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pH-sensitive poly(histidine)-PEG/DSPE-PEG co-polymer micelles for cytosolic drug delivery

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Abstract

To introduce pH sensitivity into the DSPE-PEG-based micellar system and achieve the quick intracellular drug release in response to the acidity in endosomes, a mixed polymeric micelle was developed based on three grafted copolymers, including 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol-2000(DSPE-PEG₂₀₀₀), antinucleosome antibody (mAb 2C5)-modified DSPE-PEG₃₄₀₀ (DSPE-PEG₃₄₀₀-2C5), and poly(ethylene glycol)-coupled poly(L-histidine) (PHIS-PEG₂₀₀₀). The structure of PHIS-PEG₂₀₀₀ was confirmed by ¹H NMR spectroscopy. The mixed micelles with the diameter ranging from 110 to 135nm were prepared using a dialysis method against pH 7.6 PBS. Paclitaxel (PCT) was used as a model drug, the encapsulation efficiency and loading content of PCT were 88% and 5%, respectively. The mixed micelles composed with 50wt% of PHIS-PEG₂₀₀₀ showed the desired pH-dependent drug release property with much faster drug release than micelles without PHIS-PEG₂₀₀₀. At pH around 5.5, about 75–95% of the loaded drug was released within 2 h. The MTT assay showed PCT-loaded mixed micelles had higher cytotoxicity at pH 5.8 than that at pH 7.4. Further modification of the mixed micelles with anti-cancer nucleosome- specific monoclonal antibody 2C5 significantly increased their cellular uptake efficiency and cytotoxicity. Thus, the low pH in endosomes could trigger the PCT release from the pH-sensitive mixed micelles after 2C5-mediated endocytosis. The results of this study suggest that the mixed micelles (DSPE-PEG₂₀₀₀/DSPE-PEG₃₄₀₀-2C5/PHIS-PEG₂₀₀₀) could enhance the tumor cell-specific internalization and trigger the quick drug release, resulting in the improved anti-cancer efficacy.

Keywords

DSPE-PEG; Poly(L-histidine); Mixed polymeric micelles; pH-sensitive; Cytosolic drug delivery; Tumor targeting

1. Introduction

In the past decades, polymeric micelles have been extensively studied for their potential applications in the drug delivery field [1–6]. Polymeric micelles are formed by amphiphilic block copolymers, which could self-assemble into a nanoscopic core/shell structures in an aqueous environment via hydrophobic or ion pair interactions between polymer segments [7,8]. The prominent features of such micelles are their abilities to solubilize the insoluble

drugs, avoid non-selective uptake by the reticuloendothelial system (RES), and utilize the enhanced permeability and retention (EPR) effect for passive targeting [5]. So, the drug's solubility and pharmacokinetic profiles could be significantly improved. The polymeric micelles used for drug delivery have shown the abilities to attenuate nonspecific toxicities and enhance drug delivery to desired sites resulting in the improved therapeutic efficacy [9].

The synthetic amphiphilic copolymers are ideal tools for drug delivery because they are highly versatile in terms of composition and architecture. In our previous studies, the micelles made of polyethylene glycol-phosphatidylethanolamine (PEG-PE) have been successfully used to entrap and deliver various poorly water-soluble drugs [10–17]. In aqueous environment, the hydrophobic fragments of PEG-PE form the core of a micelle where the drug molecules are entrapped. The hydrophilic shell could be further modified by various functional groups including targeting ligands, such as monoclonal antibody, or intracellular drug delivery moieties, such as cell-penetrating peptides (CPPs), and so on [18,19].

Nowadays, although nanocarriers have been reported to accumulate preferably in tumor due to passive and/or active targeting, the inefficient drug release can be another barrier that may significantly lower drug's efficacy. Longevity moiety, such as surface PEG, may inhibit the cellular uptake of long circulating nanocarriers and their following intracellular events [20,21]. Therefore, the extra functionality should be introduced into nanocarriers to minimize the effect of PEG and facilitate the drug release after endocytosis. In fact, the plain micelles have the relatively slow drug release, the intracellular drug concentration could fail to reach the optimum therapeutic threshold promptly, which may contribute to the development of multidrug resistance (MDR) in tumor cells. The rapid drug release could provide the sufficient drug concentration and kill the tumor cells before they acquire the ability to against the drugs. Therefore, the quick drug release is critical for the chemotherapeutic agents to efficiently kill the cancer cells without the acquired drug resistance.

