-Phe) (PHP) is summarized in Table 1. The  $pK_b$  value of a PHP is an important factor in determining the pH sensitivity of resulting micelles. It is known that when monomers with ionizable groups are polymerized or attached to a polymer, the  $pK$  values are shifted.[44] The apparent  $pK_b$  values, estimated from inflexion points in each acid-base titration curve, of PHP(10), PHP(16), PHP(22), and PHP(27) were  $\sim 6.7$ ,  $\sim 6.3$ ,  $\sim 5.7$ , and  $\sim 4.8$ , respectively. A hydrophobic environment is known to lower the local dielectric constant and to accordingly weaken the ionization tendency of an ionizable

group.[45,46] The copolymerization with Phe shifts the  $pK_b$  of the imidazole group in the His residue to a lower pH. It is also noted that increasing Phe content in the copolymer decreases the buffering zone of the copolymer caused by His.

### 3.2. Poly(His-co-Phe)-b-PEG micelle

In order to evaluate the pH sensitivity of the micelles, critical micelle concentrations (CMC), micelle size, and transmittance of micelle solutions were measured as a function of pH. The micelles constructed from PHP(10)-*b*-PEG, PHP(16)-*b*-PEG, PHP(22)-*b*-PEG, and PHP(27)-*b*-PEG were termed PHSM(10), PHSM(16), PHSM(22), and PHSM(27), respectively. In Figure 1(a), all micelles presented CMCs below 20  $\mu\text{g/mL}$  from pH 7 to 8. However, PHSM(10) and PHSM(16) showed significantly elevated CMC at pH 6.5 to  $\sim 85 \mu\text{g/mL}$  and  $\sim 50 \mu\text{g/mL}$ , respectively, indicating that both micelles were slightly destabilized. This destabilization is due to the conversion of nonionized histidine residues to hydrophilic ones by the protonation of the imidazole groups.

In contrast, the low CMCs of PHSM(22) and PHSM(27) were maintained at  $\sim 14 \mu\text{g/mL}$  and  $\sim 4 \mu\text{g/mL}$  at pH 6.5 and  $\sim 20 \mu\text{g/mL}$  and  $\sim 8 \mu\text{g/mL}$  at pH 6.0, respectively. This observation suggests that PHSM(10) and PHSM(16) showed appropriate pH sensitivity between pH 6.5–7, while PHSM(22) and PHSM(27) were stable below pH 6.0.

PHSM formed by a dialysis method at pH 9.0 had different sizes, depending on the composition of the PHP block. The diameter decreased from 170 nm to 110 nm as the Phe content increased. The hydrophobic nature of Phe and interactions between aromatic rings appeared to be responsible for the compact micelles.[47] Figure 1(b) reports the pH-dependent size reduction of the micelles. The particle size of PHSM(10) decreased sharply between pH 7.4 and 6.5; however, PHSM(16) reduced its size gradually between pH 7.4 and 6.0. PHSM(22) also showed a gradual reduction in size at pH 6.5–5.5, while PHSM(27) had no significant difference in particle size at all pHs tested. The result is consistent with pH-dependent CMC changes.

The transmittance of each micelle solution, which is relevant to the size of the micelle, was also monitored as a function of pH (Figure 1(c)). PHSM(10) had a transmittance transition point at pH 7.0, with PHSM(16) at pH 6.5. The transmittance transitions of PHSM(22) and PHSM(27) occurred at pH 5.8 and 5.2, respectively.

Taken together, the pH sensitivity may be controlled by the incorporation of phenylalanine comonomers into the hydrophobic core block.

### 3.3. Mixed micelle composed of poly(His-co-Phe)-b-PEG and PLLA-b-PEG

To achieve triggered drug release at early endosomal pH, there is the potential to synthesize core blocks with detailed compositions of His and Phe; however, this is not practical because of complex polymerization, purification, and characterization processes. A mixed micelle approach was previously used to control the pH sensitivity of polyHis-*b*-PEG micelles,[42] and the same method was adopted for the fine-tuning of pH-sensitivity. A candidate block copolymer of PHP(16)-*b*-PEG was selected because its micelle (PHSM(16)) was stable at pH 7 but unstable at pH 6.5. The destabilization pH is potentially adjusted by blending different amounts of PLLA-*b*-PEG (5, 10, and 20 wt%). The micelles formed by blending are denoted as m-PHSM(5, 10 and 20%) hereafter.

Figure 2(a) shows the changes in the micelle size originally formed at pH 8 as a function of pH. The m-PHSM(5%) increased in size below pH 6.5, the m-PHSM(10%) below pH 6, the m-PHSM(20%) sharply below pH 5.5. It is notable that PHP-*b*-PEG micelles showed reduced sizes as the pH fell, while in the case of mixed micelles, the average size increased. This is due

to the dissociation of PLLA-*b*-PEG from the micelles caused by incompatibility with ionized PHP blocks, followed by aggregation of PLLA-*b*-PEG. The particle size distribution was bimodal (the box in Figure 2 (a)). The smaller size distribution was 1–10 nm in size, and the bigger size distribution was 400–500 nm. This large size distribution could be due to the aggregation of PLLA-*b*-PEG after destabilization of mixed micelle at acidic pH. Previously, the study by Haiqin Yin et al.[48] proved the homogeneously-mixed core performed by using Differential scanning calorimetry (DSC) techniques. In this study, the presence of a single T<sub>g</sub> of mixed micelle in a broad composition range indicated miscibility of the poly(LHistidine)/poly(L-Lactide) blends.

The fluorescence intensity using pyrene was measured against pH using the mixed micelles. The fluorescence intensity drop was interpreted by the destabilization of the micelle core. For m-PHSM(20%), the major change in the intensity occurred between pH 6.0 and 5.5, for m-PHSM(10%) between pH 7.0 and 6.0, and for m-PHSM(5%) between pH 7.4 and 6.5. The destabilizing pH of each mixed micelle coincided with the result monitored by the change in size.

