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Tailor-Made Dual pH-Sensitive Polymer−Doxorubicin Nanoparticles for Efficient Anticancer Drug Delivery

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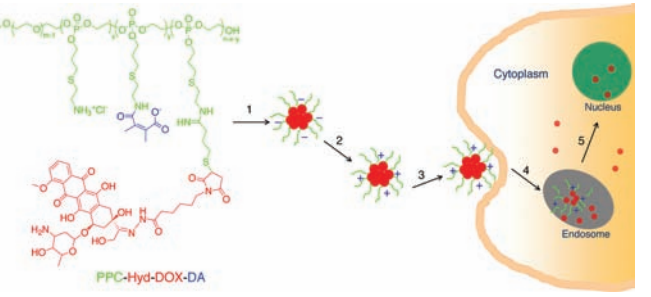
Supporting Information

ABSTRACT: Efficient delivery of therapeutics into tumor cells to increase the intracellular drug concentration is a major challenge for cancer therapy due to drug resistance and inefficient cellular uptake. Herein, we have designed a tailor-made dual pH-sensitive polymer−drug conjugate nanoparticulate system to overcome the challenges. The nanoparticle is capable of reversing its surface charge from negative to positive at tumor extracellular pH (∼6.8) to facilitate cell internalization. Subsequently, the significantly increased acidity in subcellular compartments such as the endosome (∼5.0) further promotes doxorubicin release from the endocytosed drug carriers. This dual pH-sensitive nanoparticle has showed enhanced cytotoxicity in drug-resistant cancer stem cells, indicating its great potential for cancer therapy.

In the past decade, nanoparticle-based drug delivery systems have shown exciting efficacy for cancer treatments due to their improved pharmacokinetics and biodistribution profiles *via* the enhanced permeability and retention (EPR) effect.[1] However, the EPR effect can only enhance the accumulation of nanoparticles (NPs) in tumor tissues; the poor cellular internalization as well as insufficient intracellular drug release always limits the dosages of anticancer drugs to the level below the therapeutic window, which hampers the efficacy of cancer chemotherapy.[2] To address the challenges, environment-responsive delivery systems have been attempted to improve the drug bioavailability.[3] Of these stimuli, pH-responsiveness is the most frequently used, as pH values in different tissues and cellular compartments vary tremendously. For example, the tumor extracellular environment is more acidic (pH_e 6.5) than blood and normal tissues (pH 7.4), and the pH values of endosome and lysosome are even lower at 5.0−5.5.[4] Various pH-responsive delivery vehicles have been developed for pH-triggered drug delivery.[5] Those delivery systems are responsive to a single pH condition, targeting either the tumor extracellular or the intracellular pH condition, impairing the drug delivery efficacy. For instance, the delivery systems that respond to pH_e often release the payloads extracellularly, making them inefficient in killing drug-resistant cells, while those capable of responding to intracellular environment cannot efficiently enhance the cell internalization. The development of dual pH-responsive NPs which can respond to not only pH_e but also endo/lysosomal pH for enhanced drug delivery has not been reported.

Scheme 1. Chemical Structure of the Dual pH-Responsive Polymer−Doxorubicin (DOX) Conjugate (PPC-Hyd-DOX-DA) and Schematic Illustration of Its pH Triggered Cellular Internalization and Intracellular Drug Release[a]



[a] The conjugate self-assembles into negatively charged NPs in water (**1**), and the NPs become positively charged at the extracellular pH (**2**). The positive NPs enter the cells by endocytosis (**3**, **4**), and DOX is released after cleaving the hydrazone bond at endo/lysosomal pH and diffuses into nuclei (**5**).

As a proof-of-concept, we describe here a dual pH-sensitive polymer−doxorubicin conjugate (Scheme 1) as a nanoparticulate drug delivery system. The polymeric drug carrier can respond to the tumor extracellular and intracellular pH gradients through chemically defined mechanisms, which is expected to simultaneously promote drug accumulation at the tumor site *via* EPR effect and facilitate the cell internalization and intracellular drug release, greatly enhancing the drug delivery efficiency. The efficacy of the NPs in anticancer drug delivery is evaluated in drug-resistant cancer stem cells. In the present study, a polyphosphoester is chosen since it has shown biodegradability and been widely used in biomedical fields including drug/gene delivery and tissue engineering.[6]