In order to overcome this barrier, the micellar systems with a triggered release mechanism were developed which enable the nanocarriers to release drugs in response to specific external or internal stimuli such as temperature, pH, ultrasound or enzymes [22–25]. Among of them, pH-sensitive polymeric micelle look like the most attractive candidate due to intrinsic differences between various solid tumors and the surrounding normal tissues in terms of their relative acidity [26,27]. The measured tumor extracellular pH (pHe) values of most solid tumors range from pH 6.5 to 7.2, while normal blood remains well-buffered and constant at pH 7.4. Moreover, changes in pH are also encountered once the micelle enters cells via endocytosis where pH can drop as low as 5.5–6.0 in endosomes and 4.5–5.0 in lysosomes [28].

Poly (L-histidine) (PHIS) is a pH-sensitive polymer because the imidazole ring has an electron lone pair on the unsaturated nitrogen that endows PHIS with an amphoteric nature by protonation–deprotonation. In addition, the fusogenicity of PHIS could disrupt the enveloped membrane of acidic subcellular compartments such as endosomes thus resulting in endosomal escape [29]. Thus, PHIS-based micelles have great potential as an acid triggering tumor-killing platform. However, PHIS is too sensitive to environmental pH, which could affect the stability of the core. So, PHIS was often combined with more hydrophobic polymers to form stable micelles. For example, poly(histidine-cophenylalanine) [poly(His-co-Phe)]-PEG was used instead of PHIS to increase the hydrophobicity of the core to obtain endosomal pH (w6.0)-sensitive micelles [30]. Bae et al. prepared pH-sensitive poly(L-histidine)-b-poly(ethylene glycol) and poly(L-lactide)-

bpoly(ethylene glycol) (PHIS-PEG/PLLA-PEG, 75/25 wt%) mixed micelles [26]. The properties of the mixed micelles such as stability, size, morphology, drug loading level and responsiveness, were greatly dependent on the relative content of the two copolymers. Furthermore, many functional molecules, such as targeting ligand, fluorescein isothiocyanate (FITC) and others can be separately or simultaneously attached to the surface of micelles by simple chemical methods. In other words, the combination of two or more species of block copolymers to produce the multifunctional mixed micelles is a straightforward and promising strategy.

Ligand-mediated nanocarriers are usually internalized into tumor cells by receptor-mediated endocytosis and entrapped into endosomal/lysosomal vesicles. Therefore, the task to facilitate drug escape from the endosomal/lysosomal vesicles is very important [31,32]. Although the slightly acidic conditions (pH 6.5–7.2) of the extracellular tumor space and the higher acidity in endocytic vesicles could be used to design pH-sensitive drug delivery systems for tumor treatment, many pH-sensitive drug carriers focused on response to tumor pH [33–35], which means that such acidsensitive micelles could release drug outside the tumor cells thus lowering the drug's efficacy [36].

Our previous studies have proven that the nanocarriers modified with the monoclonal antibody 2C5 have high binding affinity to the surface of a variety of tumor cells, but not normal cells [37–39]. In this study, the pH-dependent drug release characteristic was added to the DSPE-PEG immunomicelles (2C5-micelles) by incorporation of pH-sensitive PHIS-PEG (Fig. 1). This strategy could not only stabilize the micelles at neutral and tumor extracellular pH (~6.8), but also facilitate micelle's disintegration and the resultant quick drug release in response to the low endocytic pH (<6.0) upon 2C5-mediated internalization.

