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pH-Responsive Mechanism of a Deoxycholic Acid and Folate Comodified Chitosan Micelle under Cancerous Environment

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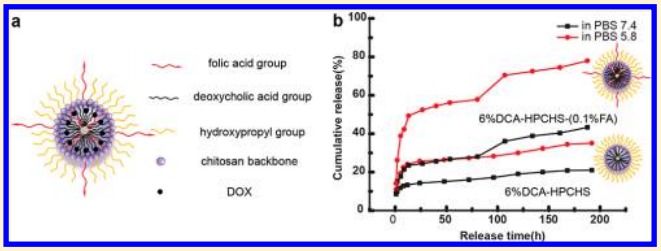
Supporting Information

ABSTRACT: Smart pH-responsive polymeric micelles have attracted much attention as one of the most promising drug delivery candidates. In this paper, a different substitution of deoxycholic acid (DCA) and folic acid (FA) comodified hydroxypropyl chitosans (HPCHS) were synthesized for doxorubicin (DOX) targeted delivery and controllable release. The results indicate that the DOX-release behavior is pH-responsive and closely related with the grafting proportions of the two hydrophobic ingredients. The pH-responsive mechanism for the optimized (6%DCA)-HPCHS-(0.1%FA) was suggested, resulting from a synergistic effect of gradual hydrolysis of the amido bond and electrostatic repulsion between the subsequently protonated DOX and the amino residue of the chitosan backbone under a cancerous microenvironment. Moreover, the DOX/(6%DCA)-HPCHS-(0.1%FA) micelle as a promising targeted drug delivery system in cancer therapy was evaluated by cell growth inhibition assays and confocal laser microscopy in vitro. The results clearly demonstrate a controlled release of its cargo and promoted curative efficacy of DOX.



1. INTRODUCTION

Smart polymeric micelle as a drug delivery system has the potential to provide safer and more effective protocol in cancer therapy.[1,2] Great efforts have addressed to design smart polymers with stimuli-responsive properties.[3−5] Among various external stimuli such as temperature, light, magnetic and electric field, and so on,[6−10] pH-triggered chemotherapy has attracted more attention in recent years.[11−13] As the pH of tumor microenvironment is slightly more acidic than healthy tissue, a pH-responsive vehicle will facilitate the controllable release of drug molecules at the tumor site.[14,15]

The sensitivity of previously reported pH-responsive polymeric micelles for antineoplastic agents' delivery either originates from ionization of the pendant groups or pH-activable linkage between the drug molecules and the backbone of the polymer.[16,17] A variety of polymer micelles with a pH-sensitive feature have been prepared for drug delivery.[18−20] For example, Zhou's group has reported an octreotide-PEG-deoxycholic acid (OPD) conjugate-modified *N*-deoxycholic acid-*O*,*N*-hydroxyethylation chitosan (DAHC) micelle as an effective carrier to deliver doxorubicin (DOX), a commonly used anticancer drug. The DOX-loaded micelle exhibited much faster drug release rate at pH 5.8 than at pH 7.4 due to the pH-dependent protonation of the pendant amino groups and the cargo molecules, and the DOX release rate was accelerated with the increase of OPD content.[21] Recently, Johnson et al. also developed a pH-activable micelle by encapsulating DOX into poly(2-hydroxyethyl methacrylate)-*b*-poly(L-histidine), which demonstrated a strong pH-dependent drug release profile.[22] Moreover, the pH responsiveness was readily controlled by regulating the length of histidine blocks.[22] Though the reported polymeric micelles have demonstrated enhanced therapeutic efficacy and limited side effects, some drawbacks still remain as following: (i) Most of the drug conjugated micelles release antitumor drugs through the cleavage of a pH-sensitive bond and result in a burst release profile, which potentially decrease the curative duration of antineoplastics.[23−25] (ii) Some polymeric micelles with pH responsiveness are of incomplete drug release and slow release rate, thus, greatly reduce bioavailability and therapeutic efficacy.[26−28] (iii) Some drug-loaded micelles release their cargo under extremely acidic or alkaline condition, which limits their application in vivo.[29−31] Therefore, further exploration of parameters accounting for pH-sensitivity is especially important. Just like the literature mentioned above, the drug release behavior may be facilely controlled by regulating the grafting proportion of the amphiphilic micelle molecules.

In addition, the modification of polymeric micelles with targeting molecule enables the more rapid advancement of controlled-release technology.[32−35] Folic acid (FA, a low molecular weight vitamin) and its conjugates have been well investigated in recent years to specifically target FA receptor

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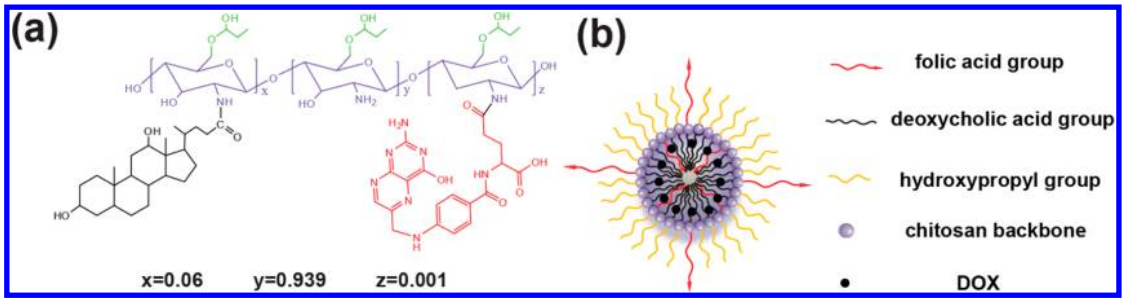

Figure 1. Chemical structure of (6%DCA)-HPCHS-(0.1%FA) (a) and schematic illustration of self-assembled DOX/(6%DCA)-HPCHS-(0.1%FA) micelle (b).