### 3.4. pH-dependent drug release

DOX loaded into m-PHSM(20%) was prepared using a dialysis method. The m-PHSM(20%) was composed of poly(His-co-Phe (16 mol%))-*b*-PEG (80 wt%)/PLLA-*b*-PEG with a folate moiety at the end of the PEG (20 wt%) (denoted as DOX/m-PHSM(20%)-f) or without folate (denoted as DOX/m-PHSM(20%)). The drug-loading efficacy was ~85%, and the drug-loading content in the micelles was ~20%.

Figure 3(a) shows the pH-dependent release profiles of DOX from m-PHSM(20%). The release rate at a given pH plateaued after 10 hr. To select an optimal candidate for the triggered release at pH 6, the amounts released over 12 hr at pH 6.5 and 6.0 from m-PHSM were compared in Figure 3(b). It is interesting to note that the released amount of DOX from m-PHSM increased as PLLA-*b*-PEG content increased. This may be relevant to the phase separation of the two block copolymers with His ionization, resulting in greater DOX release during this separation process.

The largest difference between two pHs was observed with m-PHSM(20%), although the blank mixed micelles were destabilized at pH 5.5 rather than pH 6.0. One reason for this observation may be the ionization of DOX as a result of the pH, given that it is a weak base and has a pK of 8.2.

Most DOX that was loaded was unionized when the micelle was formed at pH 9.0. The ionization of DOX in the hydrophobic micelle core is likely influenced by pH differently than free DOX. There appears to be a cooperative ionization of DOX along with the imidazole groups. Although ionized DOX is not freely soluble (a relatively hydrophobic cation, pH-dependent solubility of DOX in a base form as a function of pH),[49,50] there is a strong possibility that DOX alters the pH-sensitivity of DOX-loaded micelles, and this may explain why the 20% mixed micelle has the best triggered release of DOX at pH 6.0, deviating from the destabilization pH of the blank micelle.

### 3.5. In vitro evaluation

pH-dependent cell viability was tested using DOX/m-PHSM(20%)-f and DOX/m-PHSM (20%) against ovarian A2780 wild-type and DOX-resistant counterpart (A2780/DOX<sup>R</sup>) carcinoma cell lines that overexpress the folate receptor on the cell surface.[51,52] The purpose of this experiment was to compare the cell killing effect from each formulation. Previously, the authors found out through the cell viability test with various drug equivalent concentrations.

[53] From the result of that, the authors decided to use this DOX concentration because it is the minimum concentration of drug to show the significant cell killing effect difference between formulations at each condition.

Figures 4(a) and 4(b) show cell viability using ovarian A2780 wild-type cells with varying pH. As controls, free DOX and DOX encapsulated in pH-insensitive PLLA-*b*-PEG micelles with folate (DOX/PHIM-f) or without folate (DOX/PHIM) were used. Specifically, Figure 4(a) reports that the cell viability of free DOX and DOX/m-PHSM(20%)-f were more effective in killing ovarian A2780 wild-type cells, similar to DOX/PHIM-f made of PLLA-*b*-PEG/PLLA-*b*-PEG-folate (80/20wt/wt%).

The authors investigated the pH effect on cell growth in the absence of drug. The results indicated that there is no significant difference of cell growth from pH 7.4 to pH 5.5 (data not shown).

In this study, pH did not affect the efficacy of DOX/m-PHSM(20%)-f. The culture medium pH does not influence the folate receptor (FR)-mediated endocytosis, and once internalized, all micelles experienced the same endosomal pH. However, without folate conjugation, as shown in Figure 4(b), the cell viability of DOX/m-PHSM(20%) mixed micelles was pH-dependent, with a higher toxicity at pH 6 and 5.5. This is primarily due to the accelerated release of DOX at lower pH. In contrast, DOX/PHIM did not show altered cell viability due to slow release at all pHs tested.

Similar cell viability tests were conducted with a DOX-resistant cell line (A2780/DOX<sup>R</sup>). As seen in Figure 5(a), free DOX and DOX/PHIM-f were not effective. Free DOX was blocked for entry into the cells by the Pgp efflux pump overexpressed on the cell surface. For DOX/PHIM-f, DOX was slowly released in the endolysosomal compartments. The released DOX was in an ionized form in subcellular organelles and had low permeability through the endolysosomal membrane, which is relevant to the sequestration of a basic drug.

The rate of endosome turnover (exocytosis) in MDR cells is known to be significantly higher than in sensitive cells.[54] This also negatively contributes to the efficacy of DOX. On the other hand, DOX/m-PHSM(20%)-f showed equivalent toxicity against MDR cells as to sensitive cells at a pH of 7.4 to 6.5. The efficacy declined as pH decreased further. This may be due to a released drug fraction prior to FR-mediated internalization, which is ineffective for Pgp-expressing cells. Without folate, none of the samples worked in the tested pH range. All results suggest that both an active internalization mechanism and a triggered release at endosomal pH (double targeting) are required to treat MDR cancer cells.

To identify intracellular distribution and to demonstrate that a drug carried by the mixed pH-sensitive micelle escapes from the endosomal compartment, a green fluorescence dye (DHPE) was used to avoid the overlap of LysoTracker red with DOX fluorescence. The dye distribution in the cells is shown in Figure 6(a). DHPE fluorescence does not significantly overlap with endolysosomal compartments and even appeared in the nucleus.

When DOX was encapsulated, the cytoflowmetry in Figure 6(b) obtained from MDR cells showed that the pH-sensitive mixed micelle with folate presented with 3–4 times higher DOX intensity than pH-insensitive micelles with folate. This may be related to the recycling[54] of endosome-containing unreleased DOX back to the surface after 4 hr, or DOX fluorescence quenching by low pH and the hydrophobic micelle core, indicating DOX carried by pH insensitive micelles failed to escape from the endosomes and lysosomes. All observations confirm that the polymer components, especially the His residue, are involved in endosomolytic activity.



*In vivo* studies using this optimized pH-sensitive micelle system are in progress, and the results will be reported shortly.