2. Experimental section

2.1. Materials

Poly-L-histidine (PHIS) with a number average molecular weight of 10,000 Da (as verified by gel-permeation chromatography), semisynthetic paclitaxel (PCT) (from *Taxus* sp.), pyrene, dimethylsulfoxide (DMSO), acetic acid were purchased from Sigma-Aldrich Inc. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[succinimidyl (polyethylene glycol)-3400] (DSPE-PEG₃₄₀₀-NHS), and methoxyl PEG succinimidyl ester, MW 2000 (mPEG₂₀₀₀-NHS) were purchased from Nanocs Inc. 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)(ammonium salt, Rh-DSPE, chloroform solution, 1 mg/mL) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀, chloroform solution, 25 mg/mL), were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Cell culture media and supplements were from Cell-Gro (Kansas City, MO, USA). Cancer-specific anti-nucleosome mAb 2C5 was prepared by Harlan Bioproducts for Science (Indianapolis, IN, USA) using the cell line provided by our laboratory. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Promega (Madison, WI, USA). Cancer cell line 4T1 (murine mammary carcinoma) was purchased from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were from CellGro (Kansas City, MO). All other reagents and buffer solution components were analytical grade preparations. Distilled and deionized water produced from Milli-Q Synthesis was used in all experiments. PBS buffer was prepared by ourselves and adjusted to a predetermined pH by pH meter (Model 420A, ORION).

2.2. Polymer synthesis

2.2.1. Synthesis of PHIS-PEG₂₀₀₀—10 mg Poly (L-histidine) was dissolved in 5 mL of 10 mM acetic acid followed by adjustment of pH to 6.5 with 0.1 M NaOH. An excess amount of mPEG-NHS (1.2:1, mol/mol) was added to the above solution and the reaction was carried out under the nitrogen gas for 8 h at room temperature. The reaction mixture was monitored by the TLC and dialyzed with cellulose ester membranes with a molecular weight cut-off of 5 kDa (Spectrum Medical Industries, Rancho Dominguez, CA) against deionized water for 72 h to remove the unreacted mPEG-NHS. The solution was lyophilized after filtering through a 0.45 µm syringe filter. About 8.5 mg of white fluffy product were obtained. The synthetic scheme is shown in Fig. 2. The freeze-dried powder (2 mg) was dissolved in the deuterated (*d*)-chloroform (CDCl₃, 1 mL) and analyzed by the ¹H NMR using Varian 400 MHz (Bruker, Avance).

2.2.2. Synthesis of DSPE-PEG₃₄₀₀-2C5—To prepare antibody (mAb 2C5) modified with DSPE-PEG₃₄₀₀, the excess DSPE-PEG₃₄₀₀-NHS was incubated with the antibody (3.19 mg/mL) solution in tris-buffered saline (50 mM), pH 8.5, for 24 h at 4 °C [40]. DSPE-PEG₃₄₀₀-2C5 was then purified by the size exclusion gel chromatography using a CL-4B column.

2.3. Preparation of the mixed micelles

Since PHIS-PEG copolymer is not readily dissolved in water, a dialysis method was used to prepare the mixed micelles (DSPE-PEG₂₀₀₀/DSPE-PEG₃₄₀₀-2C5/PHIS-PEG₂₀₀₀). First, a dry lipid film was prepared by rotary evaporation of 100 µL of chloroform solution (20 mg/mL) of DSPE-PEG₂₀₀₀ (2 mg), further dried under high vacuum for at least 4 h to remove the trace of the solvent. 2 mg of PHIS-PEG was dissolved in 1 mL mixed solvent of H₂O and DMSO (1:1, v/v), a small amount of 0.1 M acetic acid was added to make sure PHIS-PEG dissolve completely, and then mixed with DSPE-PEG₂₀₀₀ dry lipid film by sonication in the ice-water bath for 15 min. The resulting solution was transferred into a pre-swollen dialysis membrane (Molecular weight cut-off, MWCO 3500) at 4 °C and then put into another dialysis membrane (MWCO 12,000–14,000) at room temperature to be dialyzed against 10 mM PBS, pH 7.6. The outer phase was replaced with fresh buffer solution at 1, 2, 4, 6, and 12 h. After 24 h, the polymers inside the membrane were self-assembled into mixed micelles. The solution was subsequently lyophilized after filtering through a 800 nm syringe filter. The yield (wt%) of micelles was calculated by weighing the freeze-dried micelle powder.

To obtain drug-loaded mixed micelles, PCT (200 µg) dissolved in methanol was added to PEG₂₀₀₀-DSPE solution in chloroform; whereas in order to prepare mixed micelles, DMSO solution of PCT (200 µg/100 µL) was added to the above PHIS-PEG in mixed solvent (DMSO/H₂O, 1:1, v/v) and then mixed with DSPE-PEG₂₀₀₀ dry lipid film by the ultrasonication in the ice-water bath for 15 min and dialyzed against 10 mM PBS, pH 7.6, at room temperature for 24 h. The final mixed micelles were ready to use after filtration using 800 nm syringe filter.