(FAR) overexpressed human tumor cells.[36,37] For example, Galbiati et al. synthesized a camptothecin-loaded chitosan-FA modified microcapsule, which could selectively inhibit the proliferation of tumor cells and therefore do negligible harm to normal cells.[38] However, except for the targeting property of FA, little was known about its influence on the critical micelle concentration (CMC) and drug release profiles of resulting polymeric micelles.

Herein, deoxycholic acid modified hydropropylchitosans (DCA-HPCHS) were employed as precursors to prepare DOX-loaded polymeric micelles with pH-responsiveness. Two different grafting proportions of DCA were designed first, which were identified as (6%DCA)-HPCHS and (12%DCA)-HPCHS by ^{1}H NMR (Figure S1, Supporting Information (SI)). The drug release behaviors of the micelles were well investigated. The results reveal that the pH-sensitive micelles largely rely on the grafting proportions of DCA. Then FA was introduced to DCA-HPCHS to investigate its contribution to the pH-responsive performance. As expected, for the first time we find that besides the active targeting function, the grafting FA proportion along with DCA contributes to the pH-responsiveness of DOX-loaded micelle. Although the DOX release rate of DOX/(12%DCA)-HPCHS is more favorable than that of DOX/(6%DCA)-HPCHS, further modification of FA on (12%DCA)-HPCHS deteriorates the biocompatibility and pH-responsive property of formed micelles (Figure S2b, SI). As a compromise, (6%DCA)-HPCHS was used as a precursor to conjugate with FA and thus endowed the formed micelles with targeting capability and drug controlled-release behavior. By systematically investigating the effect of grafting FA ratio to (6%DCA)-HPCHS on in vitro drug release, the optimal micelle molecule characterized as (6%DCA)-HPCHS-(0.1%FA) was obtained (Figure 1), which exhibited an optimized encapsulation efficiency (EE) and drug loading content (DLC) of 97.85 and 3.6%, respectively.

The mechanism of pH-responsive property of this deoxycholic acid and folic acid comodified chitosan micelle was investigated by dynamic light scattering (DLS) and ^{1}H NMR. The results reveal that the pH-responsive performance of optimized (6%DCA)-HPCHS-(0.1%FA) micelle originates from a gradual hydrolysis of amido bond, which is accompanied with the protonation of DOX and the amino residue of chitosan backbone under the cancerous environment. The resulting electrostatic repulsion between the protonated amino residue of the chitosan backbone and DOX facilitates the continuous and complete release of DOX.

The cell growth inhibition assays and confocal laser microscopy of optimized DOX-loaded micelle were also conducted. The results demonstrate that the DOX/(6% DCA)-HPCHS-(0.1%FA) micelle possesses the potential to selectively kill HeLa cells in comparison with HBE cells due to the combination of high affinity on HeLa cells mediated by FA/FAR molecular recognition and the unique pH-responsive performance under cancerous environment.

2. EXPERIMENTAL SECTION

2.1. Material. Chitosan (MW ~ 5.0 × 10^5) with a 90% degree of deacetylation was supplied by Jinan Haidebei Biochemical Co. Ltd., China. Deoxycholic acid (DCA) was provided by Alfa Aesar China Co. Ltd., China. *N*-Hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), dimethyl sulfoxide (DMSO), and triethylamine were obtained from Shanghai RichJoint Chemical Reagents Co. Ltd., China. Propylene oxide was purchased from Sinopharm Chemical Reagent Co. Ltd., China. Pyrene, doxorubicin hydrochloride (DOX•HCl), and folic acid (FA) were purchased from Sigma Company. Ltd. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Japan). All commercially available solvents and reagents were analytical grade and were used without further purification. HeLa cell lines and human bronchial epithelia (HBE) cells were kindly provided by Prof. Xiaohong Fang in Institute of chemistry, Chinese Academy of Sciences.

2.2. Synthesis of DCA-HPCHS. A mixture of DCA (0.48 g), EDC (0.11 g), and NHS (0.12 g) in 100 mL of methanol was added to 1% (wt) of acetic acid aqueous solution (100 mL) containing chitosan (1 g) under stirring condition. After reacting under room temperature for 22 h, the resulting mixture was poured into a mixed solution of methanol (7 mL) and ammonia (3 mL) to precipitate the final product, deoxycholic acid modified chitosan (DCA-CHS), which was collected by filtration and washed with acetone several times. The as-prepared DCA-CHS was dispersed in a mixture of sodium hydrate (0.8 g) and isopropanol (30 mL) to alkalize for 4 h. Then propylene epoxide (4.4 mL) was added to the above solution to react for 24 h at 45 °C. The resulting mixture was neutralized by acetic acid to precipitate the desired product. (6%DCA)-HPCHS was obtained by filtration and subsequent washing with 75% ethanol and acetone for several times and vacuum drying at 50 °C. (12%DCA)-HPCHS was synthesized following the same procedure except that the feed ratio of DCA/EDC/NHS/chitosan was fixed at 0.96:0.22:0.24:1 in mass. The composition of prepared DCA-HPCHS was determined by ^{1}H NMR spectra using a ^{1}H NMR spectrometer (AVANCE 400, Bruker).