## Conclusion

Accelerated doxorubicin release from L-histidine-based polymeric micelles triggered by an early endosomal pH of 6.0 was achieved by pH-sensitive micelles. The micelle core was composed of histidine/phenylalanine (16 mol%) copolymer (80 wt%) and poly(L-lactic acid) (20 wt%). This composition was obtained by optimization of the micelle properties in the presence of ~20 wt% doxorubicin. When this triggered release was combined with active targeting via folate receptor-mediated endocytosis, this nanosystem was able to effectively kill drug-sensitive ovarian cancer cells as well as drug-resistant counterpart cells.

## Materials and Methods

### Materials

1-Benzyl-N-carbobenzoxy-L-histidine (Z-His(Bzl)-OH), L-Phenylalanine, phosphorus(V) chloride (PCl<sub>5</sub>), thionyl chloride (SOCl<sub>2</sub>), isopropylamine, triethylamine, poly(ethylene glycol) (PEG) (Mw: 2 000 g/mol), potassium tetraborate, ammonium bicarbonate, N-hydroxysuccinimide (NHS), pyrene and N,N'-dicyclohexylcarbodiimide (DCC), phosphate-buffered saline (PBS), 1,4-Dioxane, N,N-dimethylformamide (DMF), methylene chloride (MC), chloroform, toluene, dimethyl sulfoxide (DMSO), n-hexane, and tetrahydrofuran (THF) were purchased from Sigma. All solvents were thoroughly dried and distilled before use.

### Preparation of Poly(1-benzyl-L-histidine-co-L-phenylalanine)-b-PEG (PBHP-b-PEG) diblock copolymer

Z-His(Bzl)-OH (2.5 g) was suspended in anhydrous 1,4-dioxane (10 mL), and thionyl chloride (2.5 mL) was added to the suspension to form Bz-His N-carboxyanhydride (NCA) under stirring at room temperature. The NCA was precipitated by addition of diethyl ether to give crystals of Bz-His-NCA•HCl. The precipitated NCA was filtered and dried under a vacuum. L-phenylalanine NCA was prepared using a similar method. L-phenylalanine (5.0g) was suspended in anhydrous 1,4-dioxane (50 mL) with triphogen (3.5 g) under stirring at 60°C. The mixture was stirred until the milky solution cleared. The solution was then precipitated by addition of hexane (eightfold). L-phenylalanine (Phe)-NCA was obtained by filtration and drying under vacuum for one day. In order to remove hydrochloride from Bz-His-NCA•HCl, triethylamine (0.5 mL) was added to The NCA (1.0 g) solution in DMF (10 mL). Triethylammonium hydrochloride was filtered out, and a clear yellowish solution of Bz-His-NCA was used for polymerization. The solution of Bz-His-NCA was mixed with Phe-NCA with various feeding ratios. The polymerizations of mixed NCAs were initiated by isopropylamine. The polymerization was kept for 3 d at room temperature.

The coupling reaction of PBHP with PEG was carried out by using activated monocarboxylated PEG. It was prepared as previously described.[50] Briefly, the preactivation of PEG (NHS-PEG) was prepared by reacting with PEG (1 mole), NHS (1.5 mol), and DCC (1.25 mol) in methylene chloride at room temperature. The coupling reaction between PHHP copolymer and activated PEG in DMSO was conducted for 2 days. After the reaction, the block copolymer (PBHP-*b*-PEG) was obtained by precipitation in diethyl ether and subsequent filtration and drying under a vacuum for 2 days. The yield was 80 wt%.

### Poly(Histidine-co-phenylalanine)-b-PEG (PHP-*b*-PEG)

To remove benzyl groups from the imidazole ring, anhydrous liquid ammonia was needed. Anhydrous ammonia gas was collected at low temperatures using dry ice in isopropyl alcohol.

PBHP-*b*-PEG (2.0 g) was suspended in anhydrous liquid ammonia (40 mL), and finely cut metallic sodium (0.5g) was added. The suspension color changed from yellow to blue, which persisted for 15 min. The excess sodium was removed by adding ammonium chloride, and the liquid ammonia was evaporated. One molar HCl (15 mL) was added to dissolve the dried polymers, and the solution was filtered through filter paper. The filtrate was extracted with diethyl ether (20 mL). The aqueous layer was neutralized with 1M NaOH. The deprotected copolymer-PEG was then precipitated during neutralization, and the precipitate was obtained from the freezing dry. The yield of final copolymer was 70 wt%, and the degree of deprotection was 98 % .

### PLLA-*b*-PEG-folate

The synthesis of PLLA-*b*-PEG-folate has been described elsewhere.[42] Briefly, PLLA-*b*-PEG was prepared by the conventional method.[55] The PLLA-*b*-PEG-COOH was activated using DCC and NHS in methylene chloride. PLLA-*b*-PEG-folate was prepared by conjugation of folate-NH<sub>2</sub> with activated PLLA-*b*-PEG-COOH in DMSO for 2 days. The unreacted amine-folate was removed by dialysis (MWCO 2000).

### NMR and acid-base titration

The molecular weight (MW) and composition of the copolymers were determined by <sup>1</sup>H NMR (MDSO-d<sub>6</sub> with tetramethylsilane) spectra. The pK<sub>b</sub> of the synthesized polymers was determined by gradually adding 0.1N-HCl solution to each polymer solution of which the initial pH was adjusted to pH 12. PHP copolymers (without PEG) (2 mM) and NaCl in deionized water (10 mL) were first adjusted to pH 11 with 1M NaOH. The solution was titrated by stepwise addition of 0.1 M HCl solution to obtain the titration profile.

### Preparation of mixed micelles

The pH-sensitive mixed micelles were prepared by blending of PHP-*b*-PEG with PLLA-*b*-PEG or PLLA-*b*-PEG-folate at different weight ratios (95/5, 90/10, 80/20 wt/wt% of PHP-*b*-PEG/PLLA-*b*-PEG or PHP-*b*-PEG/PLLA-*b*-PEG-folate). The polymers were dissolved in DMSO and transferred to a preswollen dialysis membrane (MWCO 15,000). The dialysis proceeded for 24 hr against HCl-Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> buffer solution (pH 9.0), and the buffer solution was exchanged every 2 hr. The final mixed micelles were ready to use after filtration using a 0.45- $\mu$ m syringe filter. Before drug loading into the polymeric micelle, DOX•HCl was stirred with 2 mole ratio of triethylamine in DMSO overnight to remove HCl from DOX•HCl in order to use free base of drug (DOX). For free base of drug (DOX)-encapsulated mixed micelles, the polymers and drug were dissolved in DMSO and were dialyzed for 24 hr. To measure the amount of entrapped drug, the micelle solution was lyophilized, and the DOX-encapsulated micelle powder was dissolved in DMSO. The UV absorbance of the DMSO solution was measured at 481 nm to determine the amount of DOX.