To prepare antibody-bearing micelles, 0.02 µmol of DSPE-PEG₃₄₀₀-2C5 was added to the above-mentioned forming micelles solution. Whenever fluorescent tagging was desired, 5 nmol of the fluorescent Rh-PE was added to the micelle composition. Following the lyophilization, the samples were sealed and stored at 4 °C until use.

2.4. Determination of encapsulation and loading efficiencies (EE and LE)

The loading capacity and efficiency of PCT in micelles were determined by HPLC. The mixed micelles were solubilized with acidic DMSO (pH 6.0) before HPLC analysis. The

D-7000 HPLC system equipped with a diode array and fluorescence detector (Hitachi, Japan) and Spherisorb ODS2 column (Waters, Milford, MA, USA) was used. The column was eluted with acetonitrile/water (60:40, v/v) at a flow rate of 1.0 mL/min. PCT was detected at 227 nm. Each run was done in triplicate. The PCT loading was determined using a calibration curve obtained in the same conditions using standard concentrations of PCT (ranging between 0.7 and 140 µg/mL); the correlation coefficient R^2 was 0.9994. PCT loading efficiency and encapsulation efficiency were calculated as follows:

$$EE(\%) = (\text{Weight of loaded drug} / \text{Weight of drug input}) \times 100\% \quad (1)$$

$$LE(\%) = (\text{Weight of loaded drug} / \text{Weight of drug} - \text{loaded micelles}) \times 100\% \quad (2)$$

2.5. Particle size and zeta potential of the mixed micelle at different pH values

For investigating the pH-sensitivity of the mixed micelles, they were dissolved in 10 mM PBS at different pH values (7.4, 6.5 and 5.5) and diluted to 2 mg/mL (for the zeta potentials measurements) and 1 mg/mL (for the particle size measurements), respectively. After 10 min, zeta potential and particle size of the mixed micelles were measured. The zeta plus dynamic light scattering equipment (Brookhaven Instruments, Holtsville, NY) was used to determine the zeta potentials, particle size and size distribution of the mixed micelles at different pH. The particle size distribution and zeta potential of all samples were measured in triplicate.

2.6. Critical micelle concentration (CMC) determination

The CMC of the mixed micelles was estimated by the standard pyrene method [18]. Briefly, a freeze-dried mixed micelle sample was dispersed in PBS (pH 7.4, ionic strength 0.15). Varied concentrations of micellar solutions ranged from 0.1 µg/mL to 10 µg/mL were mixed with pyrene for 6 h with shaking at room temperature. The fluorescence of the solubilized pyrene was measured at the excitation wavelength of 339 nm and emission wavelength of 373 (I_1) and 394 nm (I_5), respectively, using a F-2000 fluorescence spectrometer (Hitachi, Japan). The CMC was estimated by the intensity ratio of the fifth and the first (I_5/I_1) highest energy bands in the emission spectra profile against of the micelle concentration. The CMC of mixed micelles was obtained from the crossover point at low polymer concentration in this plot.

2.7. Drug release at different pH values

The *in vitro* pH-sensitive drug release was determined using a dynamic dialysis method. The release experiments were conducted at 37 °C. Typically, 5 mg of drug-loaded micelles in 1 mL water were placed into a dialysis bag (MWCO: 15,000) and dialyzed against 25 mL of PBS (pH 7.4, 6.0, 5.0) containing 0.02% Tween 20 with magnetic stirring at 200 rpm. Tween-20 was added to the release medium to improve drug solubility. At hourly intervals, a 0.5 mL sample was taken out from the release medium and the same volume of PBS was added to the release medium. Then the samples were assayed for the drug content by HPLC. Each study was conducted in triplicate and the results of triplicate test data were used to calculate the accumulated drug.

2.8. Stability test

To verify the stability of mixed polymeric micelles at pH 7.6, the turbidity change of micelle solutions (1 mg/mL) was followed from the light transmittance at λ_{500} nm. To further study the plain and PCT-loaded mixed micelles' stability at different storage conditions, a sample of each preparation (plain and PCT-loaded micelles) was stored as a micelle suspension at 4

°C or at 25 °C. Changes in the micelle size and size distribution as well as polymer coagulation and drug content were examined after 2 weeks and 3 months.

