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pH-triggered blooming of 'nano-flowers' for tumor intracellular drug delivery†

Zhefan Yuan, Zhenyang Que, Sixue Cheng, Renxi Zhuo and Feng Li*

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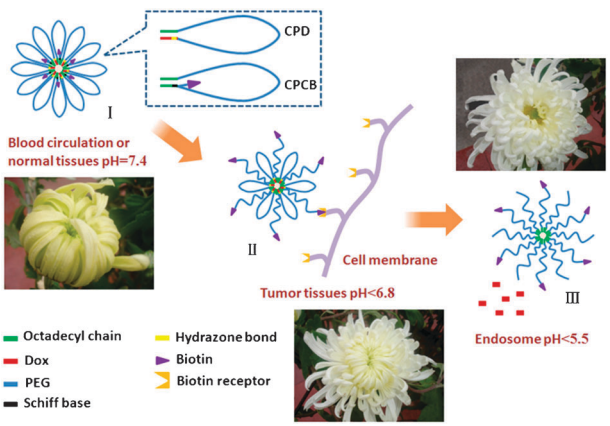
With the decrease in pH value, the 'nano-flower' exhibited a half-open state to expose the target ligands on the surface under tumor acidic conditions and fully bloomed to release Dox under endosomal acidic conditions.

In the past decades, drug delivery systems (DDSs) have been extensively investigated for cancer therapy.[1] To achieve the ability of site-specific delivery, target ligands were introduced to functionalize the DDSs for enhancing the drug endocytosis of cancerous cells.[2–4] To our knowledge, the conventional method for ligand conjugation is attaching target ligands onto the surface of DDSs to maximize contact opportunities with cell membranes. However, this strategy also enhances the specific or non-specific contact opportunities with normal tissues and cells.

In Nature, the flower, the reproductive portion of angiosperms, consists of a floral axis that bears the essential organs of reproduction (stamens and pistils) and usual accessory organs (sepals and petals). Within the flower, the pollens are usually protected by the round petals incipiently and exposed and released ultimately when the flower fully opens. Besides the common way of flowering, some flowers show the special flowering behavior with environmental stimuli responsive characteristics. For example, *Nymphaea alba* stretches its petals in daylight and folds them at night due to its sensitivity to sunshine,[5] while *Cestrum nocturnum* L. usually blooms at night, which has folk names like night cestrum.[6] Inspired by these phenomena, we desire to design a so-called 'smart' nanocarrier. On the one hand, the carrier possesses an ability to protect the target ligands from contacting non-targeted tissues. On the other hand, it can expose the target ligands in response to the tumor micro-environment and then release the drugs intracellularly to kill the cancerous cells. To achieve tumor-specific exposure of target ligands, it is important to choose appropriate local stimuli. Among the biological stimuli (pH,[7,8] redox environment,[9] and enzymes *etc.*),[10,11] pH sensitivity is the most widely exploited because the extracellular pH value (~6.8) in most tumors is lower than that in normal tissues (~7.4).[12] Utilizing this property, some groups obtained quite a few encouraging results. For example, Wang's group reported one kind of DDS with 2,3-dimethylmaleic anhydride modified amino groups. This DDS could convert the charge from negative to positive (first reported by Kataoka *et al.*)[13] at pH 6.8, resulting in intracellular drug delivery mediated by electrostatic interaction.[14] Recently, Bae's group prepared a super pH-sensitive multi-functional polymeric micelle based on poly(L-histidine)-containing block copolymers, which could expose target ligands on the micelle surface under tumor acidic conditions (7.0–6.5) and dissociate at a lower early endosomal pH (<6.5).[15] However, since the micellar core mainly formed by poly(L-histidine) blocks started partially hydrophobic-to-hydrophilic conversion under tumor acidic conditions, the premature leakage of drugs outside the cancerous cells might occur.

Herein we demonstrated a novel smart DDS that might protect the target ligands and drugs in the blood circulation, expose the target ligands in tumor tissues and then release drugs at endosomal pH (Scheme 1).

