Dual Acid-Responsive Micelle-Forming Anticancer Polymers as New Anticancer Therapeutics

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Cinnamaldehyde, a major active compound of cinnamon, is known to induce apoptotic cell death in numerous human cancer cells. Here, dual acid-responsive polymeric micelle-forming cinnamaldehyde prodrugs, poly[(3-phenyl-prop-2-ene-1,1-diyl)bis(oxy)bis(ethane-2,1-diyl)diacrylate]-co-4,4'(trimethylene dipiperidine)-co-poly(ethylene glycol), termed PCAE copolymers, are reported. PCAE is designed to incorporate cinnamaldehyde via acid-cleavable acetal linkages in its pH-sensitive hydrophobic backbone and self assemble to form stable micelles which can encapsulate camptothecin (CPT). PCAE self assembles to form micelles which release CPT and cinnamaldehyde in pH-dependent manners. PCAE micelles induce apoptotic cell death through the generation of intracellular reactive oxygen species (ROS) and exert synergistic anticancer effects with a payload of CPT in vitro and in vivo model of SW620 human colon tumor-bearing mice. It is anticipated that dual acid-sensitive micelle-forming PCAE with intrinsic anticancer activities has enormous potential as novel anticancer therapeutics.

1. Introduction

Cancer is a major cause of mortality world-wide and is responsible for approximately 13% of all deaths, according to the World Health Organization.[1,2] Apoptosis is the process of programmed cell death occurring in multicellular organisms and is controlled by a diverse range of cell signals. Therefore, insufficient levels of apoptosis can promote the accumulation of abnormal cells, which can contribute to pathogenesis of cancer and tumor growth. One of promising approaches for effective cancer therapy is systemic chemotherapy using anticancer drugs, which are able to trigger apoptosis by activating key elements of the apoptosis program. Several lines of evidence suggest that many of well-known triggers of apoptosis are stimulators of intracellular generation of ROS such as hydrogen peroxide, superoxide anion and hydroxyl radicals, but the central mechanisms are not known.[3–6] Apoptosis is known to be inhibited by antioxidants such as catalase, *N*-acetyl-cysteine (NAC) and glutathione, further supporting that ROS play an essential role in apoptosis.[7,8] Therefore, there have been great efforts for the development of anticancer drugs that are able to induce apoptosis selectively in malignant cancer cells. However, selective induction of apoptosis in malignant cancer cells has remained challenging.

Cinnamaldehye is a major component in cinnamon which is an important dietary factor and food additive. Cinnamaldehyde contains α,β-unsaturated carbonyl moiety, known as active Michael acceptor pharmacophore.[9,10] It is Food and Drug Administration (FDA)-approved for use in foods and is given the Generally Recognized as Safe (GRAS) status in United States. Interestingly, a number of studies have shown that cinnamaldehyde and its analogues inhibit growth of various human cancer cells and induce apoptotic cell death through ROS generation, ROS-mediated mitochondrial permeability transition and caspase activation.[11–13] Despite its potent anticancer activities, the use of cinnamaldehyde in clinical applications is limited by its poor stability and lack of specificity toward diseased tissues, which are the common drawbacks of small molecule drugs.[14,15] In particular, its rapid clearance with a half-life of ~4 min necessitates frequent dosing to achieve therapeutic relevance.[15]

Nanoscale drug carriers and polymer-drug conjugates have been the common approaches to maximize therapeutic efficacy of drugs and minimize their undesirable side effects by decreasing the dose. A new rational strategy for controlled drug delivery involves polymeric prodrugs, in which therapeutic drugs are covalently incorporated into the backbone of biodegradable polymers.[16–18] For example, salicylic acid-derived poly(anhydride-ester), termed PolyAspirin, is a polymeric prodrug of aspirin, which covalently incorporates salicylic acid in its backbone, not attached to the side groups and release salicylic acid during its degradation due to the hydrolytically labile anhydride and ester bonds within the backbone.[16] These polymeric prodrugs allowed for a high percentage (62%) of deliverable drugs that are available as the polymer degrades.