2.9. Cell tests in vitro

2.9.1. Cell cultures—The *in vitro* cell viability tests were performed with 4T1 murine breast cancer cells. 4T1 cells were maintained in DMEM medium supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a 5% CO₂ humidified atmosphere.

2.9.2. In vitro cytotoxicity of PHIS-PEG/DSPE-PEG mixed micelles—To evaluate the pH-sensitive characteristics of PHIS-PEG/DSPE-PEG mixed micelles, the cytotoxicity was determined in 4T1 tumor cells at different pH values. Briefly, 4T1 cells were seeded at 8×10^3 cells per well density in 96-well plates (Corning Inc., NY, USA) and incubated at 37 °C for 24 h. The pH of the culture medium was adjusted with 0.1 M HCl to a desired pH (pH 7.4, 6.8, and 5.8) before treatment. The various formulations (PCT-loaded DSPE-PEG/PHIS-PEG mixed micelles, PCT-loaded DSPE-PEG micelles and free PCT in DMSO solution) containing three different PCT concentrations (0.1, 1, and 10 µg/mL) were added to cells. The empty mixed micelles (20 µg/mL) were used as control. After 18 h incubation, cells were washed twice with PBS, and then the cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. For this purpose, 10 µL of MTT stock solution (5 mg/mL) were diluted 10-fold with the complete growth medium. Then, the diluted MTT was added to each well and incubated for 4 h until the purple precipitates were visible. 200 µL of DMSO was added to each well, and the plates were incubated at 37 °C for 15 min until all the crystals were dissolved. The absorbance intensity at 560 nm was recorded using a Labsystems Multiskan MCC/340 microplate reader (Labsystems and Life Sciences International, UK), and the cytotoxicity was expressed as the percentage of the control.

2.10. 2C5-mediated cellular uptake

2.10.1. Endocytosis of micelles by fluorescence microscope—4T1 cells were seeded in a 6-well plate (1×10^6 cells/well) and incubated for 24 h before experiment. Rh-PE labeled DSPE-PEG₃₄₀₀-2C5/PHIS-PEG₂₀₀₀ mixed micelles and DSPE-PEG₃₄₀₀/PHIS-PEG₂₀₀₀ mixed micelles, were first pre-incubated in PBS, pH 7.4, for 1 h and then incubated with 4T1 cells for 1 h at 37 °C at the final micelle concentration of 0.1 mg/mL. After the incubation, the cells were washed 3 times with PBS and observed at 100× magnification using a Nikon Eclipse E400 microscope (Nikon, Japan) under the bright light or under the epifluorescence with a rhodamine filter.

2.10.2. Flow cytometry study—To quantitate the fluorescence intensity in cells, the treated 4T1 cells were harvested with 0.2% (w/v) trypsin-0.1% (w/v) EDTA solution. The cells were centrifuged and washed with ice-cold PBS 3 times before the fluorescence-activated cell sorting (FACS) analysis using a BD FACS Caliber flow cytometer (FACSCAN, Becton Dickinson). The cells were gated using forward versus side-scatter to exclude debris and dead cells before the analysis of 10,000 cell counts. The data were analyzed with BD Cell Quest Pro Software.

2.11. Cytotoxicity of 2C5-modified mixed micelles

To testify the interaction of 2C5-modified mixed micelles with cancer cells and pH-dependent drug release, the *in vitro* cell viability test was performed with 4T1 cells. The cells were seeded (5×10^3 cells/well) in a 96-well plate and incubated for 24 h at 37 °C and 5% CO₂. The cells were washed with 100 µL/well of the fresh DMEM complete medium

and incubated with dilutions of free PCT, PCT-loaded DSPE-PEG₃₄₀₀-2C5/DSPE-PEG₂₀₀₀/PHIS-PEG₂₀₀₀ mixed micelles, and DSPE-PEG₃₄₀₀-2C5/DSPE-PEG₂₀₀₀ micelles with no pH-sensitive component. The equivalent PCT concentrations (0.01, 0.1, and 1 µg/mL) of each formulation were prepared by dilution with the cell culture media. After an additional 48 h incubation at 37 °C, the cell viability was measured using the MTT assay (same protocol as above).