2.3. Synthesis of DCA-HPCHS-FA. A total of 0.15 mg of FA reacted with 0.067 mg of EDC in aqueous solution for 2 h. Then the resulting mixture was added dropwise to 50 mg of (6%DCA)-HPCHS and reacted for another 16 h. The mixture

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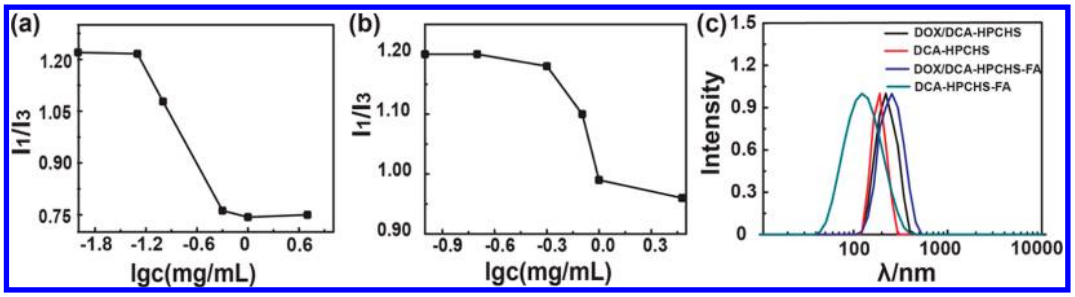

Figure 2. Variation of fluorescence intensity ratio (I_1/I_3) for pyrene in PBS solution (pH 7.4) in the presence of (6%DCA)-HPCHS-(0.1%FA) (a) or (6%DCA)-HPCHS (b). Particle size distributions of (6%DCA)-HPCHS, (6%DCA)-HPCHS-(0.1%FA), DOX/(6%DCA)-HPCHS, and DOX/(6%DCA)-HPCHS-(0.1%FA) micelles (c).

was ultrafiltrated using an ultrafiltration tube (M_w cut off = 3000 Da, Sartorius) at 10000 rpm for 20 min several times to remove the free FA. (6%DCA)-HPCHS-(0.1%FA) was obtained by dehydration with the rotary evaporator. The synthesis of (6%DCA)-HPCHS with 0.001, 0.01, 1, and 10% FA grafted degree was followed the same procedure except that the feed ratio of FA to (6%DCA)-HPCHS in mass was fixed at $3{:}10^5$, $3{:}10^4$, $3{:}10^2$, and 3:10, respectively. Likewise, (12% DCA)-HPCHS-(0.1%FA) was synthesized under the same condition as (6%DCA)-HPCHS-(0.1%FA) case, except for the substitute of (6%DCA)-HPCHS for equivalent (12%DCA)-HPCHS. The UV−vis absorption was measured using a UV-4802H spectrophotometer.

2.4. CMC Determination of DCA-HPCHS-FA. The CMC of (6%DCA)-HPCHS and (6%DCA)-HPCHS-(0.1%FA) were determined by fluorescence measurement using pyrene as a probe.[39] Modified chitosan solutions containing 1×10^{-6} M pyrene in a series of gradient concentrations were prepared and monitored by a fluorescence spectrophotometer with an excitation wavelength at 335 nm, excitation slit, and emission slit at 5 nm. The intensities of the emission ranging from 350 to 550 nm were recorded. The CMC can be evaluated by measuring the intensity ratio (I_1/I_3) of the first (373 nm) and the third (384 nm) highest energy band in the emission spectra of pyrene and determined by the interception of two straight lines at low concentration region.

2.5. Preparation and Characterization of Blank and DOX-Loaded Micelles. A total of 30 mg of modified chitosan in 1 mL of DMSO was added dropwise to 5 mL of deionized water, and the resulting solution was stirred for 10 h for the formation of the blank micelle. Then the product was ultrafiltrated using an ultrafiltration tube (M_w cut off = 3000 Da, Sartorius) at 10000 rpm for 20 min to concentrate and then diluted to the desired concentrations. The DOX-loaded micelles were prepared as the following procedures. A 5 μL aliquot of triethylamine was added to a mixture of doxorubicin hydrochloride (1 mg) and DCA-HPCHS (30 mg) or DCA-HPCHS-FA (30 mg) in 1 mL of DMSO. The resulting mixture was stirred at room temperature for 2 h to produce hydrophobic doxorubicin, which subsequently was added dropwise to 5 mL of deionized water and stirred for 10 h. Finally, the mixture was ultrafiltrated at 10000 rpm for 20 min for several times to remove the unloaded DOX, and the solution was redispersed to desired concentrations. The DOX concentration and drug loading contents were determined by fluorescence spectrophotometry analysis. The fluorescence intensity of the DOX-loaded micelles solution was measured at an excitation wavelength of 490 nm, the emission wavelength at 590 nm, the excitation and emission slits at 5.0 nm. The DOX content was calculated according to a standard curve obtained from doxorubicin hydrochloride aqueous solution. The size and zeta potential of micelles were characterized using a Malvern Zetasizer Nano ZS 90 at 25 °C.

2.6. In Vitro DOX Release from DOX-Loaded Micelles. The in vitro DOX release behaviors of the DOX-loaded micelles were evaluated. Generally, 1 mL of DOX/DCA-HPCHS or DOX/DCA-HPCHS-FA micelle solution was added into a dialysis bag (M_w = 3500 Da, Spectrum Laboratories Inc., CA, U.S.A.) and was immersed in 50 mL of phosphate buffer solution (PBS) at pH 7.4 or 5.8. The experiments were conducted at 37 °C with a horizontally shaking at 120 rpm for 8 days. A 1 mL aliquot of the solution in the tube was sampled at predetermined time intervals, and the same amount of fresh PBS was replenished. DOX content in the withdrawn samples was determined by a fluorescence spectrophotometry as previously reported.[28] All the experiments were conducted in triplicate.