The primary goal of this work is the development of polymeric prodrugs of cinnamaldehyde to explore the therapeutic potential of anticancer cinnamaldehyde by taking advantages of polymeric prodrug strategy. We developed micelle-forming

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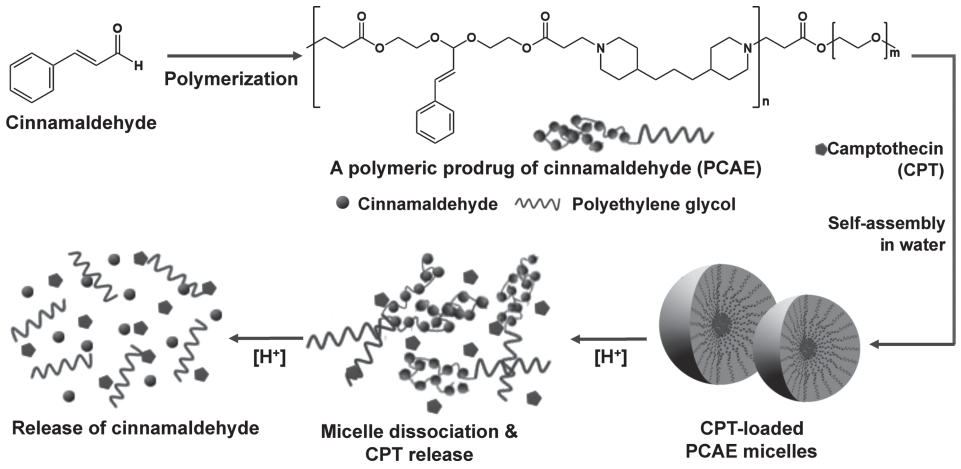


Scheme 1. A diagram of dual pH-responsive micelle-forming polymeric prodrug of cinnamaldehyde as new anticancer therapeutics.

PCAE as a polymeric prodrug of cinnamaldehyde, which incorporates cinnamaldehyde in its backbone and has dual acid-sensitive mechanism. Here, we report the physiochemical properties and therapeutic potential of PCAE micelles as anticancer drugs and drug delivery systems.

2. Results and Discussion

We molecularly engineered dual acid-sensitive polymeric prodrug of cinnamaldehyde as novel anticancer therapeutics, which incorporates cinnamaldehyde covalently in the backbone of biodegradable poly(*β*-amino ester) (PAE). PAE was exploited as a platform of polymeric prodrugs because of its excellent tunability, biocompatibility and ease of preparation.[19] PAE is also known to rapidly undergo acid-induced hydrophobic-hydrophilic transition due to the presence of tertiary amine groups in its backbone.[20] We developed PCAE as micelle-forming polymeric prodrugs of cinnamaldehyde, poly[(3-phenylprop-2-ene-1,1-diyl)bis(oxy)bis(ethane-2,1-diyl) diacrylate]-*co*-4,4'(trimethylene dipiperidine)-*co*-poly(ethylene glycol). PCAE was designed to possess a hydrophobic PAE backbone incorporating cinnamaldehyde *via* an acid-labile acetal linkage and hydrophilic PEG segment. Key design features of PCAE are the rapid hydrophobic-hydrophilic transition and cleavage of acetal linkages upon exposure to the mildly acidic environment. Therefore, it was hypothesized that PCAE micelles have dual pH-sensitive mechanism in response to a single triggering event of a decrease in pH in tumoral environments because of the presence of tertiary amine groups and acid-labile acetal linkages in the backbone.

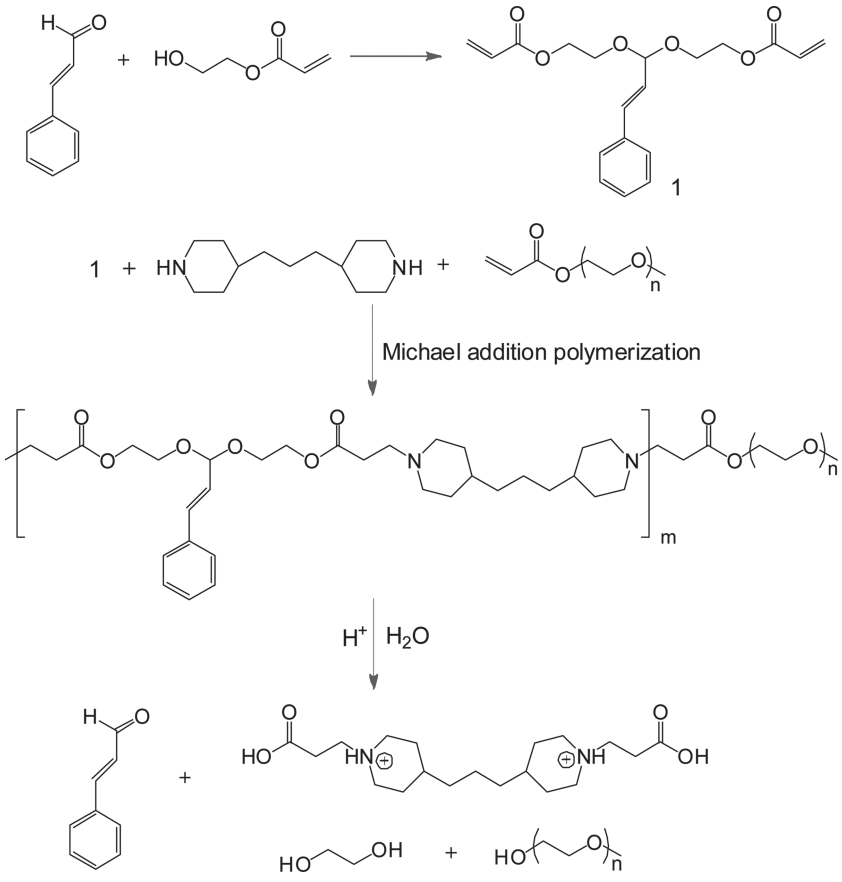
As shown in **Scheme 1**, amphiphilic PCAE copolymers are self-assembled in an aqueous solution to form stable micelles, which can encapsulate hydrophobic anticancer drug, camptothecin (CPT). Stable CPT-loaded PCAE micelles would rapidly dissociate and liberate anticancer drug payloads due to sharp hydrophobic-hydrophilic transition of the tertiary amine backbones in acidic environments. Then, PCAE releases anticancer cinnamaldehyde during the acid-triggered cleavage of acetal linkages, leading to the synergistic therapeutic effects with a payload of anticancer drug, CPT.