2.12. Statistical analysis

The data were tested for statistical significance using the paired Student's *t* test using GraphPad Prism 4 (GraphPad Software, Inc.; San Diego, CA). Any *p* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Synthesis and characterization of PHIS-PEG

Synthesis of PHIS-PEG is shown in Fig. 2. In this reaction, methoxyl PEG-NHS with the end-capping group was used. PHIS was dissolved in 10 mM acetic acid. To yield stable product, 0.1 M NaOH was added to adjust pH of the solution to above 6.5, because acidic conditions are not favorable for this reaction. The excessive mPEG-NHS was used to make the PHIS to react completely. The unreacted mPEG-NHS and other reagents are easily removed by dialysis. Successful synthesis of mPEG-PHIS was confirmed by ¹H NMR spectroscopy using a Bruker Avance 400 spectrometer (400 MHz), and CDCl₃ was used as the solvent (Fig. 3). According to the peak area of PHIS (a, 0.07) and mPEG (b, 0.18), the molar ratio of PHIS and mPEG in the product was approximately 1:1 [0.07/(10000/137): 0.18/((2015/44) × 4)].

3.2. Preparation of mixed micelles

The mixed micelles containing PHIS-PEG₂₀₀₀ (50 wt%) were prepared using a modified dialysis method. PHIS-PEG was first dissolved in the mixed solvent of H₂O and DMSO (1:1, v/v). A preswollen dialysis membrane (MWCO 3500) was used to remove DMSO slowly at 4 °C and then another dialysis membrane (MWCO 12,000–14,000) was used to remove unmicellated polymers. After the dialysis against PBS, pH 7.6, mixed micelles with the mean diameter of 112 nm are. The diameter of PCT-loaded mixed micelles is about 138 nm. The yield of mixed micelles upon the dialysis was about 90% (w/w). Based on the equations (1) and (2), PCT encapsulation and loading efficiencies were ca. 88 wt% and 5 wt %, respectively.

3.3. Size distribution and zeta potential at different pH

The average diameter of PHIS-PEG/DSPE-PEG mixed micelles (1:1, mass ratio) was around 112 nm without filtering. In general, the size of individual core-shell type micelles is in the range of few tens of nanometers. However, micelles with a size range of several hundred nanometers are often observed due to intermicellar aggregation in amphiphilic block copolymer systems. One can speculate that the micelles made of PHIS-PEG/DSPE-PEG mixtures could be secondary aggregates of smaller individual micelles. Fig. 4 presents the average particle size of mixed micelles at three different pH values (7.4, 6.5, 5.5). The micelle size increased as pH decreased. When pH drops from 7.4 to 6.8, size distribution of micelle does not change significantly (data not shown). When pH drops to 6.5, PHIS of mixed micelles becomes partly protonated and its hydrophilicity increased. As a result the micelles began to swell to balance the increasing electrostatic repulsions. However, the micelle entity wouldn't destabilize because the intra-micellar hydrophobic interactions were still strong enough to maintain a homogeneously-mixed core. So, a small increase in the

micelle size takes place (Fig. 4). This result suggests that PHIS-PEG/DSPE-PEG mixed micelles (1:1, m/m) could be stable at tumor pH and be endocytosed as the micelle entity. As pH goes down to 5.5 (like in endosomes), there is a significant micelle size increase. Because further disruption of the micelle core forces ionized PHIS-PEG unimers to dissociate from the micelles, making micelles disintegrate and aggregate into bigger formation. The pH dependency of the PHIS-PEG/DSPE-PEG mixed micelles is influenced by the mixing ratio of the two polymer components. As was shown in our experiments with different component mass ratios (7:3 and 3:7), the higher the PHIS-PEG content, the easier the destabilization of mixed micelles as pH drops (data not shown).

The fabrication of polymeric micelles by mixing a pH-sensitive polymer and a pH-insensitive polymer may provide an innovative way of tuning the pH-sensitivity of a mixed system. At the same time, the charge of mixed micelle changed from -11.86 mV at pH 7.4 to approximately $+6.4$ mV at pH 5.5. The slightly positive zeta potential at pH 5.5 was due to the protonation of unsaturated nitrogen of the imidazole rings in PHIS. These results also indicated that the PHIS-PEG/DSPE-PEG mixed micelles should be stable at the tumor pH and would disintegrate at endosomal pH.