2.7. Cell Culture. HeLa cell line as a FAR positive model and HBE cell as a FAR negative model were used to evaluate the functionality of modified chitosan micelles. The cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37 °C under a humidified atmosphere with 5% CO_2.

2.8. Cell Growth Inhibition Assays. Cell viabilities of HBE and HeLa cells incubated with either free DOX or DOX/(6%DCA)-HPCHS-(0.1%FA) micelle solution were determined by CCK-8 assays, respectively. First, HeLa or HBE cells with a 5×10^4 cells per well density were seeded into a 96-well culture plate with 200 μL of DMEM supplemented with 10% FBS and 1% PS in each well, and incubated at 37 °C under a humidified atmosphere with 5% CO_2 for 24 h. After the aged culture medium was replaced by freshly prepared culture medium containing either free DOX or DOX/(6%DCA)-HPCHS-(0.1%FA) micelle solution in a serial of gradient concentrations, the resulting cells were incubated for predetermined time (24, 48, and 72 h). Then the culture medium was removed and 100 μL of DMEM without phenol red and 10 μL of CCK-8 were added into each well. After incubating for another 1 h, the absorbance of each well was measured at 450 nm by a microplate reader.

2.9. Confocal Laser Microscopic Characterization. HeLa cells were seeded into a confocal dish with 5×10^4 cells per well and allowed to grow until 60% confluence. Cells were washed twice with PBS and then incubated with either (6%DCA)-HPCHS-(0.1%FA) or DOX/(6%DCA)-HPCHS-(0.1%FA), or DOX/(6%DCA)-HPCHS micelle solution (with equivalent DOX at a concentration of 20 μg mL^{-1}) or

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free DOX (20 μg mL^{-1}) in DMEM for 24 h at 37 °C. Then the cells were washed twice with ice-cold PBS for visualizing under a laser scanning confocal microscope.

3. RESULTS AND DISCUSSION

3.1. CMC Characterization. From Figure 2a and b, CMC values of (6%DCA)-HPCHS-(0.1%FA) and (6%DCA)-HPCHS can be calculated as 0.05 and 0.5 mg mL^{-1}, respectively. The lower CMC value of (6%DCA)-HPCHS-(0.1%FA) (Figure 2a) relative to that of (6%DCA)-HPCHS (Figure 2b) indicated that the introduction of FA moiety enhanced the hydrophobic property of (6%DCA)-HPCHS molecule. Because the CMC of the two polymers were lower than 1 mg mL^{-1}, the formation for either the blank or drug-loaded micelles at a concentration of 5 mg mL^{-1} was guaranteed.

3.2. EE and DLC of DOX-Loaded Micelles. The preparation of blank and DOX-loaded micelles was followed by the procedures mentioned in the Experimental Section. DOX was loaded in the inner core of the polymeric micelles (6%DCA)-HPCHS, (12%DCA)-HPCHS, and (6%DCA)-HPCHS-(0.1%FA) through hydrophobic−hydrophobic interaction, with the EE and DLC of 88.01 and 2.4%, 90.85 and 2.9%, and 97.85 and 3.6%, respectively. Obviously, the EE and DLC values of DOX are closely related with the grafting degree of DCA and FA. Considering DOX is accommodated by the hydrophobic core of the micelle, it makes sense that (12% DCA)-HPCHS and (6%DCA)-HPCHS-(0.1%FA) with higher hydrophobic component possess higher EE and DLC than the (6%DCA)-HPCHS case. In contrast, Jeong's group reported that the EE value of DOX within dexoycholic acid-modified chitosan (DCA-CHS) was merely 27.5%,[40] which might ascribe to the following reasons: (i) the used DOX was not alkalized by triethylamine to give hydrophobic DOX, hence, the interaction between DOX and micelle core was weakened; (ii) the encapsulation process was a direct dissolution of DCA-CHS followed by sonication, which may lead to the leakage of DOX from the drug-loaded micelle; (iii) the absence of hydrophilic group (hydroxypropyl) may reduce the stability of micelle since the high solubility of unmodified chitosan is not a desirable feature.

3.3. DLS Characterization. The size distributions of (6% DCA)-HPCHS, DOX/(6%DCA)-HPCHS, (6%DCA)-HPCHS-(0.1%FA), and DOX/(6%DCA)-HPCHS-(0.1%FA) micelles were obtained by DLS as about 190, 220, 122, and 255 nm, respectively (Figure 2c). Generally, the drug-loaded micelles are larger than their counterparts, which is an indirect but potent indicator for the successful encapsulation of DOX since loaded drugs in micelles may contribute a little to the size increase in formed micelles. More importantly, the diameter of drug-loaded micelles falls into the range of 200−300 nm, which is a favorable size for an enhanced permeability and retention (EPR) effect. Therefore, these DOX-loaded micelles may circulate longer in vivo and maintain the DOX concentration above the curative effect for a long time, which will unambiguously contribute to the effective accumulation in tumor sites and subsequently controlled drug release and adverse effects remission.