The PPC-Hyd-DOX-DA conjugate was obtained through a multiple synthesis process as shown in Figure S1, and the details are described in the Supporting Information. First, the parental diblock copolymer monomethoxyl poly(ethylene glycol)-*b*-poly(allyl ethylene phosphate) (mPEG-*b*-PAEP) was prepared by

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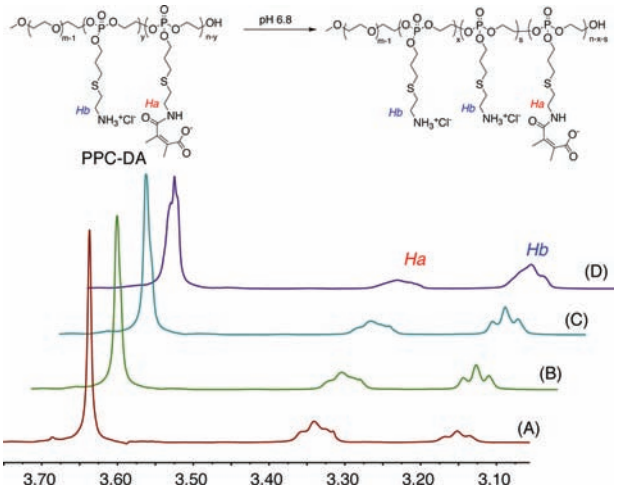

Figure 1. ^{1}H NMR spectra of PPC-DA after incubation at pH 6.8 in D_2O/DCl (25 °C) for different time periods: (A) 0 min, (B) 10 min, (C) 30 min, (D) 60 min (ppm).

ring-opening polymerization. The average degree of polymerization of PAEP was calculated to be 75 according to its ^{1}H NMR spectra (Figure S2). Then, a UV-induced thiol−ene "click" method[7] was utilized to synthesize cysteamine modified mPEG-*b*-PAEP (mPEG-*b*-PAEP-Cya, PPC). Indicated by the ^{1}H NMR spectrum in Figure S3, all of the allyl groups have been transformed to amino groups. Subsequently, partial amino groups were converted to sulfhydryl groups by a reaction with 2-iminothiolane,[8] which was confirmed by Ellman assay.[8] To conjugate doxorubicin (DOX) onto the polymer through an acid-labile hydrazone bond, the sulfhydryl-reactive derivative of DOX (Mal-DOX)[9] was incubated with the freshly prepared sulfhydryl-functionalized polymer, resulting in the DOX conjugated PPC-Hyd-DOX polymer. HPLC analysis showed that no unreacted Mal-DOX or free DOX remained in the polymer solution (Figure S4). As expected, the conjugate released the intact DOX after acid treatment (Figure S4). The remaining amino groups of the fresh prepared PPC-Hyd-DOX polymer were further reacted with 2, 3-dimethylmaleic anhydride (DMMA) to obtain the pH-dependent charge-conversional PPC-Hyd-DOX-DA conjugate. Resonances at δ 7.80, 7.52 in its ^{1}H NMR spectrum (Figure S5) were the characteristic resonances of DOX, while that at 1.85 ppm was assigned to the methyl groups of DMMA residue. According to its UV−vis absorbance at 490 nm, the DOX content in the conjugate was calculated to be 8.32 wt %, corresponding to a conjugation efficiency of 76%, in agreement with the ^{1}H NMR result in Figure S5. Due to the hydrophobic nature of the DOX residue,[10] the PPC-Hyd-DOX-DA conjugate self-assembled into NPs in aqueous solution. The dynamic light scattering measurement suggested that the NPs were ∼27 nm in diameter (Figure S6).

It has been demonstrated that the amide bond formed between an amino and DMMA is cleavable under slightly acidic conditions such as at pH 6.8.[11] To verify the acid-responsive cleavage of the amide bond in this study, we synthesized a model polymer PPC-DA by reacting PPC with DMMA and recorded its ^{1}H NMR spectra at different time points after incubation at pH 6.8 (Figure 1). About 75% of the amino groups were converted to carboxyl groups in PPC-DA according to the integral ratio of *Ha* to *Hb* (1:0.35, Figure 1A). However, with the incubation at pH 6.8, the integral ratio of *Ha* to *Hb* gradually decreased, reaching a