We first synthesized cinnamaldehyde containing diacrylate 1, (3-phenylprop-2-ene-1, 1-diyl) bis(oxy)bis(ethnane-2,1-diyl) diacrylate and methoxy polyethylene glycol (PEG) monoacrylate in order to take advantages of poly(*β*-amino ester) chemistry such as mild reaction conditions and vast libraries of diacrylates and amines.[21] PCAE was then synthesized from a Michael-type addition polymerization of **1**, methoxy PEG monoacrylate and trimethylene dipiperidine in a 0.9:0.1:1.0 mixture, as shown in **Scheme 2**. We also reasoned that PCAE hydrolytically degrades to release cinnamaldehyde and non-toxic compounds and its hydrolytic degradation is accelerated under acidic conditions.[22,23]

The chemical structure of PCAE block copolymers was confirmed by ^{1}H NMR. As shown in Figure S2, acetal protons are at ~5.3 ppm and ethylene protons between amine and ester groups are observed 2.5~3.8 ppm, respectively, demonstrating the successful polymerization of PCAE containing acetal linkages. The weight average molecular weight was determined to be ~10 000 *Da* with a polydispersity of ~1.3 using gel permeation chromatography. The hydrolytic degradation of PCAE and subsequent cinnamaldehyde release were also confirmed by ^{1}H NMR. After hydrolysis of PCAE in CD_3COOD/D_2O for 2 days, acetal protons disappeared, but aldehyde protons were observed at ~9.3 ppm, demonstrating that cinnamaldehyde was released from the hydrolytic degradation of PCAE (Figure S2B). It was also determined that 1 mg of PCAE copolymer contains ~200 μg of cinnamaldehyde.

Amphiphilic PCAE copolymers are able to self-assemble in an aqueous solution to form stable micelles that have PCAE segments in the hydrophobic core and a PEG (MW 2000 *Da*) corona. The micelle formation was evidenced by the ^{1}H NMR spectrum (Figure S3). All protons on the hydrophobic PCAE segment almost disappeared in the ^{1}H NMR spectrum monitored in D_2O, suggesting that the hydrophobic segments form a solid core in aqueous media, causing broadening effects due to the restricted mobility in NMR spectroscopy.[24]

PCAE copolymers were self-assembled to form monodispersed micelles with a mean hydrodynamic diameter of ~90 nm (PDI ~ 5.0×10^{-2}), as evidenced by dynamic light scattering and transmission electron microscopy (**Figure 1**). The micelles are



Scheme 2. A synthetic route and degradation of dual pH-responsive PCAE block copolymer.

expected to avoid rapid renal clearance and unwanted reticuloendothelial system, resulting in accumulation in tumor tissues by enhanced permeability and retention (EPR) effects.[25] The zeta potential of PCAE micelles was determined to be ~1.0 m*V* at pH 7.4.