3.4. In Vitro Drug Release of DOX from Drug-Loaded Micelles. As described in the Experimental Section, the DOX release from micelles was conducted in a dialysis bag. Figure 3 compares the contribution of DCA in different grafted degrees to DOX release. The release curve of DOX/(12%DCA)-HPCHS (Figure 3a) shows that the drug-loaded micelles possess excellent pH-responsive property. The micelle released about 85% cargo within 96 h, while the remaining 15% exerted an effect as long as 100 h in the plateau period at pH 5.8, whereas DOX release was slow and restrained at pH 7.4. In contrast, DOX/(6%DCA)-HPCHS micelles (Figure 3b) exhibited much slower release rate at both pH values, suggesting a poor pH-sensitive performance.

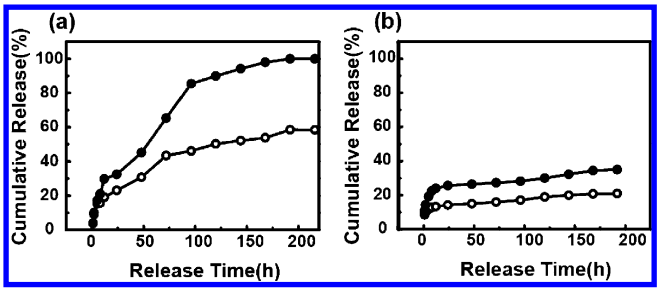

Figure 3. In vitro release of DOX from DOX/(12%DCA)-HPCHS (a) and DOX/(6%DCA)-HPCHS (b) in PBS 5.8 (-●-) and PBS 7.4 (-○-).

Interestingly, FA-modified (6%DCA)-HPCHS can dramatically enhance pH-responsive performance of formed micelles compared with its precursor. In contrast, further modification of FA on (12%DCA)-HPCHS deteriorates the biocompatibility and the pH-responsiveness as well, which may reduce the potency of DOX to cancer cells (Figure S2b, SI). Therefore, a different substitution proportion of FA to the (6%DCA)-HPCHS backbone ranging from 10 to 0.001% was investigated. The results demonstrate that only those with a substitution proportion below 0.1% could ensure good biocompatibility of micelles and good encapsulation efficiency of DOX as well (Figure S2c, SI). Notably, the pH-responsive property of formed micelles is proportional to the grafted FA proportion ranging from 0.001 to 0.1%. As a compromise, (6%DCA)-HPCHS-(0.1%FA) was revealed as an excellent candidate with good biocompatibility, sufficient tumor targeting ability, and good pH-responsive property (Figure 4). In comparison with DOX/(12%DCA)-HPCHS, DOX/(6%DCA)-HPCHS-(0.1% FA) released its cargo with a doubled release rate in pH 5.8 at 18 h due to the synergistic effect of DCA and FA. Also, the remaining 25% was released within the following plateau period, which lasted as long as 150 h. Though DOX release accelerated at pH 7.4 as well, its total cargo release dropped to 45% at plateau, again confirming an excellent pH-responsive property. More recently, Zhou's group reported a pH-sensitive DOX delivery platform based on *N*-deoxycholic acid-*O*,*N*-hydroxyethylation chitosan (DAHC) micelle with 60% DOX release at pH 5.8 and 40% DOX release at pH 7.4 after 100 h.[21] Compared with DAHC micelle, DOX/(6%DCA)-HPCHS-(0.1%FA) micelles exhibited a faster and more durable drug release profile, which not only ensured a relatively high DOX concentration at the tumor site, but also facilitated the maintenance of a high concentration as long as possible. These advantages meet the prerequisite for drug controlled release.

3.5. pH-Responsive Mechanism of DOX-Loaded Micelle. The pH-responsive mechanism of (6%DCA)-HPCHS-(0.1%FA) was investigated by zeta potential and ^{1}H NMR. As Table 1 illustrates, (6%DCA)-HPCHS-(0.1%FA) along with its precursor (6%DCA)-HPCHS is electropositive in PBS 5.8 and electronegative in PBS 7.4, indicating that the

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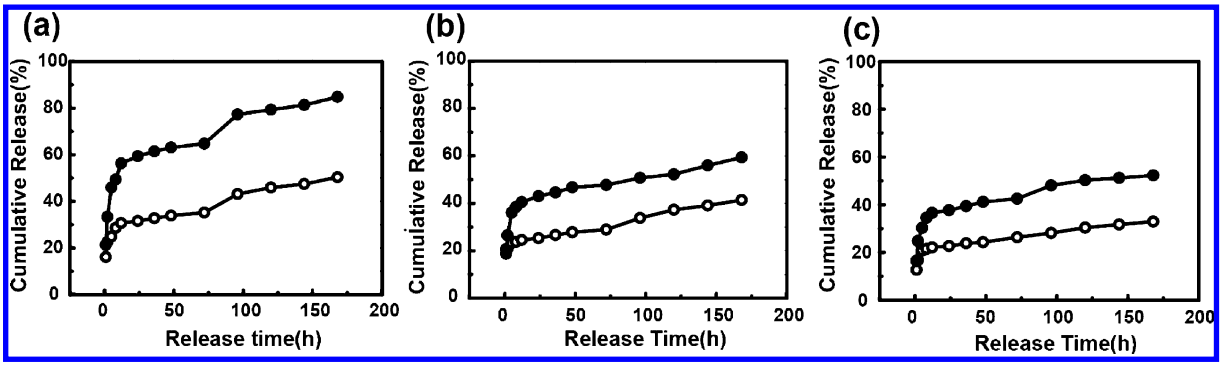

Figure 4. In vitro release of DOX from DOX/(6%DCA)-HPCHS-(0.1%FA) (a), DOX/(6%DCA)-HPCHS-(0.01%FA) (b), and DOX /(6%DCA)-HPCHS-(0.001%FA) (c) in PBS 5.8 (-●-) and PBS 7.4 (-○-).