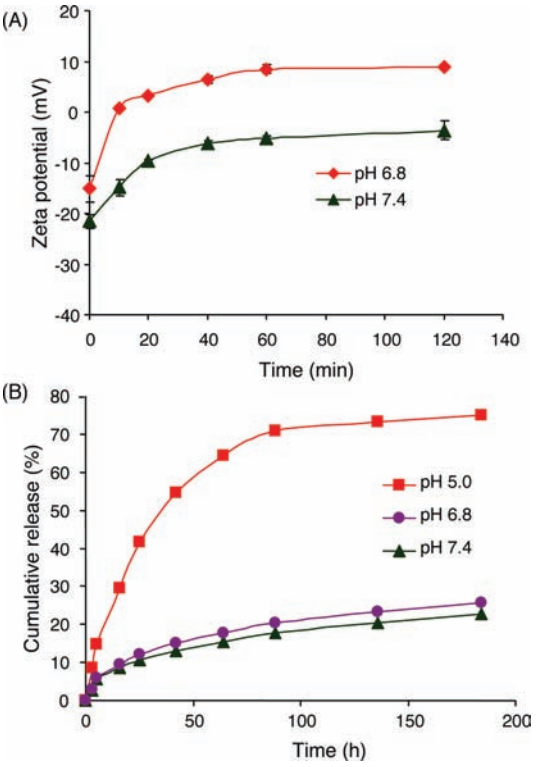

Figure 2. (A) Zeta potential change of PPC-Hyd-DOX-DA NPs after incubation at pH 7.4 or 6.8 for different time periods. (B) Time-dependent cumulative release of DOX from PPC-Hyd-DOX-DA NPs at different pH values.

value of 1:2.27 by 60 min, corresponding to 60% transformation of the carboxyl groups back to amino groups, which indicated the rapid acid-responsive cleavage of the amide bonds.

PPC-Hyd-DOX-DA NPs were thus expected to show charge-conversion behavior in the tumor extracellular environment. As shown in Figure 2A, initially, PPC-Hyd-DOX-DA NPs were negatively charged at pH 6.8 and 7.4. The zeta potential of NPs increased significantly when incubated at pH 6.8 and became positive within 10 min, corresponding to 30% of the carboxyl groups transforming to amino groups according to the integral ratio of *Ha* to *Hb*. Despite the zeta potential of NPs slowly increasing with incubation at pH 7.4, it remained negative within 2 h. Given that cell membranes are generally negatively charged, the charge-conversion behavior of PPC-Hyd-DOX-DA NPs at tumor extracellular pH will enhance their internalization by tumoral cells.[11b,12]

Since DOX was conjugated to the polymer by an acid-labile hydrazone bond, it should show endo/lysosomal pH-sensitive DOX release. To demonstrate this, the NPs were incubated at different pH values and the release of DOX was monitored. As shown in Figure 2B, 22.60 ± 0.33% of DOX were released by 184 h when the NPs were incubated at pH 7.4. Incubation of NPs at pH 6.8 resulted in a slightly increased cumulative release of DOX within the same period (25.56 ± 0.25%). However, when the pH was lowered to 5.0, more than 75% of DOX were released from the NPs by 184 h, indicating the sensitivity of PPC-Hyd-DOX-DA to endo-/lysosomal pH. From this point of view, PPC-Hyd-DOX-DA NPs can reduce premature drug release during circulation but specifically enhance intracellular drug release, which will be definitely beneficial to effective cancer treatment.

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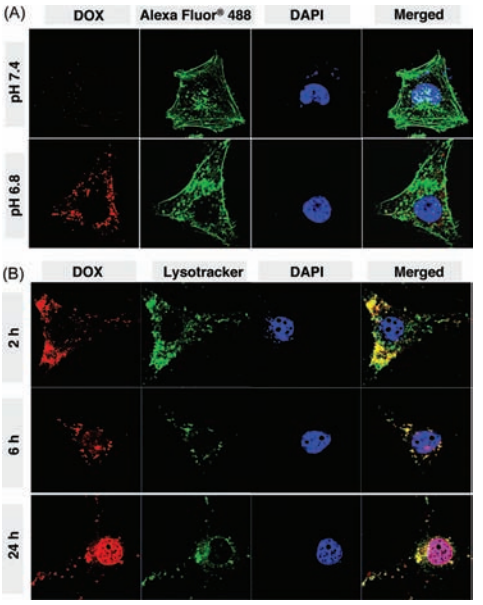