The critical micelle concentration (*cmc*) of PCAE copolymers was determined using pyrene, which has been a widely used hydrophobic fluorescence probe for micelle formation.[26] Pyrene was added to the various concentrations of PCAE copolymers and the ratio of fluorescence intensity at 384 and 373 nm was measured as an index of micelle hydrophobicity ($I_{384}I_{373}$). The plot of I_{384}/I_{373} versus the logarithm of the PCAE copolymer concentrations demonstrates that PCAE copolymers form thermodynamically stable micelles encapsulating the fluorescent probe in their hydrophobic core at a concentration higher than ~5 μg/mL (**Figure 2**A). In order to investigate the pH-responsiveness of PCAE micelles, their demicellization behavior was studied over a wide range of pH values, as shown in Figure 2B. As the pH decreased, the index of micelle hydrophobicity gradually decreased and no further reduction was observed at pH values lower than 6.6. These observations suggest that the tertiary amine groups (pK_b = 6.5) in the hydrophobic block are protonated in acidic tumoral environments (<pH ~ 6.8) and become hydrophilic, leading to rapid micelle dissociation.[20,26]

The stability of PCAE micelles was also studied by measuring the change of their hydrodynamic size as a function of time, in the presence or absence of FBS (fetal bovine serum). The PCAE micelles remained stable in saline solution over a period of 18 h without a significant change in size and the size was not significantly influenced by the presence of FBS (Figure 2C). However, changing the pH to 6.4 by the addition of HCl solution caused a rapid drop of their size, indicating that the micelles were rapidly dissociated due to sharp hydrophobic-hydrophilic transition of tertiary amine groups in the PCAE backbone.

We also studied the effects of pH on the cinnamaldehyde release from PCAE micelles (Figure 2D). At physiological pH, cinnamaldehyde was slowly released from PCAE micelles, with ~25% released at 72 h. In contrast, at pH 5.4, PCAE micelles showed a significantly faster release kinetics, with ~100% release at ~40 h because of the acid-triggered cleavage of acetal linkages.[22] In acidic environments, PCAE micelles dissociated rapidly and released cinnamaldehyde in a sustained and continuous manner for ~40 h. Next, drug release profiles of PACE micelles were also studied using CPT as a model drug at different pH. As shown in Figure 2E, a magority of CPT was released within ~10 h at pH 5.4. However, a limited amount (~20%) of CPT was released even after 48 h of incubation at neutral pH, in a good agreement with previously reported PAE micelles.[27] These observation support our hypothesis that PCAE micelles have dual pH-responsiveness and have potential as drug delivery systems with pH-triggered drug release profiles.

Cinnamaldehyde is known to induce the generation of intracellular ROS that mediates apoptosis of cancer cells.[9,13] We therefore

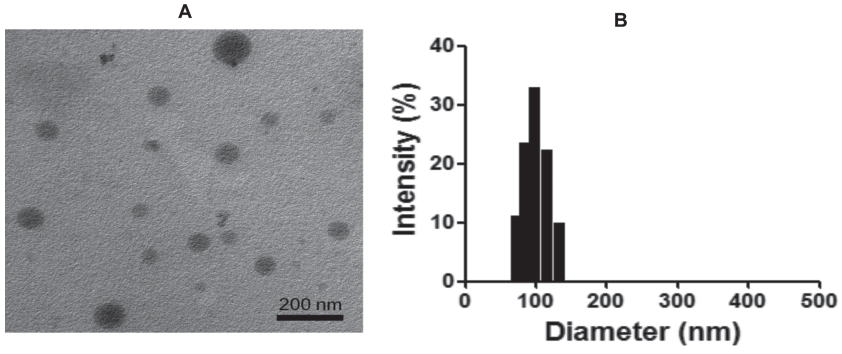


Figure 1. Characterization of PCAE micelles. A representative TEM image (A) and dynamic light scattering (B) of PCAE micelles in pH 7.4 phosphate buffer.