Interestingly, this DDS was not only like a flower in morphology but also showed a pH-triggered blooming process. So we called this DDS as a 'nano-flower'. The 'nano-flower' consisted of two polymer components: octadecyl-polyethylene glycol (biotin)-octadecyl ester (CPCB) and octadecyl-poly-ethylene glycol-doxorubicin (CPD) (Fig. 1a). The ABA-type



Scheme 1 Schematic illustration of the process of pH-triggered blooming of the nano-flower with different biological functions at various blooming stages: (I) unopened state, (II) half-open state, (III) fully-open state.

Polymers of Ministry of Education, College of Chemistry and Molecular Science, Wuhan University, Wuhan, 430072, P. R. China. E-mail: lfsj2004@hotmail.com

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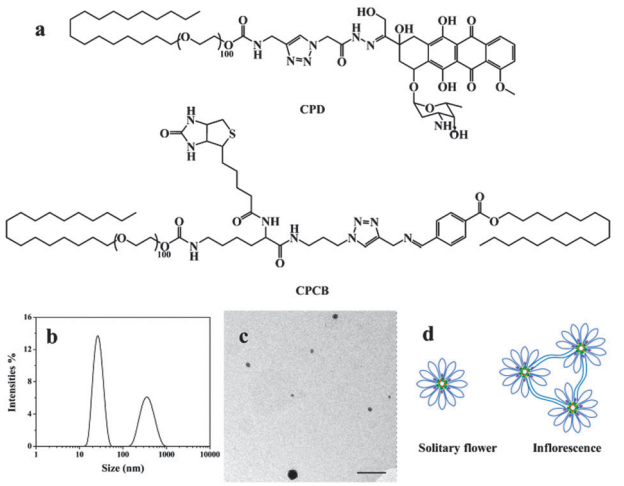

Fig. 1 (a) The formulas of CPCB and CPD. (b) Size distribution of CPCB/CPD mixed-micelles. (c) The TEM image of CPCB/CPD mixed-micelles. The scale bar is 500 nm. (d) The possible structure of CPCB/CPD mixed-micelles.

(A: hydrophobic, B: hydrophilic) structure of CPCB made the polyethylene glycol block bend in aqueous solution and hid the biotin in the surface of the hydrophobic core. CPCB bore a benzoic-imine bond that was stable in the physiological environment and hydrolyzed at pH 6.8.[16] The hydrolysis of benzoic-imine bonds could cut off the hydrophobic chain adjacent to biotin in CPCB and release the biotin to the micelle surface. In CPD, doxorubicin (Dox) was attached to the polymer covalently by a hydrazone bond. Here Dox was chosen not only as a model drug but also as the hydrophobic core of micelle. Moreover, the hydrazone bond between Dox and polymer was reported to show less than 5% of decomposition at pH 6.5–7.4 for 72 h.[17] By using this conjugation strategy for Dox, we expected to prevent the premature leakage of Dox from the inner core of DDS in normal tissue efficiently.

Cu-catalyzed Azide–Alkyne Cycloaddition (CuAAC) click chemistry was utilized in the synthesis of CPCB and CPD. Generally, an azide-modified biotin moiety was introduced into carbonyldiimidazole activated Brij 100 and alkyne was linked to the octadecyl chain by a benzoic-imine bond. Finally the CPCB was successfully synthesized by CuAAC click chemistry. Similarly, CPD was prepared by azido acetyl hydrazine modified doxorubicin and alkyne modified Brij 100 (Fig. 1a).

As indicated by the ^{1}H NMR spectrum of CPCB, 86.9% of Brij 100 was successfully modified with the benzoic-imine bond. According to the UV-visible absorbance of CPD at 480 nm, 91.2% of Brij 100 was successfully modified with Dox. The critical micelle concentrations of CPCB and CPD were approximately 15.0 mg L^{-1} and 67.0 mg L^{-1}, respectively, which were measured by a fluorescence probe method. The nano-flower was prepared by mixing equal masses of CPCB and CPD in DMSO and then dialyzed with PBS (pH = 7.4). The final concentration of CPCB and CPD was fixed at 0.1 mg mL^{-1} respectively. Because of the insolubility of the octadecyl chain and doxorubicin, the flexible PEG segment might bend in water and the biotin moieties were pulled to the outer layer of the micellar core subsequently. The DLS measurement showed two average particle sizes of 25 nm and 300 nm, respectively (Fig. 1b). The TEM image also proved the existence of two sorts of particles with different radiuses (Fig. 1c). In aqueous solution, the folded PEG chain tended to expand due to its hydrophilicity, while the hydrophobic Dox and alkyl chain were inclined to enter the hydrophobic micellar core. Therefore, some bridges between neighboring micelles were formed, which might result in the generation of large-sized particles (Fig. 1d).[18] The single micelle might be thought as a 'solitary flower' and the large bridge-linked particle was just like 'inflorescence'. The similar phenomena were observed in the single-component micellar solution of CPCB and CPD, respectively (Fig. S10 and S11, ESI†).