Figure 3. (A) Cellular uptake of PPC-Hyd-DOX-DA NPs (red) at pH 6.8 or 7.4 after incubation with MDA-MB-231 cells for 1 h. DAPI (4′, 6-diamidino-2-phenylindole, blue) and Alexa Fluor488 phalloidin (green) were used to stain cell nuclei and F-actin, respectively. (B) Subcellular distribution of PPC-Hyd-DOX-DA NPs (red) at pH 6.8. DAPI (blue) and Lysotracker Green (green) were used to stain the cell nuclei and acidic organelles. Cells were imaged using a 60× water-immersion objective.

To further demonstrate whether PPC-Hyd-DOX-DA NPs can be more efficiently internalized by cancer cells at the pH_e, we compared the cellular uptake behaviors of PPC-Hyd-DOX-DA NPs at pH 7.4 and 6.8. We incubated the NPs with MDA-MB-231 breast cancer cells at each pH for 1 h, and their cellular distribution was evaluated by confocal laser scanning microscopy (CLSM) analysis. It was demonstrated in Figure 3A that PPC-Hyd-DOX-DA NPs were remarkably internalized at pH 6.8 and distributed intensively in the cytoplasm, which was rarely observed in the cells incubated with the identical NPs at pH 7.4. To avoid the possible concern of DOX release at pH 6.8 due to cleavage of the hydrazone bond, we performed an experiment using a control conjugate PPC-Ami-DOX-DA (Figure S7), which possessed a similar structure with PPC-Hyd-DOX-DA except that the DOX was conjugated *via* a noncleavable amide bond. The CLSM image (Figure S8) showed a similar phenomenon to that in Figure 3A. The fluorescent activated cell sorting (FACS) analyses (Figure S9) further confirmed the enhanced internalization of PPC-Hyd-DOX-DA NPs at pH 6.8, which was evidenced by the remarkably enhanced cellular fluorescence. Taken together, it is concluded that the charge-conventional PPC-Hyd-DOX-DA NPs indeed exhibit significantly enhanced cellular internalization at pH_e.

Following cellular internalization, another key issue is whether the covalently conjugated DOX molecule can be efficiently released from the NPs, triggered by the intact intracellular endo-/lysosomal pH. To demonstrate this, we incubated MDA-MB-231 cells with PPC-Hyd-DOX-DA NPs at pH 6.8 for 2 h and then replaced the culture medium with a fresh one at pH 7.4. The cells were continuously cultured and subjected to CLSM observations. As shown in Figure 3B, after 2 h of incubation PPC-Hyd-DOX-DA NPs were dominantly localized in Lysotraker-labeled acidic organelles, with rare DOX observed in the nuclei.

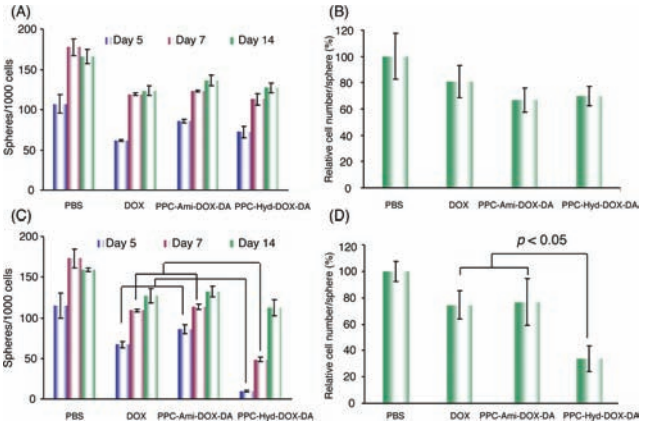

Figure 4. Time-dependent sphere formation of SK-3rd cells after incubation with various formulations (A and C) and the relative cell numbers/sphere measured at 14 days (B and D). Cells were first incubated with the formulations in culture media for 2 h at pH 7.4 (A and B) or 6.8 (C and D), and then the media were replaced with fresh media and further incubated at pH 7.4. $p < 0.005$. PBS control was set as 100%. The dose of DOX or its equivalent was 2 μg/mL.