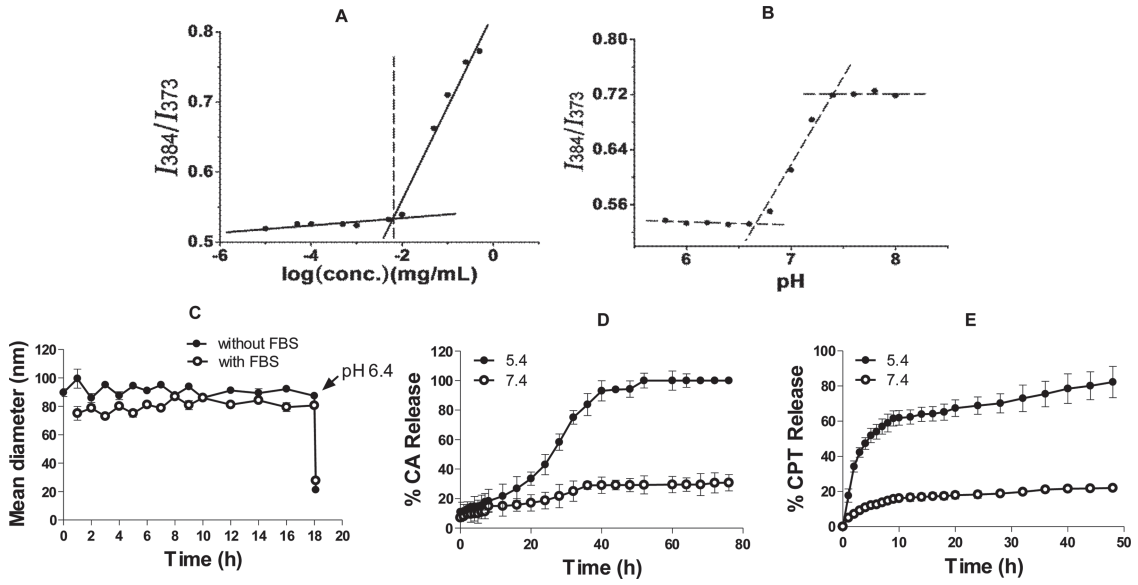


Figure 2. pH-responsiveness of PCAE micelles. The intensity ratio (I_{384}/I_{373}) from pyrene emission spectra (A) as a function of PCAE concentrations in phosphate buffer (pH 7.4) and (B) as a function of pH. (C) Stability of PCAE micelles in the absence or presence of FBS, (D) Release kinetics of cinnamaldehyde (CA) from PCAE micelles at pH 7.4 and 5.4. (E) Release kinetics of CPT from PCAE micelles at pH 7.4 and 5.4. Data presented are means ± s.d (n = 4).

assessed the ability of PCAE micelles to induce the generation of ROS by confocal laser scanning microscopy (CLSM) using DCFH-DA (2',7'-dichlorfluorescein-diacetate). DCFH-DA was used as a fluorescent probe because it is non-fluorescent, but becomes fluorescent DCF (2',7'-dichlorfluorescein) upon the activation by ROS including H_2O_2.[28] **Figure 3** shows the representative CLSM images and relative quantification of DCF fluorescence intensity. SW 620 cells treated with 100 μM of cinnamaldehyde for 24 h showed green fluorescence, indicating that cinnamaldehyde induces the generation of intracellular ROS, which converts DCFH-DA to fluorescent DCF. Anticancer drug CPT also induced ROS generation and the combination of CPT and cinnamaldehyde showed enhanced ROS generation. Treatment with PCAE micelles also induced the ROS generation in SW 620 cells, in a dose dependent manner. For comparison purposes, we also developed amphiphilic poly(β-amino ester)-*co*-poly(ethylene glycol) (PAE) as previously reported.[26] PAE has the chemical structure similar to PCAE, but has no cinnamaldehyde in its backbone. Cells treated with PAE micelles showed no green fluorescence at the same concentration, suggesting that the generation of intracellular ROS is attributed mainly to cinnamaldehyde released from PCAE micelles.

In order to further confirm the generation of ROS, cells were pre-treated with an antioxidant, NAC. Pre-treatment with NAC completely inhibited intracellular ROS generation induced by both cinnamaldehyde and PCAE micelles. The results demonstrate that PCAE micelles release cinnamaldehyde which triggers intracellular ROS generation.