### The particle sizes of the polymeric micelles by dynamic light scattering (DLS)

The particle size of the polymeric micelles was determined by DLS using an average value of particle size (Z-average value) as a mean particle size. The DLS experiment was performed with an argon ion laser system at a wavelength of 488 nm. Each sample was filtered through a 0.45- $\mu$ m filter into a pre-cleaned 10 mm diameter cylindrical cell.

### pH-dependent critical micelle concentrations (CMC)

Fluorescence was used to measure the critical micelle concentration of each micelle with different pHs. A stock solution of pyrene (6.0  $\times 10^{-2}$  M) was prepared in acetone for the measurement of steady-state fluorescence spectra. It was diluted with deionized water to give a pyrene concentration of 1.2  $\times 10^{-7}$  M. Acetone in the solution was then evaporated under

vacuum at 60°C for 1 hr. The acetone-free pyrene solution was mixed with the solution of polymeric micelles, the concentration of which ranged from  $1 \times 10^{-4}$  to  $1 \times 10^{-1}$  g/L. The initial pH of each micelle solution used for CMC study was established by dialysis. The CMC of each micelle was estimated by plotting  $I_1$  (intensity of the first peak) of the pyrene emission spectra profile at 339 nm.

### **pH-dependent stability**

The pH-dependent stability of micelles was measured by the turbidity change of the micellar solution at different pHs. The solution concentration was 0.05 g/L, and the initial pH was 12. The pH was gradually decreased by adding 0.1 M HCl solution. The turbidity change was determined by the light transmittance of solutions, using a microplate reader (SpectraMax M2®, Molecular Devices, Sunnyvale, CA, USA) at  $\lambda=500$  nm. The micelle samples were equilibrated with different pH buffer solutions (HCl- $\text{Na}_2\text{B}_4\text{O}_7$  buffer) for 24 hr before measurement.

The pH sensitivity of each mixed micelle was measured by plotting  $I_1$  (intensity of first peak) of the pyrene emission spectra profile at 339 nm. The method was similar to measurements of CMC.

### **pH-dependent drug release test**

The DOX loaded mixed micelles were dispersed in 1 mL of phosphate-buffered saline at different pHs. The solutions were transferred in a dialysis membrane tube (MWCO 10000), and the membrane was immersed in a vial containing 10 ml phosphate buffer solution adjusted to each pH. The release of DOX from micelles was performed by mechanical shaking (100 rev/min) at 37°C. The outer phase of the dialysis membrane was taken and replaced with fresh buffer solution at predetermined times to maintain sink condition. The released DOX concentration was measured by a UV-Vis spectrophotometer (SpectraMax M2®, Molecular Devices, Sunnyvale, CA, USA).

### **Cytotoxicity test**

Ovarian A2780-sensitive or DOX-resistant cells growing in flasks were treated by 0.2 % (w/v) trypsin-0.1 % (w/v) EDTA solution in order to detach the cells. The detached cells ( $5 \times 10^4$  cells/mL) were seeded in a 96 well plate and were incubated for one day. The equivalent DOX concentrations of each formulation PLLA-*b*-PEG/PLLA-*b*-PEG-folate and PHP-*b*-PEG/PLLA-*b*-PEG-folate including free DOX were prepared by diluting with RPMI 1640 cell culture medium. The pH of the culture medium was adjusted with 0.1 M HCl, or 0.1 M NaOH at a desired pH prior to use. No considerable pH change in the culture medium was observed during 48 hr. After 48-hr treatments, 20  $\mu\text{L}$  of MTT solution (500 $\mu\text{g/L}$ ) was added to each well and then incubated for 4 hr. The medium was removed, and DMSO (100  $\mu\text{L}$ ) was added to each well and incubated for 10 min. The absorbance of each well was read with a microplate reader (SpectraMax M2®, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm.

### **In vitro efficacy tests of mixed micelles**

To measure the *in vitro* efficacy of mixed micelles, several tests were used. The first investigation looked at endosomal disruption by mixed micelles. The A2780/DOX<sup>R</sup> MDR tumor cell line grown on a Lab-Tek II live cell chamber slides (Nalge Nunc, Naperville, IL, USA) was treated with the hydrophobic DHPE fluorescent dye, which encapsulated the mixed micelle (DHPE/Micelle = 1/10 wt/wt% and micelle concentration: 20  $\mu\text{g/mL}$ ), and LysoTracker Red DND-99 (80 nM) for 30 min. The micelle-treated cells were washed three



times with phosphate-buffered solution (pH 7.4), and the RPMI 1640 medium was replaced with phosphate buffer solution.

Both DHPE fluorescence (green) and lysotracker fluorescence (red) inside live cells were examined by a confocal microscope (Leica TCS NT, Leica, Germany). DHPE dye has a 496 nm excitation and a 519 nm emission wavelength, while lytracker dye has a 577 nm excitation and a 590 nm emission wavelength. The cellular DOX uptake was measured by flow cytometry. A2780/DOX<sup>R</sup> MDR tumor cells ( $1 \times 10^6$  cells/well) were cultured with RPMI 1640 media with 10% FBS and 1% penicillin-streptomycin in a 6 well-plate overnight. The appropriate DOX concentration (20  $\mu\text{g}/\text{mL}$ ) of each formulation including DOX-free was given to the cells and incubated for 30 min. The cells were then washed three times with PBS solution and were detached using trypsin-EDTA solution. The detached cells were fixed with 2.5% glutaraldehyde. The cell fluorescence was measured by flow cytometry.