It is known that the complex of 4-hydroxyazobenzene-2-carboxylic acid (HABA) and avidin has a strong absorption at 500 nm. Since the affinity between HABA and avidin is relatively weaker ($K_d = 5.8 \times 10^{-6}$ M) than that between biotin and avidin ($K_d = 1 \times 10^{-15}$ M), HABA can easily be replaced by biotin from the HABA–avidin complex, resulting in an absorption decrease at 500 nm. The HABA/avidin assay was used here to verify the exposure of biotin from the 'nano-flower'.

As shown in Fig. 2a, after addition of CPCB micelles into PBS (pH 7.4), the absorption of the HABA–avidin complex at 500 nm obviously decreased. There was about 25.9 nmol per milligram sample of the biotin moiety available to avidin. However, if the HABA/avidin complex was treated with CPCB micellar solution at pH 6.8, the amount of available biotin moiety was up to 45.9 nmol per milligram of sample. In comparison with the result in the ^{1}H NMR spectrum (155.5 nmol biotin per milligram sample of CPCB), only 16.6% of biotin was available to avidin when the pH value of CPCB solution was 7.4. In contrast, when the CPCB micelle was incubated under acidic conditions (pH 6.8), almost twice the amount of biotin (about 29.5%) replaced the HABA from the HABA/avidin complex, implying that the 'nano-flower' began 'blooming' under weak acidic conditions. Although the shell formed by folded PEG chains could reduce the unfavorable contacts between ligands and proteins, the shielding effect of PEG was not dominant.

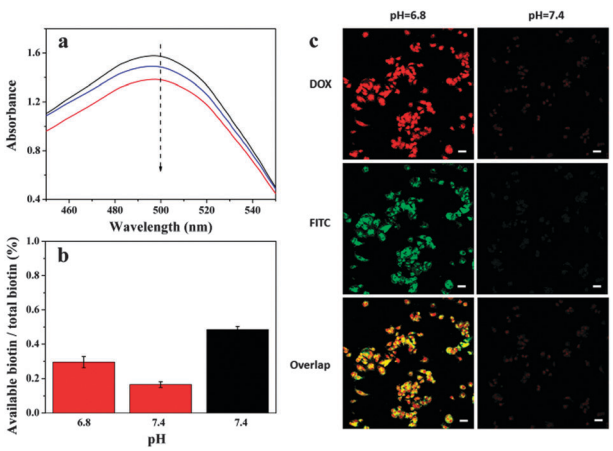

Fig. 2 (a) The UV-vis absorption spectra of the HABA/avidin solution before (black line) and after being treated with CPCB micellar solution at pH 6.8 (red line) or 7.4 (blue line). (b) Available biotin of CPCB micelle (red) and CPB micelle (black) for avidin. The results were normalized with the biotin amount calculated using ^{1}H NMR spectra. (c) Confocal images of MCF-7 cells treated with nano-flowers ($w_{CPCB}/w_{CPD}/w_{CPFITC}$ = 1/1/0.1) for 4 h. The scale bar is 40 μm.

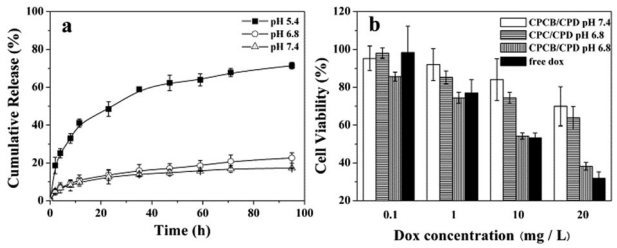

Fig. 3 (a) The cumulative release of Dox from CPCB/CPD mixed-micelles. (b) Cytotoxicity of the mixed-micelles in MCF-7 cells for 24 h incubation.