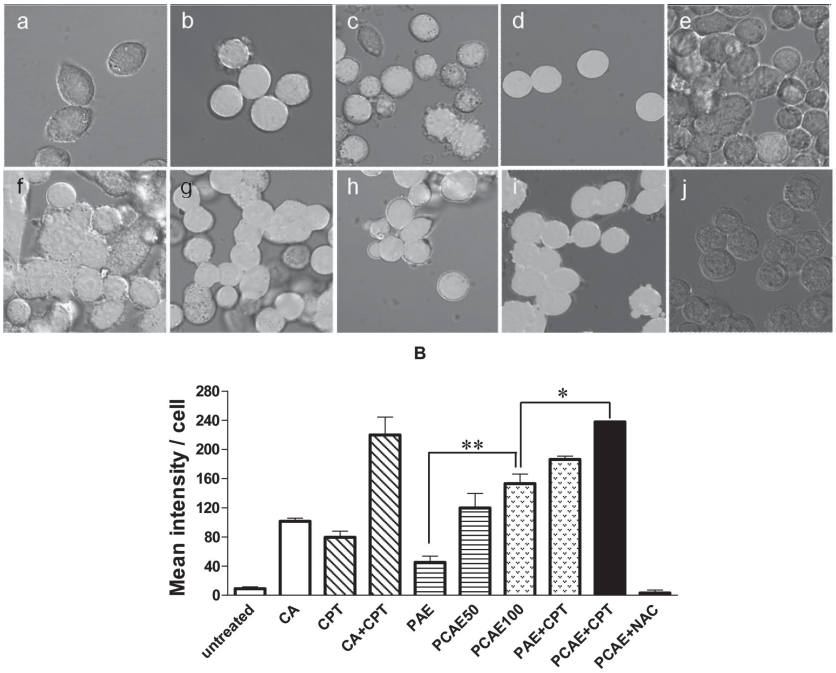


Figure 3. Generation of ROS in SW620 cells by PCAE micelles. (A) Representative CLSM images. a) untreated cells, b) 100 μM of cinnamaldehyde, c) 10 μg of CPT micelles, d) 10 μg of CPT with 100 μM of cinnamaldehyde, e) 100 μg of PAE micelles, f) PCAE 50 μg of PCAE micelles, g) 100 μg of PCAE micelles, h) 100 μg of CPT-loaded PAE micelles, i) 100 μg of CPT-loaded PCAE micelles, j) 100 μg of PCAE micelles in the presence of antioxidant NAC. A given amount of CPT and PCAE micelles was added to 1 mL of culture medium. (B) Relative quantification of intracellular ROS generation. CA stands for cinnamaldehyde (100 μM). $^*P < 0.05$, $^{**}P < 0.01$ (n = 4 ± s.d).

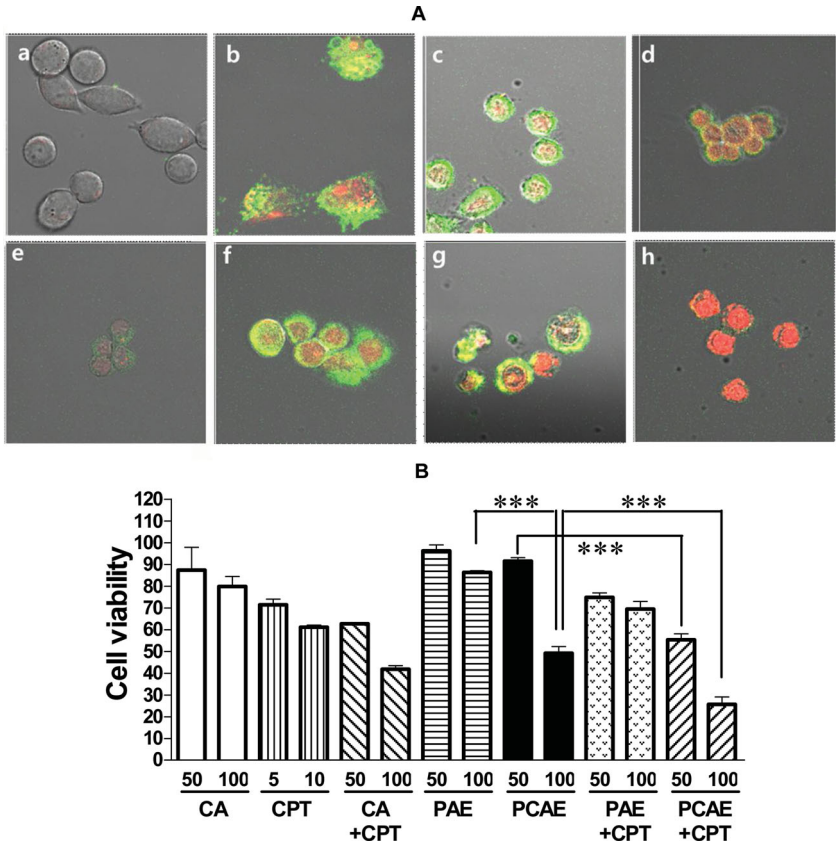


Figure 4. Induction of apoptotic cell death by PCAE micelles. (A) Representative CLSM images of SW620 cells stained with annexin V-FITC and PI. a) Untreated cells, b) 100 μM of cinnamaldehyde, c) 10 μg of CPT, d) 10 μg of CPT with 100 μM of cinnamaldehyde, e) 100 μg of PAE micelles, f) 100 μg of CPT-loaded PAE micelles, g) 100 μg of PCAE micelles, h) 100 μg of CPT-loaded PCAE micelles. A given amount of CPT and PCAE micelles was added to 1 mL of culture medium. (B) Cell viability determined by MTT assay. CPT was loaded at 10wt% of PAE or PCAE micelles. ***$P < 0.001$ ($n = 3 \pm$ s.d.).