Table 1. Zeta Potential Values of (6%DCA)-HPCHS and (6%DCA)-HPCHS-(0.1%FA) under pH 7.4 and 5.8, Respectively

zeta potential	in PBS 7.4	in PBS 5.8
(6%DCA)-HPCHS	−4.29 mV	2.81 mV
(6%DCA)-HPCHS-(0.1%FA)	−6.55 mV	3.94 mV

driving force for the pH-responsive drug release should be relative to the interaction between the micelle and its positive-charged cargo. Noticeably, (6%DCA)-HPCHS-(0.1%FA) micelles showed a more dramatic charge conversion under pH 5.8 in comparison with (6%DCA)-HPCHS due to the introduction of grafted FA moiety. Therefore, the enhanced pH-responsive property of (6%DCA)-HPCHS-(0.1%FA) should be partially attributed to the introduced FA because the amido linkage was susceptible to the microenvironment of a tumor site as well. As a consequence, the grafted ratio of hydrophobic DCA and FA groups and their compromise on the chitosan backbone not only endows the formed micelles with multifunctionality, but also facilitates the continuous drug release by the synergistic contributions from the protonation degree of free amine groups and hydrolysis degree of formed amide bonds under a cancerous environment. The ^{1}H NMR of (6%DCA)-HPCHS in PBS 7.4 and in PBS 5.8 (Figure 5) confirms the suggested pH-responsive mechanism. Because the intensity of the carboxyl group peak located at 8.5 ppm is closely related with the hydrolysis degree of (6%DCA)-HPCHS, as Figure 5 reveals, (6%DCA)-HPCHS demonstrates enhanced hydrolysis degree in PBS 5.8 relative to PBS 7.4 through the comparison of integral ratio between the carboxyl group of deoxycholic acid at 8.5 ppm and the chitosan C2 at 2.7 ppm. Therefore, the pH-responsive property of (6%DCA)-HPCHS should be attributed to a different hydrolysis degree in PBS 5.8 and 7.4 because the acidic environment could promote the hydrolysis of the amido bond. In addition, the contents of obtained amino groups and deoxycholic acid as a result of hydrolysis also influence the pH-responsive property of (6% DCA)-HPCHS. Once the amino group is positively charged and DCA (pK_a = 6.58) keeps nonionized under acidic environment (PBS 5.8), the resulting electrostatic repulsion between the micelle core and DOX in PBS 5.8 will facilitate the drug release. In contrast, the weak hydrolysis degree under physiological condition along with the less positive-charged amine groups of (6%DCA)-HPCHS and negative-charged DCA restricts the DOX release from its carrier. Likewise, the

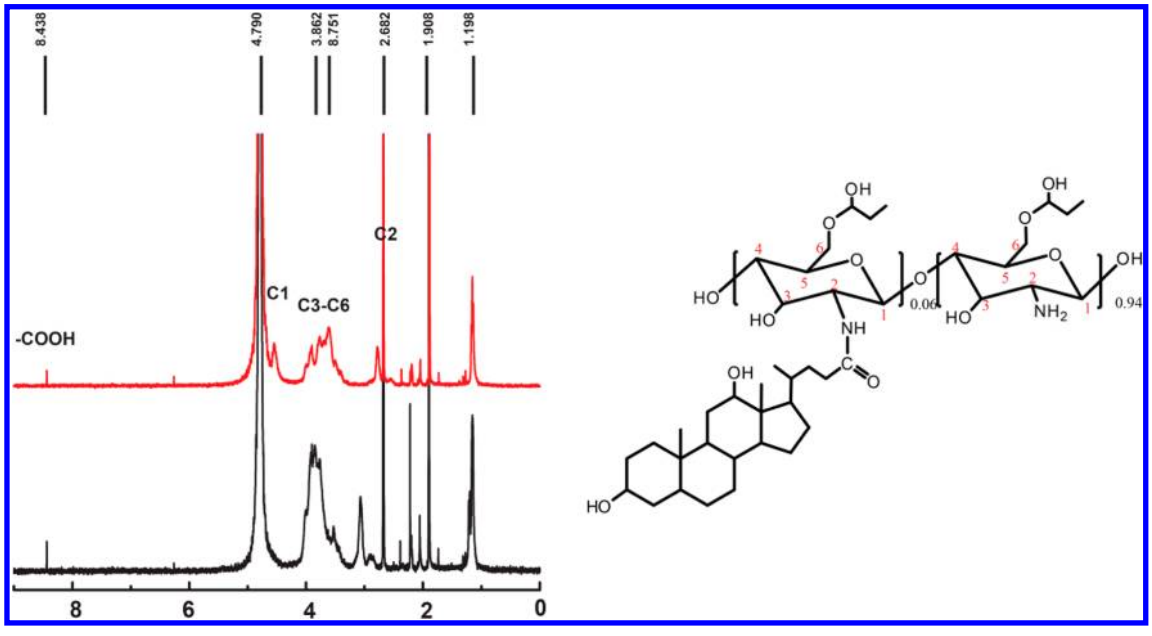

Figure 5. ^{1}H NMR of (6%DCA)-HPCHS in PBS 5.8 (black line) and in PBS 7.4 (red line).