3.4. Critical micelle concentration (CMC) of mixed micelle

The micelle formation by the self-assembly of PHIS-PEG/DSPE-PEG was monitored by the fluorometry in the presence of pyrene as a fluorescent probe, which is highly hydrophobic and preferentially migrates into the lipophilic micellar core in an aqueous solution (Fig. 5). The increase in emission intensity versus polymer concentration indicates the change from the unimer to the micellar structure. The CMC was estimated by the intensity ratio measurement of the fifth and the first (I_{394}/I_{373}) highest energy bands in the emission spectra profile against of the micelle concentration ($\mu\text{g}/\text{mL}$). The CMC value was determined from the crossover point at low concentrations. The CMC of mixed micelles was about $3.6 \mu\text{g}/\text{mL}$. When pH dropped from 7.4 to 5, the emission intensity of pyrene decreased 5 times (data not shown). This confirmed that mixed micelles were disrupted as pH drops to about 5.

3.5. Stability of mixed micelles

Stability is an important property for micelles, especially for mixed micelles. It can be expected that compared to PHIS-PEG micelles, the intra-micellar hydrophobic interactions in the mixed micelles will be strengthened by the presence of DSPE-PEG.

To evaluate the micellar stability, the turbidity was measured at pH 7.6. Within 7 days, the absorbance and transmittance had almost not change, being ca. 0.022 and 95.4%, respectively. In addition, it was found that the micelle size and drug content remained unchanged for 3 months at 4°C indicating that the micelles had considerably high thermodynamic stability. At room temperature however, there was some size change after 2 weeks, but the drug content remained the same.

3.6. In vitro drug release studies

When the micelle enters cells via endocytosis, the experienced pH will drop from neutral to the pH as low as 5.5–6.0 in endosomes and 4.5–5.0 in lysosomes. With this in mind, the *in vitro* release profiles of PTC-loaded PHIS-PEG/DSPE-PEG mixed micelles were tested at three different pH values – 5.0, 6.0, and 7.4 – at 37°C . As shown in Fig. 6, the drug release was pH-dependent. The drug release was much faster at either pH 5.0 or pH 6.0 than at pH 7.4. Within the first 2 h, the accumulated PCT release of the mixed micelles at pH 5.0 and pH 6.0 was more than 60% and 40%, respectively, while at pH 7.4 it was only about 10%. This pH-sensitivity was attributed to the imidazole groups of PHIS. The drug release rate

can be altered rapidly via the deformation of the inner core induced by the changes in environmental conditions. At pH 7.4, the structure of the mixed micelles was stable and only a small amount of drugs was released. When pH dropped to 5.0 or so, PHIS in the mixed micelles becomes almost completely protonated, and the hydrophilicity and electrostatic repulsions became so strong that the PHIS blocks could no longer hold together inside the core. The phase separation occurs in the core, and PHIS-PEG unimers dissociate from the mixed micelles, so that drug is released. The result suggests that the mixed micelles could react on relatively minor differences in pH by destabilizing and releasing drug(s) inside tumor cells (Fig. 6).

3.7. pH-dependent cytotoxicity

The *in vitro* cytotoxicity of PCT-loaded PHIS-PEG/DSPE-PEG mixed micelles at three different pH values characteristic of normal tissue (pH 7.4), tumor extracellular space (pH_e 6.8), and early endosomal compartment (pH < 6.0), were evaluated using the MTT assays (Fig. 7). (In preliminary studies, we found that pH 5.0 could affect tumor cell viability. To avoid this, pH 5.8 was used in cytotoxicity assay.) At pH 7.4 and 6.8, PHIS-PEG/DSPE-PEG mixed micelles and DSPE-PEG micelles demonstrated low cytotoxicity and no significant difference between these pH values was noted ($n = 3$, $p > 0.05$). At pH 5.8, PCT-loaded DSPE-PEG micelles showed mild cytotoxicity (63–88% cell viability). However, PHIS-PEG/DSPE-PEG mixed micelles showed much higher cytotoxicity (24–56% cell viability) at all concentrations of PCT. No cytotoxicity was observed in blank mixed micelles at three different pH values and at the polymer concentration of up to 20 $\mu\text{g/mL}$ (Fig. 7). The results are well consistent with the data observed in *in vitro* drug release, indicating that the pH-sensitive micelles facilitate the drug release and efficiently kill the tumor cells.