We also investigated the ability of PCAE micelles to deliver anticancer drugs using CPT as a model drug, which is known to trigger ROS generation to induce apoptotic cell death.[29] CPT was loaded at a concentration of 10 wt% of PCAE with ~90% encapsulation efficiency and CPT-loaded PCAE micelles had a mean hydrodynamic size of ~95 nm (PDI ~5.9×10^{-2}). CPT-loaded PCAE micelles induced ROS generation significantly more than CPT-loaded PAE micelles and empty PCAE micelles, suggesting that PCAE micelles have potential as drug carriers and are able to exert synergistic effects with CPT on ROS generation.

In order to determine whether PCAE micelles induce apoptosis and exert synergistic effects with a payload of CPT on the induction of apoptotic cell death, flow cytometry was performed using fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) and propidium iodide (PI) as an apoptosis marker and a cell viability marker, respectively. As shown in Figure S4, treatment with PCAE micelles remarkably induced apoptosis, evidenced by the increasing population of cells in the upper right quadrant, which corresponds to late apoptotic cells.[30] Almost half of the cells were undergoing and had undergone apoptosis after 24 h of treatment with 100 μg/mL of PCAE micelles. However, the same dose of PAE micelles induced minimal apoptosis. CPT-loaded PCAE micelles induced significantly higher apoptotic effects than empty PCAE micelles and CPT-loaded PAE micelles, indicating that PCAE micelles have great potential as drug carriers and their apoptotic activity can be enhanced with a payload of CPT.

In order to further demonstrate the apoptotic cell death by PCAE micelles and synergistic effects with CPT, cells were observed using CLSM (**Figure 4**A and Figure S5) after annexin V-FITC and PI staining. Untreated cells were annexin V-FITC and PI negative, suggesting that cells were intact. However, cells treated with 100 μM of free cinnamaldehyde or CPT were annexin V-FITC and PI positive, indicating that cells are in late apoptosis, with the loss of membrane integrity.[31] Cells treated with PAE micelles showed negligible apoptosis, but CPT-loaded PAE micelles induced remarkable apoptosis, evidenced by strong green and red fluorescence.[32] In contrast, the same dose of PCAE micelles caused significant apoptosis and CPT-loaded PCAE micelles exhibited even higher degree of apoptosis, suggesting the synergistic apoptotic effects of PCAE micelles with a payload of CPT.

We performed the MTT assay to evaluate the cytotoxic effects of PCAE micelles on SW 620 cells. As shown in Figure 4B, PCAE micelles exhibited cytotoxic effects in a dose dependent manner, with ~40% of cell death within 48 h at a dose of 100 μg/mL, but PAE micelles at the same doses showed negligible cytotoxicity. CPT alone at the equivalent amounts loaded in the PAE or PCAE micelles showed a dose-dependent cytotoxicity. Combination of CPT with cinnamaldehyde showed a combined anticancer effects. It is important to note that CPT-loaded PCAE micelles exhibited significantly higher cytotoxic effects than empty PCAE micelles and CPT-loaded PAE micelles. CPT-loaded PCAE micelles also had significantly higher cytotoxic effects than equivalent CPT alone. These findings suggest that PCAE micelles have synergistic anticancer effects with a payload of CPT. Therefore, it can be reasoned that anticancer activity of PCAE micelles reduces the dose of anticancer drugs to exert therapeutic effects and minimize drug-induced side effects.

Finally, in order to extrapolate our encouraging in vitro findings, we evaluated potential of PCAE micelles as novel anticancer therapeutics using a xenograft tumor mouse model. Various formulations of CPT were intravenously injected into SW 620 human colon tumor-bearing mice through a tail every 3 days. **Figure 5** shows the growth curves of the tumor after the injection of saline, free cinnamaldehyde (2 mg/kg), free CPT (1 mg/kg), empty PCAE micelles (10 mg/kg), CPT-loaded PCAE micelles (10 mg/kg) with 1 mg/kg of CPT. Untreated