There were some other factors, such as the strength of binding affinity, the density of the PEG shell and the conformation of PEG, which might also influence shielding effects on the interaction of ligand–protein.[19,20] Additionally, we measured the CPB micelle (the reactant of CPCB before click chemistry), in which all the biotin moieties were exposed on the micelle surface, and found that 81.8 nmol per milligram of CPB was available to avidin. By calculation, it was equivalent to 48.5% of biotin available to avidin (Fig. 2b). It indicated that the aggregated conformation of micelle might sterically prohibit interaction of biotin with avidin. Although we did not estimate the optimum percentage of CPCB in mixed micelle for tumor targeted here, the strategy of single-component micelle (one biotin per macromolecule) seemed to be a waste of target ligands, which was the main reason for choosing a mixed micelle rather than a single component micelle loaded with drugs.

The cell uptake capacity of 'nano-flower' on MCF-7 cells was measured here to estimate acid-triggered 'blooming' again. To acquire desired fluorescence effects, 5 wt% of Brij modified with FITC was used to form the mixed-micelle ($w_{CPCB}/w_{CPD}/w_{CPFITC}$ = 1/1/0.1). We compared the cell uptake behaviors of the mixed-micelles at pH 6.8 and 7.4. In Fig. 2c, strong fluorescence was observed in the MCF-7 cells incubated at pH 6.8, while very slight fluorescence was found at pH 7.4. This result was consistent with that of the HABA/avidin assay. The biotin receptor-mediated endocytosis was significantly enhanced under tumor acidic conditions that triggered the 'nano-flower' blooming. A controlled trial was also carried out by treating the MCF-7 cells with other mixed micelles consisting of CPC/CPD/CPFITC at the same weight ratio as CPCB/CPD/CPFITC mixed micelles. In Fig. S12 (ESI†), there was no obvious fluorescence observed in cells either at pH 6.8 or 7.4. It further proved that the internalization process of the 'nano-flower' was mediated by the interaction between biotin and its receptor. Moreover, it was also noted that Dox was not significantly internalized by the MCF-7 cell without the aid of biotin even at pH 6.8, which indicated that the hydrolysis of the hydrazine bond needed lower pH value or more time in terms of dynamics. A long term cumulative drug release measurement was carried out and the results are shown in Fig. 3a. After 4 days of release at 37 °C, only 17.2% Dox was released from the CPCB/CPD mixed-micelles at pH 7.4 and the value increased to 22.7% at pH 6.8. When the pH was set to 5.4, which was similar to that under the acidic conditions of endosomes, 71.5% Dox was released finally. Since Dox was covalently linked onto the polymer chains, the drug release behavior could be controlled by the hydrolysis kinetics of the hydrazine bond. Based on the results, it might be inferred that the 'nano-flower' had a half-open state in the extracellular environment of tumor tissues, held the cargos through the cell membrane, and bloomed fully under endosomal acidic conditions to release the drugs finally. In Fig. 3b, the MTT assay was used to estimate the cytotoxicity of the mixed-micelles in MCF-7 cells. As the Dox concentration increased, the mixed micelles of CPCB/CPD incubated under acidic conditions showed significantly higher cytotoxicity than that under neutral conditions. It was also found that the cytotoxicity of CPC/CPD mixed micelles at pH 6.8 was slightly higher than that of the CPCB/CPD sample at pH 7.4. Although the hydrolysis of hydrazine bonds at pH 6.8 resulted in a slight release of Dox, the biotin receptor-mediated endocytosis was the main reason for the apoptosis of cancer cells.

In summary, the 'nano-flower' prepared by CPCB/CPD mixed-micelles here was proven to be a smart drug carrier. Due to the covalent conjugation of Dox on polymer chains and the shielding of target ligands by folded PEG chains, the 'nano-flower' might effectively prevent the premature leak of Dox and decrease the specific contact with receptors outside the tumor tissues. With the decrease in pH value, the 'nano-flower' exhibited a half-open state to expose the target ligands on the surface under tumor acidic conditions and fully bloomed to release Dox under endosomal acidic conditions. The pH-triggered blooming behavior made the 'nano-flower' accomplish a process of tumor-triggered cell uptake and intercellular drug release, which might be a good candidate for chemotherapy.

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