3.8. Cellular uptake of non-targeted and 2C5-targeted micelles

To determine the cellular uptake of the micelles, the cells were incubated with different Rh-labeled micellar formulations for 1 h. Fig. 8a showed that the cells treated with non-targeted pH-sensitive mixed micelles showed mild scattered red fluorescence. In contrast, as shown in Fig. 8b, after the incubation of 4T1 cells with 2C5-modified mixed micelles, much more red fluorescence was observed.

The treated cells were also evaluated with FACS analysis (Fig. 9). Based on the data, the non-targeted pH-sensitive mixed micelles (PEG-PHIS/DSPE-PEG) showed the lower fluorescence with the geometric mean of about 150% compared to that of the untreated cells (negative control, as 100%) while the modification with 2C5 significantly increased the number of the fluorescent cells with the fluorescence geometric mean of about 300%. This is in consistent with the data of fluorescence microscopy, indicating that 2C5 could enhance the cellular uptake of the micellar nanocarrier.

3.9. In vitro cytotoxicity assay of targeted micelles

Targetability and pH-sensitivity can provide a synergistic effect. After internalization, pH-triggered PCT release at early endosomal pH and the fusogenic activity of PHIS could facilitate the PCT release from the endosomal compartment to cytosol. Fig. 10 demonstrates such combined effect. The PCT-loaded DSPE-PEG₃₄₀₀-2C5/DSPE-PEG₂₀₀₀/PHIS-PEG targeted and pH-sensitive mixed micelles induced an enhanced cytotoxicity (less than 20% cell viability at 1 $\mu\text{g/mL}$ of PCT), while the targeted but not pH-sensitive DSPE-PEG₃₄₀₀-2C5/PE-PEG₂₀₀₀ micelles results in about 40% cell viability at 1 $\mu\text{g/mL}$ of PCT. The difference can be explained by the quick drug release and its efficient endosomal escape in case of targeted and pH-sensitive mixed micelles.

4. Conclusion

A pH-sensitive polymeric mixed micelle was prepared by combining PHIS-PEG and DSPE-PEG polymers. The mixed micelle composed of PHIS-PEG and DSPE-PEG (1:1, m/m) are stable at tumor pH_e (above 6) and could be endocytosed as the intact micelle. When pH drops to around 5.5, a destabilization of micelles is observed caused by phase separation in the micelle core and dissociation of the ionized PHIS-PEG molecules. *In vitro* cytotoxicity study demonstrated that the PCT-loaded mixed micelles showed pH-dependent cytotoxicity especially pronounced at lowered pH values because of pH-triggered PCT release and the fusogenic activity of PHIS assisting the endosomal drug release. The conjugation of tumor-specific mAb 2C5 to pH-sensitive mixed micelles further enhances the tumor cell killing effect via the 2C5-mediated specific recognition and internalization. The combined use of active targeting and pH-triggered quick drug release could significantly increase the intracellular drug concentration and efficiently kill tumor cells without any risk of developing drug resistance. We hope that our future animal experiments will support this hypothesis.

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Fig. 1.
The acid-triggered drug release from the pH-sensitive mixed micelles.

Fig. 2.
Synthesis scheme of PHIS-PEG conjugate.

Fig. 3.
 ^1H NMR spectrum of PHIS-PEG.

Fig. 4. The particle size (upper) and zeta potential (lower) of the pH-sensitive mixed micelles at different pH (7.4, 6.5, 5.5).

Fig. 5.
Critical micelle concentration (CMC) of the pH-sensitive mixed micelle.

Fig. 6.
The cumulative PCL release from the pH-sensitive mixed micelles at different pH.

Fig. 7.
The cytotoxicity of paclitaxel-loaded micelles in 4T1 cells.

Fig. 8.
The fluorescence microscopy of Rh-PE labeled mixed micelles.

Fig. 9.
FACS analysis of pH-sensitive mixed micelles in 4T1 cells.

Fig. 10.
Cytotoxicity of 2C5-mixed micelles in 4T1 cells.