Nevertheless, from 6 to 24 h, gradually increasing DOX resided in the cell nuclei, suggesting the efficient release of DOX from the NPs. To further demonstrate the DOX release is closely related to the endo-/lysosomal pH-responsibility of the hydrazone linkage, the control conjugate PPC-Ami-DOX-DA was used again. Following incubation of PPC-Ami-DOX-DA NPs with MDA-MB-231 cells under identical conditions to PPC-Hyd-DOX-DA, DOX was seldom observed in the nuclei even after 24 h of incubation (Figure S10), indicating that DOX could not be sufficiently released from the NPs in the cells and in turn could not enter the cell nuclei. The different subcellular distributions of PPC-Hyd-DOX-DA and PPC-Ami-DOX-DA NPs indicated the endo-/lysosomal pH-triggered DOX release from PPC-Hyd-DOX-DA NPs. Based on these combined results, it can be summarized that PPC-Hyd-DOX-DA NPs possess the tailor-made dual pH-sensitive feature.

The superiority of the dual pH-sensitive NPs in cancer therapy was evaluated in SK-3rd, a cancer stem cell line.[13] Recent advances in tumor biology have proposed that the minor cancer stem cells in solid tumors are responsible for tumor progression and metastasis,[14] but they are highly resistant to chemotherapy.[15] Even a small portion of the cancer stem cells survive chemotherapy and support tumor recurrence, leading to therapy failure. Therefore, effectively killing the cancer stem cells has become a new strategy for efficient cancer therapy.[16] SK-3rd cells maintain the "stemness" by suspension culture of its sphere, which possesses the characteristic $CD44^+CD24^-lin^-$ marker of cancer stem cells and the self-renewal capacity.[12] The number of spheres reflects the quantity of cells capable of *in vitro* self-renewal, and the number of cells/sphere measures the self-renewal capacity of each sphere-generating cell. We incubated SK-3rd cells in the culture medium with PPC-Hyd-DOX-DA NPs at pH 7.4 or 6.8, with free DOX and PPC-Ami-DOX-DA treatments as the controls. After 2 h of incubation, the drug or NPs were removed and the cells were further incubated to allow the formation of spheres. The number of spheres was counted at day 5, 7, and 14, and the results were depicted in Figure 4. At pH 7.4, it was found that treatments with three drug-containing formulations did not show a significant difference in the sphere numbers (Figure 4A) or cell

numbers/sphere (Figure 4B). It indicates the inefficient delivery of DOX into the SK-3rd spheres by PPC-Hyd-DOX-DA, due to its negatively charged feature at pH 7.4. In contrast, at pH 6.8, the number of spheres was significantly fewer at days 5 and 7 when the cells received PPC-Hyd-DOX-DA treatment, which was compared with the treatments of free DOX and PPC-Ami-DOX-DA (Figure 4C, $p < 0.005$). Furthermore, although the sphere numbers were significantly elevated at day 14 following the treatment of PPC-Hyd-DOX-DA, the cell numbers in the spheres was significant lower when compared with the treatments of free DOX and PPC-Ami-DOX-DA ($p < 0.05$, Figure 4D). PPC-Hyd-DOX-DA, with the charge-conversional property at pH_e, enhanced the cellular internalization, subsequently released DOX in the cells in response to the endo/lysosomal pH, and thus inhibited the progression of cancer stem cells.

In conclusion, this work has demonstrated an efficient anticancer drug delivery system using tailor-made dual pH-sensitive polymer−drug conjugate NPs. The NPs can respond to both extracellular and intracellular pH environments to simultaneously enhance cellular uptake and promote acid-triggered intracellular drug release. With dual pH sensitivities, the NPs have showed enhanced inhibition to the progression of drug-resistant SK-3rd cancer stem cells. Until today, a number of endo-/lysosomal pH-triggered drug release NPs have been developed as delivery systems. Recently, a novel endo-/lysosomal pH-responsive catechol polymer−boronic acid conjugate has been designed for enhanced intracellular drug delivery.[17] Since such endo-/lysosomal pH-responsiveness can be realized by a variety of chemical structures, our proof-of-concept design by combining the tumor extracellular and endo-/lysosomal pH responsivenesses provides a novel and versatile approach for efficient cancer chemotherapy.

■ ASSOCIATED CONTENT

Supporting Information. Synthesis, characterization, and other experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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