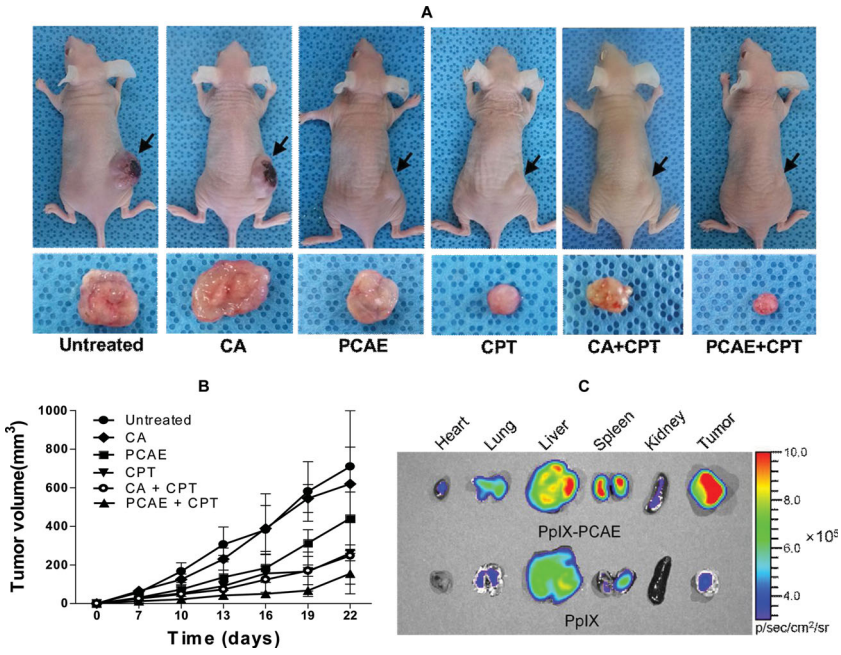


Figure 5. Anticancer effects of PCAE micelles in vivo. (A) Representative images of tumor bearing mice treated with various formulations, (B) Change in tumor volumes of tumor bearing mice. CA stands for cinnamaldehyde. (C) Representative images showing the biodistribution of protoporphyrin-loaded PCAE micelles in tumor bearing mice. Data presented are means ± S.D. *$p < 0.001$ relative to untreated group, †$p < 0.05$ relative to CPT group, ‡$p < 0.05$, relative to PCAE group, **$p < 0.05$ relative to untreated group, n = 4.

group given saline only showed gradual and continuous tumor growth. Cinnamaldehyde-treated mice showed a tumor growth rate similar to the untreated group, indicating no therapeutic effects on tumor growth due probably to its low blood half-life.[15]

Interestingly, reduction in tumor volume was observed with empty PCAE micelles which can release cinnamaldehyde, equivalent to 1 mg/kg. It can be explained that PCAE micelles with a hydrodynamic diameter of ~90 nm passively target the tumor site by EPR effects and then release cinnamaldehyde which induces apoptotic cell death. The passive targeting of PCAE micelles to tumor site was confirmed by the study of biodistribution. As shown in Figure 5C, a large amount of PCAE micelles was localized in tumors after 24 h of injection. CPT alone also showed anticancer effects, with 65% reduction in tumor volume at day 22 post-injection compared to untreated group. In contrast to in vitro studies, no synergistic anticancer effects were observed with the combination of CPT and free cinnamaldehyde. However, treatment with CPT-loaded PCAE micelles exhibited more reduction in tumor volume than free CPT and empty PCAE micelles.

The anticancer efficacy of CPT-loaded PCAE micelles was also evaluated by the TUNEL (terminal deoxynucleotidyl transferase) assay, which detects apoptosis at the tumor tissues (Figure S6). Apoptotic cells were rarely detectable in untreated groups and cinnamaldehyde-treated group, but were clearly detected in PCAE and CPT-treated groups. However, CPT-loaded PCAE micelle-treated group showed the most apoptotic cells in tumor tissues, reinforcing that PCAE micelles have great potential as drug carriers and exert synergistic anticancer effects with a payload of CPT.

3. Conclusions

We have developed, for the first time, polymeric prodrug micelles which have potent intrinsic anticancer activity and are also able to serve as drug carriers. PCAE developed through the rational design of monomers and controllable polymerization was designed to incorporate cinnamaldehyde in its pH-sensitive hydrophobic backbone via acid-cleavable acetal linkages. PCAE self-assembled to form stable micelles which encapsulate CPT and show pH-dependent demicellization and cinnamaldehyde release. PCAE micelles induced apoptotic cell death through the generation of intracellular ROS and their apoptotic activities were significantly enhanced with a payload of CPT in vitro and in vivo model of SW 620 colon tumor-bearing mice. We anticipate that dual acid-responsive PCAE micelles are able to serve as anticancer drugs as well as drug carriers and therefore have enormous potential as novel anticancer therapeutics.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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