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## Notation

PHP(x), poly(His-co-Phe)(x=Phe mole % in a copolymer product having numerical values of 10, 16, 22 and 27); PHSM(x), pH-sensitive micelle made of PHP(x)-b-PEG; PHIM, pH-insensitive micelle without folate made of PLLA-b-PEG; PHIM-f, pH-insensitive micelle composed of PLLA-b-PEG (80 wt%) and PLLA-b-PEG-f (20 wt%); DOX/PHIM-f, DOX loaded PHIM-f; m-PHSM(y%), mixed pH-sensitive micelle composed of PHP(16)-b-PEG and PLLA-b-PEG (y=PLLA-b-PEG wt%); m-PHSM(z%)-f, mixed pH-sensitive micelle composed of PHP(16)-b-PEG and PLLA-b-PEG (z=PLLA-b-PEG-folate wt%); DOX/m-PHSM(z%)-f, DOX loaded m-PHSM(z)-f.

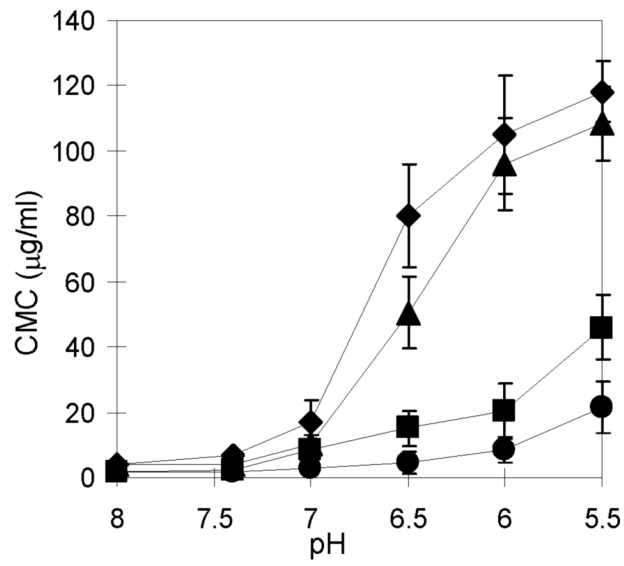
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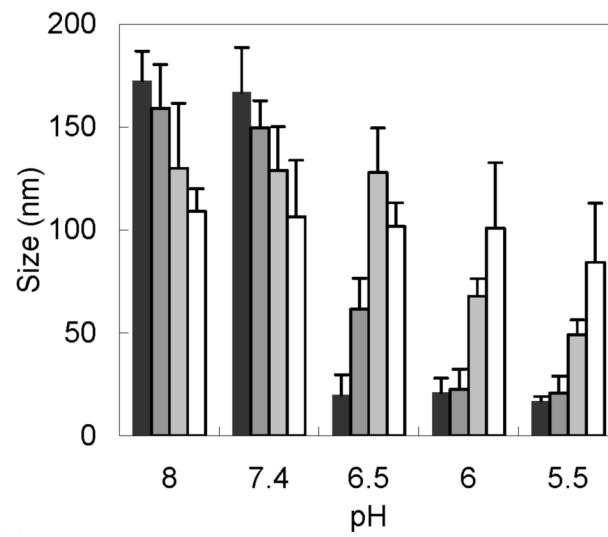
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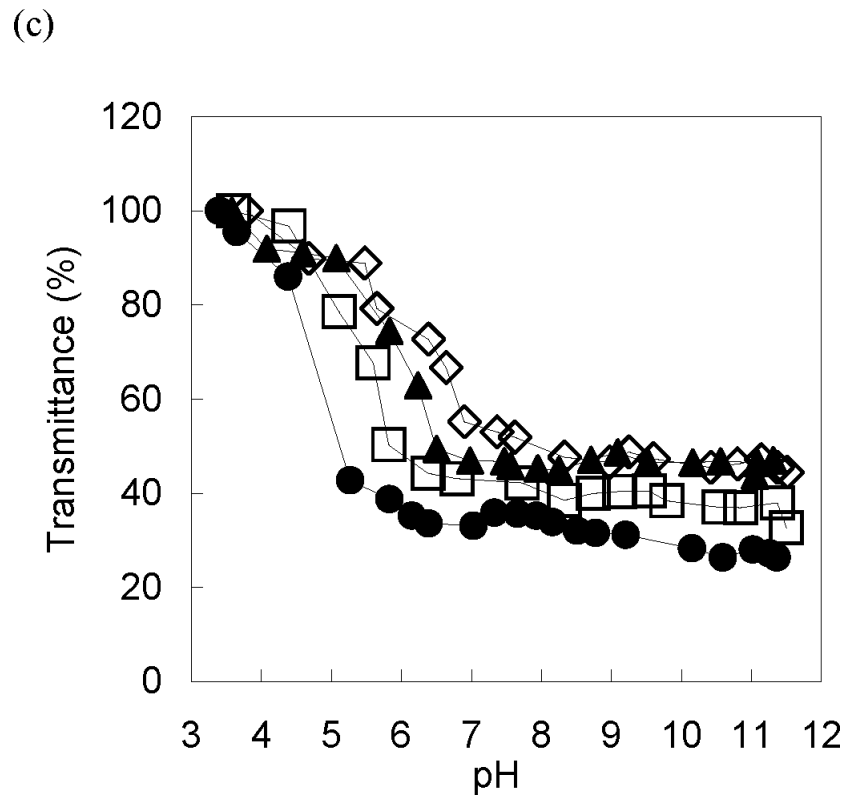
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(a)