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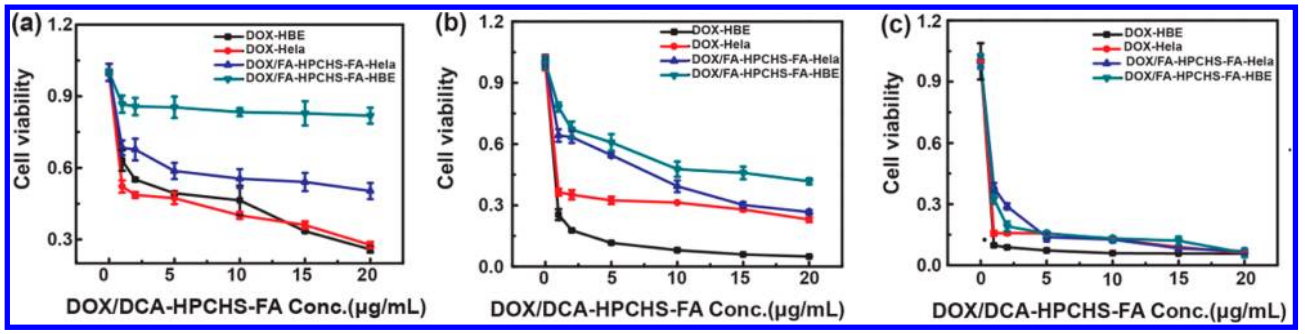

Figure 6. Cell viabilities of HeLa and HBE cells incubated with free DOX or DOX/(6%DCA)-HPCHS-(0.1%FA) for 24 (a), 48 (b), and 72 h (c), respectively.

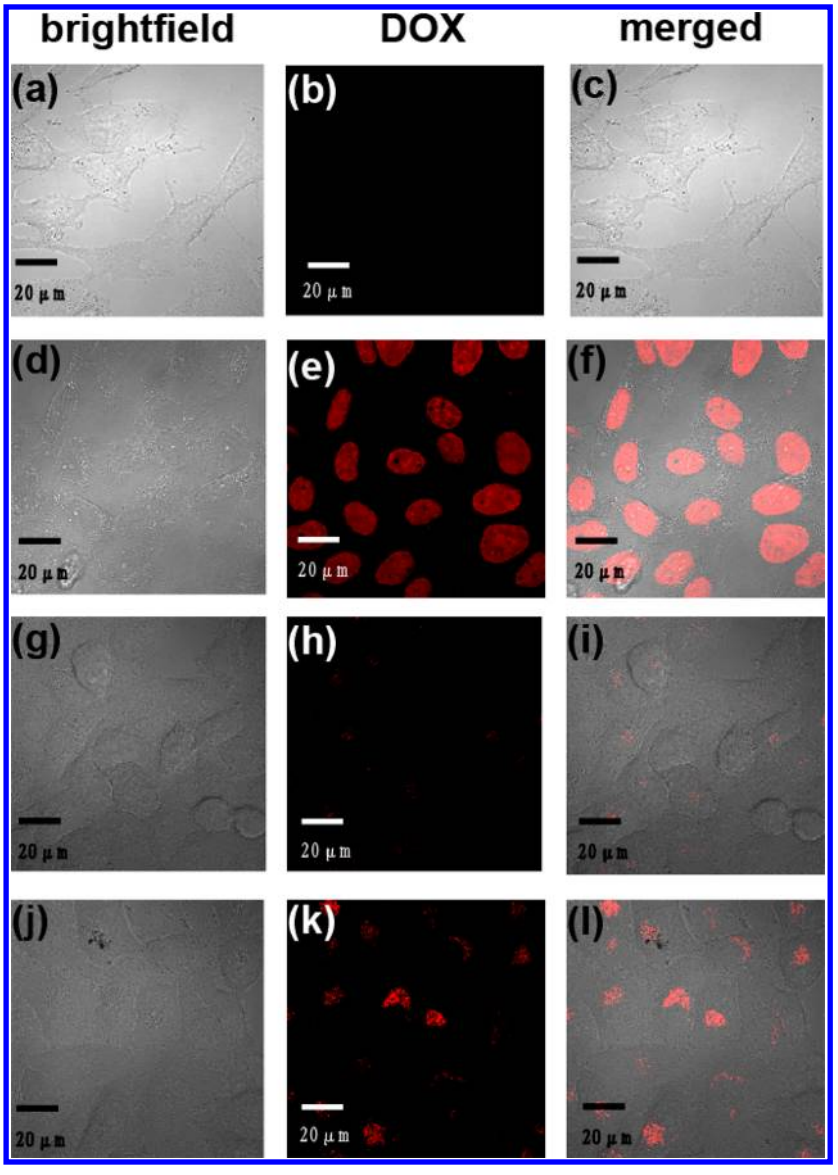

Figure 7. Confocal laser microscopy images of HeLa cells incubated with (6%DCA)-HPCHS-(0.1%FA) (a–c), free DOX (d–f), DOX/(6%DCA)-HPCHS (g–i), and DOX/(6%DCA)-HPCHS-(0.1%FA) micelle solutions (j, k), respectively, for 24 h.

grafted FA on (6%DCA)-HPCHS via amidation linkage to chitosan plays the same role as DCA does during controllable drug release process. Because the free carboxy group of grafted FA possesses a pK_a2 of 6.76 similar to deoxycholic acid mentioned above, the obtained amine groups derived from the hydrolysis of amidation bonds containing -NHCO-FA and -NHCO-DCA favor protonating and therefore promote the electrostatic repulsion between the micelle and its cargo in PBS 5.8. Compared with the pH-responsive micelle system developed by You and co-workers,[37] the pH-responsive performance of (6%DCA)-HPCHS-(0.1%FA) results from gradual hydrolysis of amido bond rather than cleavage of pH-

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sensitive hydrazine bond, thus, guarantees a controlled release of its cargo rather than a bust release profile. This superiority will unambiguously promote the efficacy of DOX.