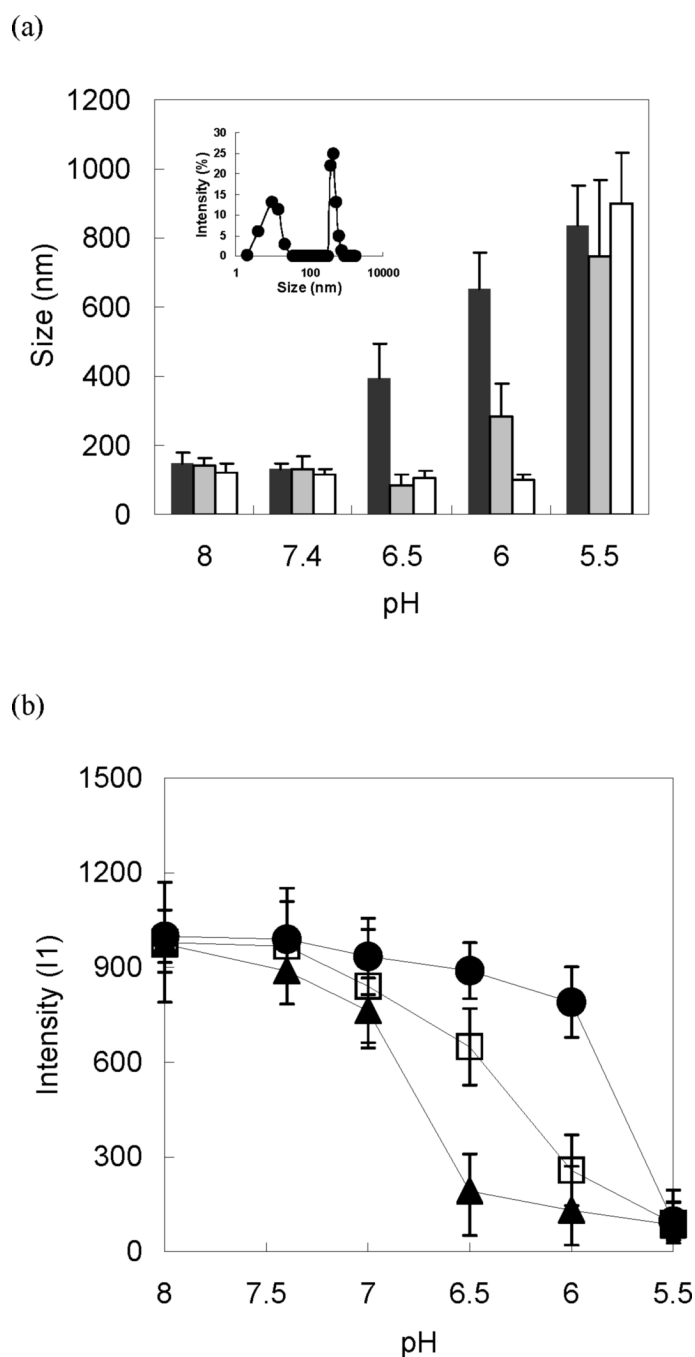
(b)





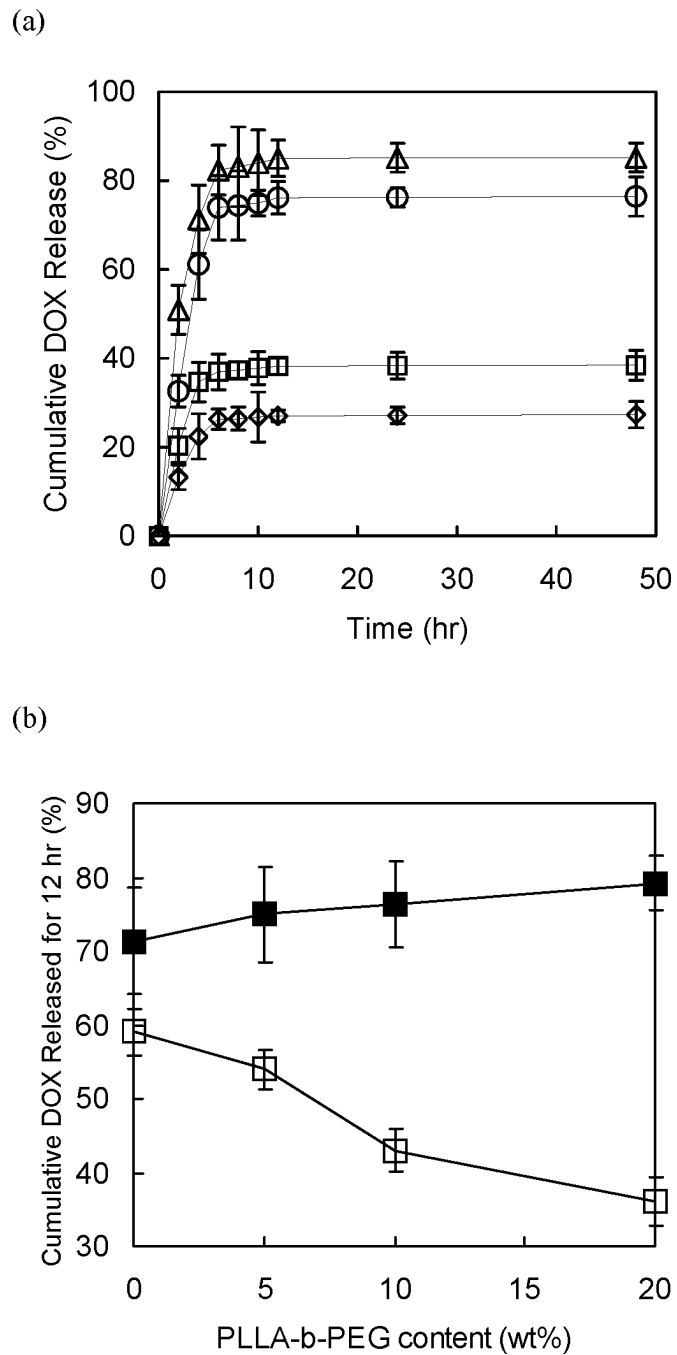
**Figure 1.** (a) pH-dependent critical micelle concentration (CMC) of the micelles constructed of poly (His-co-Phe)-*b*-PEG: PHSM(10)(◆), PHSM(16) (▲), PHSM(22) (■), and PHSM(26) (●); (b) pH-dependent particle size: PHSM(10) (black column), PHSM(16) (dark gray), PHSM(22) (pale gray), PHSM(27) (white) and (c) transmittance changes with pHs: PHSM(10) (◇), PHSM(16) (▲), PHSM(22) (□), and PHSM(27) (●).



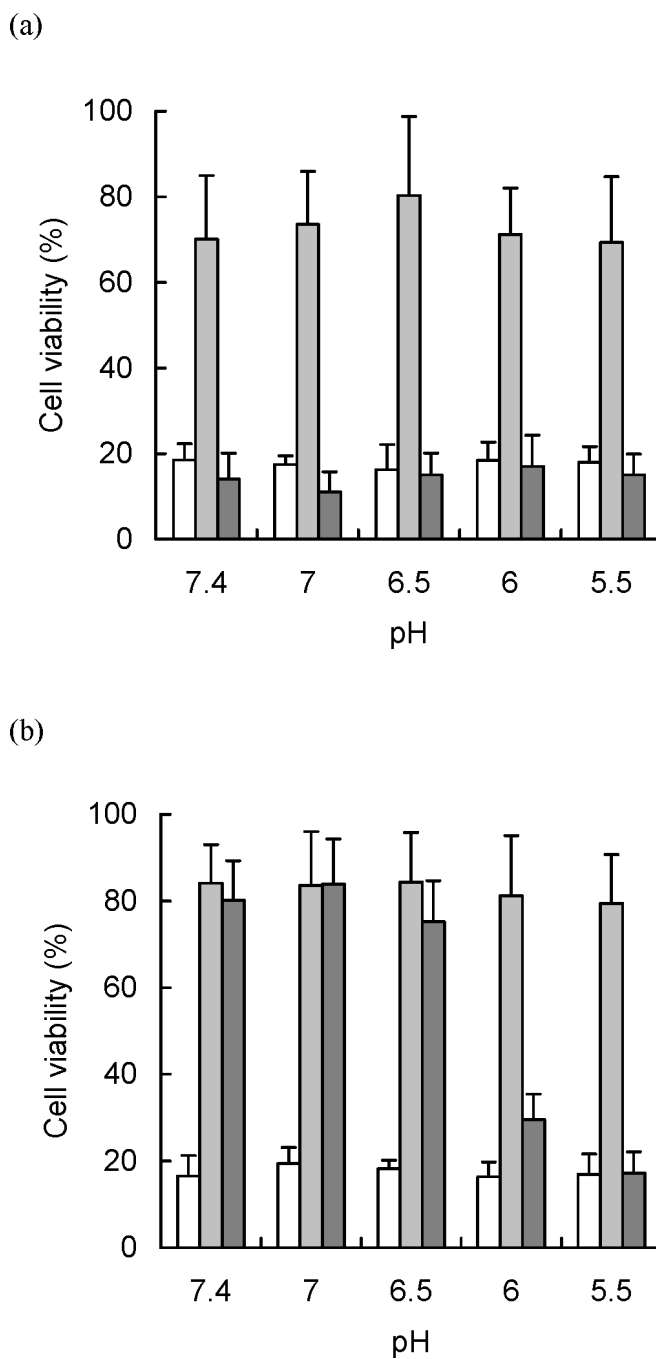


**Figure 2.**

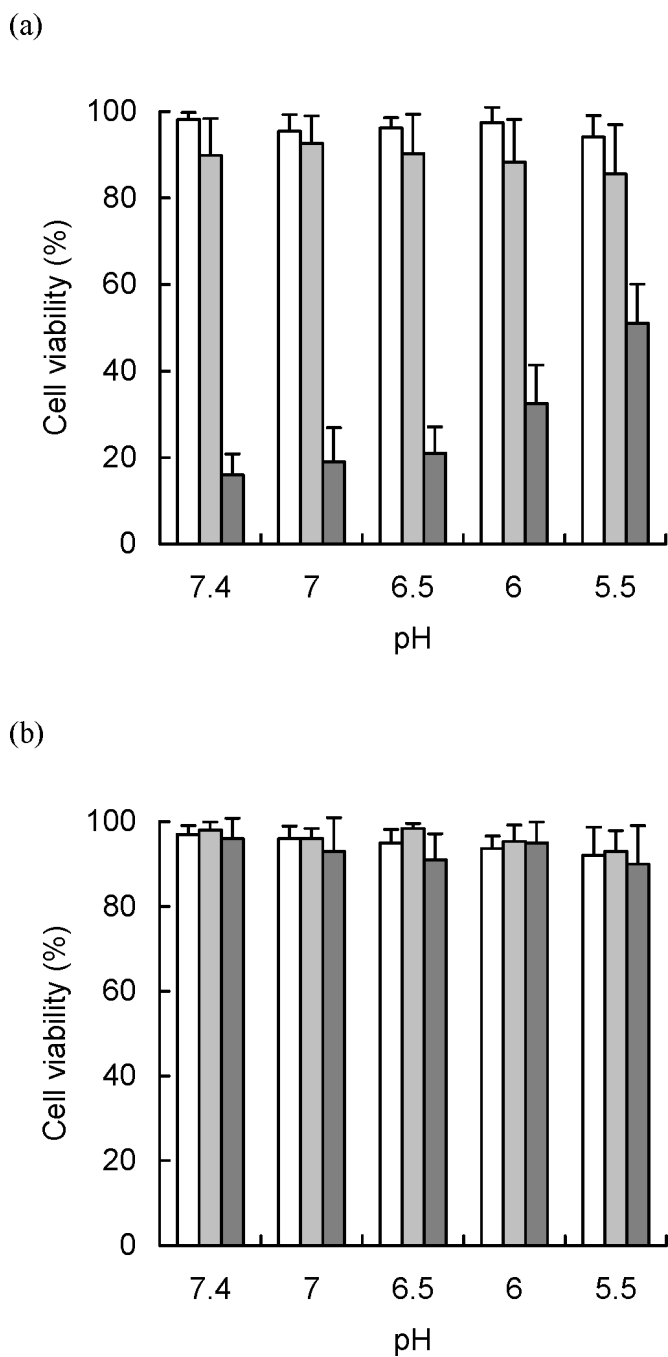
(a) pH-dependent particle size of mixed micelles composed of PHSM(16) and different amounts of PLLA-*b*-PEG. The weight ratios (wt/wt%) of PHSM(16) and PHIM were 95/5 (black), 90/10 (gray), and 80/20 (white) denoted by m-PHSM(5, 10, and 20%). Small box capture indicated a bimodal particle size distribution at pH 6.5 using m-PHSM(5%). (b) The change of pyrene fluorescence intensity ( $I_1$ ) with pH at a constant micelle concentration (0.05g/l). m-PHSM(5%) ( $\blacktriangle$ ), m-PHSM(10%) ( $\square$ ), and m-PHSM(20%) ( $\bullet$ ) was monitored in NaOH (or HCl)- $\text{Na}_2\text{B}_4\text{O}_7$  buffer solution (pH 8.0) with exposure to each pH for 24 hr.