3.6. Biocompatibility of Blank Micelles. Figure S3 reveals that the obtained micelle is of excellent biocompatibility. The viabilities of HeLa and HBE cells are all above 80% after incubated with blank (6%DCA)-HPCHS-(0.1%FA) micelle below the concentration of 1 mg mL^{-1} for 24 h, indicating that the blank micelles have no obvious cytotoxicity to HeLa and HBE cells in this dosage. It is in good accordance with expectation that the used ingredients for the formation of (6% DCA)-HPCHS-(0.1%FA) micelle are biocompatible as reported.[40]

3.7. Cell Growth Inhibition Assays. Figure 6 demonstrates that the cell growth inhibitions of HeLa and HBE cells incubated with DOX/(6%DCA)-HPCHS-(0.1%FA) micelle are time- and DOX dose-dependent. Generally speaking, with an increase of DOX concentration and incubation time, the viabilities of HeLa and HBE cells decrease dramatically. Notably, after incubating with DOX/(6%DCA)-HPCHS-(0.1%FA) for 24 h (Figure 6a), HBE cells still keep a viability of 80%, while the viability of HeLa cells dramatically drops to 50% at a DOX concentration of 20 μg mL^{-1}. In comparison with free DOX, which inhibits HeLa cells and HBE cells almost equally, DOX/(6%DCA)-HPCHS-(0.1%FA) micelle demonstrates an excellent tumor cell specific inhibition property, which should ascribe to its cancer cell targeting property and pH-responsiveness. Specially, the FA groups on the micelle backbone promote the drug-loaded micelles to anchor specifically onto the FAR overexpressed cancer cells and subsequently internalize into cells via FAR-mediated endocytosis, where DOX in the micelle is controllably and continuously released at the tumor site due to the pH-responsive property. As a result, the inhibition of DOX against HeLa cells is enhanced and its cytotoxicity against normal cells is reduced. Though the inhibition rate of the micelle solution against HeLa cells is just 50% due to the incomplete release of DOX from the DOX/(6%DCA)-HPCHS-(0.1%FA) micelle, prolonging the incubation time would improve this phenomenon as described below. As the incubation time is set longer, the inhibition against HeLa and HBE cells becomes obvious with attenuated differences (Figure 6b,c). Especially with an incubation time of 72 h, the inhibition rates of the drug-loaded micelle to HeLa and HBE cells finally reach nearly 100% as the free DOX does (Figure 6c). This may ascribe to the gradual release of its cargo from the DOX/(6%DCA)-HPCHS-(0.1% FA) micelle, leading an accumulated dosage to the lethality of cells. During the effective and controlled release period of DOX/(6%DCA)-HPCHS-(0.1%FA) micelle in the range of 48 to 72 h, the drug release behavior is less influenced by the ingredients in the cell medium. Thus, it may potentially extend to in vivo as a promising drug carrier.

3.8. Confocal Laser Microscopy. Figure 7 shows the confocal laser microscopy images of HeLa cells incubated with (6%DCA)-HPCHS-(0.1%FA) (Figure 7a–c), free DOX (Figure 7d–f), DOX/(6%DCA)-HPCHS (Figure 7g–i), and DOX/(6%DCA)-HPCHS-(0.1%FA) (Figure 7j–l) micelle solution for 24 h, respectively. The dose of DOX was 20 μg mL^{-1}. Red fluorescence with different intensities was observed clearly in HeLa cells incubated with DOX/(6%DCA)-HPCHS-(0.1%FA), free DOX, and DOX/(6%DCA)-HPCHS, while no fluorescence could be detected in HeLa cells incubated with blank micelle (6%DCA)-HPCHS-(0.1%FA), indicating that the red fluorescence was from DOX specifically. Compared with those incubated with DOX/(6%DCA)-HPCHS, HeLa cells incubated with DOX/(6%DCA)-HPCHS-(0.1%FA) demonstrate much stronger fluorescence intensity. This result again confirms that the introduced FA moieties in (6%DCA)-HPCHS-(0.1%FA) improve the cellular uptake of final DOX. Moreover, DOX/(6%DCA)-HPCHS-(0.1%FA) exhibits superior controllable DOX release behavior compared with free DOX as only part of DOX is released within 24 h, which is well consistent with the results from in vitro release and cell inhibition experiments.

4. CONCLUSION

In summary, for the first time, we reveal that the hydrophobic DCA and FA groups play a synergistic role in the pH-responsive property of deoxycholic acid and folic acid comodified hydropropylchitosans (DCA-HPCHS-FA). The mechanism of pH-responsive performance of this micelle is suggested, resulting from the combinations of gradual hydrolysis of amido bond and electrostatic repulsion between the subsequently protonated DOX and the amino residue of chitosan backbone under cancerous microenvironment. By systematically investigating the effect of grafting FA ratio to (6%DCA)-HPCHS on in vitro drug release, the optimal micelle molecule characterized as (6%DCA)-HPCHS-(0.1%FA) is obtained, which exhibits an optimized EE and DLC of 97.85 and 3.6%, respectively. The cell growth inhibition assays and confocal laser microscopy experiments clearly demonstrate that the optimized DOX-loaded micelle significantly enhances the therapeutic efficacy and limits the side effect of DOX.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectra, UV–vis spectra, in vitro release of DOX, and cell growth inhibition of HeLa cells and HBE cells incubated with (6%DCA)-HPCHS-(0.1%FA). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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