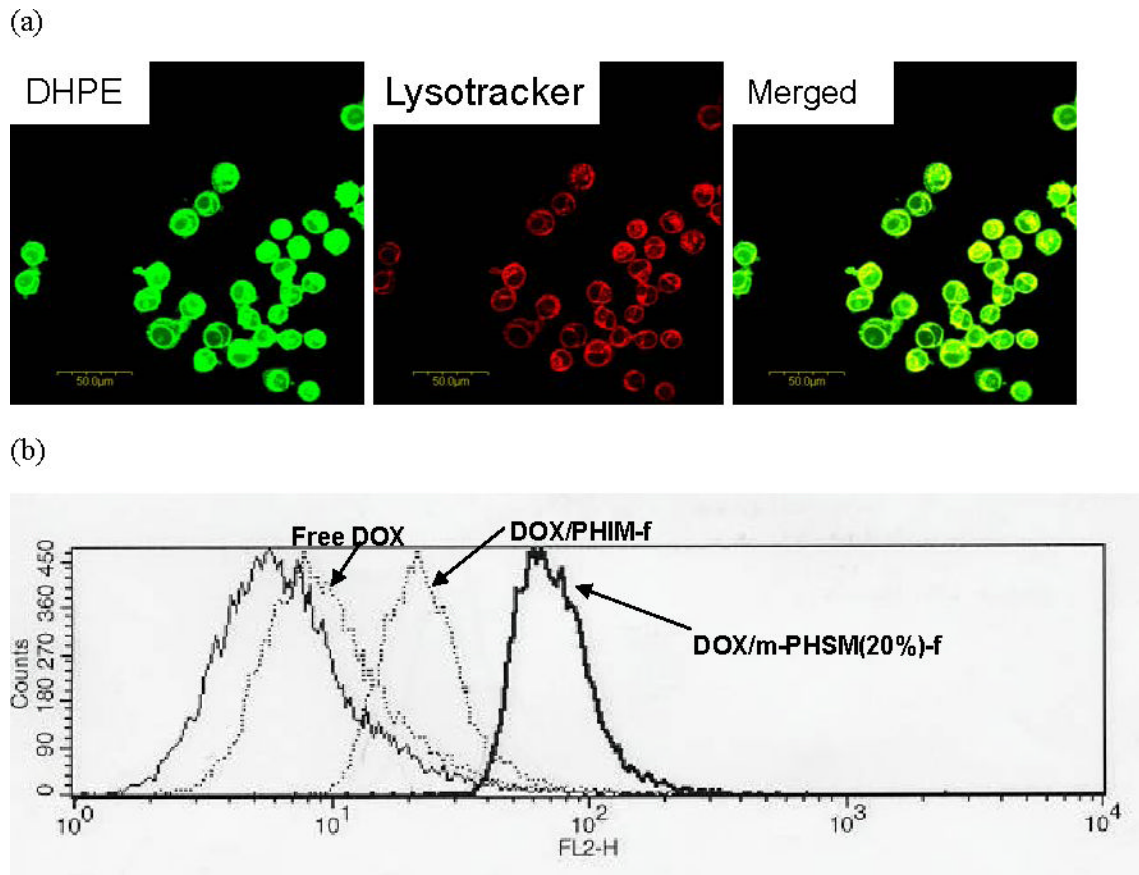
**Figure 3.** (a) pH-dependent DOX release profiles from m-PHSM(20%): pH 5.5 ( $\Delta$ ), pH 6.0 ( $\circ$ ), pH 6.5 ( $\square$ ), pH 7.4 ( $\diamond$ ); (b) Cumulative amount of DOX for 12 hr released from m-PHSM constructed with the different amounts of PLLA-*b*-PEG at pH 6.5 ( $\square$ ) and pH 6.0 ( $\blacksquare$ ).



**Figure 4.** Cell viabilities determined by MTT assay of ovarian A2780 wild-type carcinoma cells treated with (a) micelles with folate: free DOX (white), DOX/PHIM-f (gray), and DOX/m-PHSM (20%)-f (dark gray), (b) micelles without folate: free DOX (white), DOX/PHIM (gray), and DOX/m-PHSM(20%) (dark gray). DOX dose was equivalent to 1000 ng/mL in each formulation.



**Figure 5.** Cell viabilities determined by MTT assay of ovarian A2780/DOX<sup>R</sup> multidrug (MDR) resistant carcinoma cells treated with (a) micelles with folate: free DOX (white), DOX/PHIM-f (gray), and DOX/m-PHSM(20%)-f (dark gray) and (b) micelles without folate: free DOX (white), DOX/PHIM (gray), and DOX/m-PHSM(20%) (dark gray). DOX dose was equivalent to 1000 ng/mL in each formulation.



**Figure 6.** Using ovarian A2780/DOX<sup>R</sup> multidrug (MDR) resistant carcinoma cells, (a) Intracellular localization of DHPE (a green fluorescence dye) carried by m-PHSM(20%) and (b) DOX uptake study by flow cytometry using DOX/m-PHSM(20%)-f, DOX/PHIM-f, and free Drug were performed.



Table 1

Characterization of poly(Histidine-co-Phenylalanine).

Polymer	Monomer mole feed ratio		$\bar{M}_n^*$	Phe mol% in polymer	pK <sub>b</sub>	Buffering pH range
	His	Phe				
PHP(10)	99	1	5260	10	6.7	8.2-4.8
PHP(16)	95	5	5080	16	6.3	7.6-4.7
PHP(22)	90	10	5300	22	5.7	6.5-4.0
PHP(27)	80	20	5480	27	4.8	5.8-3.2

\* The number average molecular weight of polymers was determined by <sup>1</sup>H-NMR.