

pH/Glutathione (GSH) Co-triggered Degradable Polyprodrug as Drug Self-delivery System for Tumor-specific Doxorubicin Delivery: Effect of Aggregation States

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 Electronic Supplementary Information

Abstract Numerous efforts have been devoted to altering the dynamic covalent linkers between the drug structural units in polyprodrugs from the viewpoint of molecular structure; however, the effect of their aggregation states has not yet been explored. Here, the effect of aggregation states on the *in vitro* drug release and cytotoxicity was investigated using a pH/glutathione (GSH) co-triggered degradable doxorubicin (DOX)-based polyprodrug (PDOX) as a model, which was synthesized by the facile polymerization of a pH/GSH dual-triggered dimeric prodrug (DDOX_{ss}) and 2,2-dimethoxypropane (DMP) by forming acid-labile ketal bond. Owing to the pH/GSH dual-triggered disulfide/ α -amide and acid-labile ketal linkers between the DOX structural units, the resultant PDOX exhibited excellent pH/GSH co-triggered DOX release. With a similar diameter, the PDOX-NPs1 nanomedicines *via* fast precipitation showed faster DOX release than PDOX-NPs2 *via* slow self-assembly, regardless of their polymerization degree (DP). The effect of aggregation states is expected to be a secondary strategy for a more desired tumor intracellular microenvironment-responsive drug delivery for tumor chemotherapy, in addition to the molecular structures of polyprodrugs as drug self-delivery systems (DSDSs).

Keywords Tumor chemotherapy; Drug self-delivery system; pH/GSH co-triggered polyprodrug; Aggregation states; Doxorubicin

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INTRODUCTION

Polymer prodrugs have attracted more attention as drug self-delivery systems (DSDSs) in tumor chemotherapy, owing to their better controlled drug release performance than non-covalent drug-loaded drug delivery systems (DDSs).^[1,2] However, the drugs are conjugated onto the side or end groups of the polymers as carriers *via* stimuli-responsive dynamic covalent bonds, resulting in a lower drug content. In addition to the immunogenicity of the polymer carriers,^[3] the premature drug leakage is still a challenge because the conjugated drugs leak once the single conjugation is cleaved.

In contrast to conventional polymer prodrugs, the drugs were incorporated into the main chain of the polyprodrugs as structural units with stimuli-responsive dynamic covalent bonds on both sides.^[4,5] As DSDSs, the drug content can be distinctly increased without the immunogenicity of the polymer carriers. They can degrade and release drugs only when both dynamic covalent bonds are cleaved, better on-demand drug release can be achieved *via* such duplicated conjugation

with minimized drug leakage. Most importantly, various stimuli-responsive dynamic covalent bonds can be easily integrated for dual-stimulus co-triggered drug release for tumor-specific chemotherapy.^[6–8]

Although numerous efforts have been devoted to adjusting the tumor intracellular microenvironment-responsive drug delivery of polyprodrugs for better tumor chemotherapy by altering the dynamic covalent linkers between the drug structural units from the viewpoint of molecular structure,^[4] the effect of their aggregation states has not been explored thus far. In the present study, the effect of aggregation states on *in vitro* drug release and cytotoxicity was investigated using a pH/glutathione (GSH) co-triggered degradable doxorubicin (DOX)-based polyprodrug (PDOX) as a model, by controlling their aggregation states *via* fast precipitation and slow self-assembly approaches, respectively. Aggregation states have been revealed as a secondary strategy for a more desired tumor intracellular microenvironment-responsive drug delivery.

EXPERIMENTAL

The pH/GSH dual-triggered disulfide/ α -amide-bridged dimeric prodrug (DDOX_{ss}) was synthesized by coupling DOX with 2,2'-

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dithiodiacetic acid (DSDD) according to a previous report.^[9] (Yield: 83.10%; (¹H-NMR (400 MHz, DMSO-d₆, δ): 12.75 (s, 2H), 3.65 (s, 4H)).

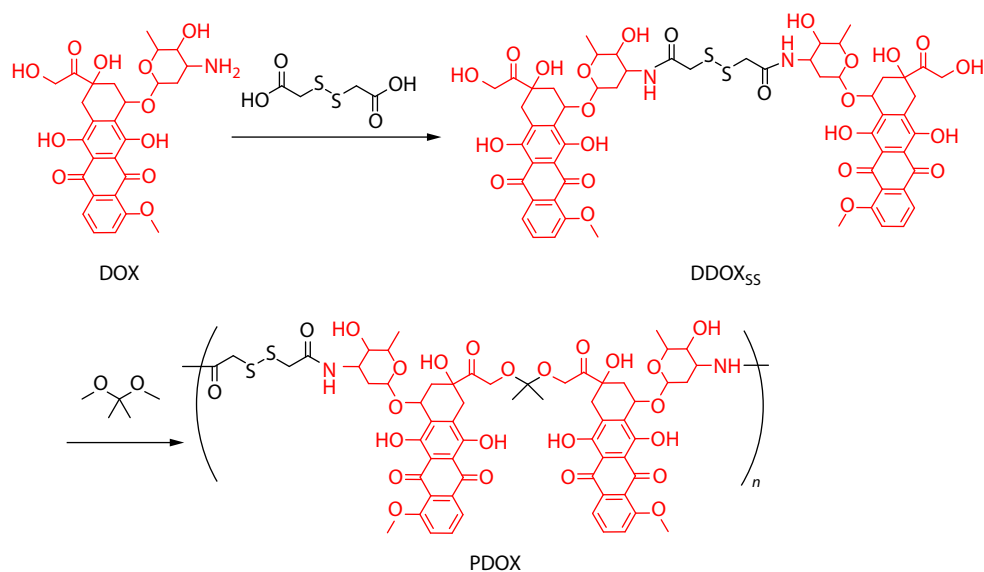
PDOX was synthesized by facile polymerization of DDOX_{ss} and 2,2-dimethoxypropane (DMP) with a yield of 58.61% (Scheme 1). Typically, 2.5 μL (0.02 mmol, 1.0 equiv.) of 2,2-dimethoxypropane (DMP) and 2.0 μg of *p*-toluenesulfonic acid (TsOH) were added in a DMF solution containing 25.0 mg (0.020 mmol, 1.0 equiv.) of DDOX_{ss}. After stirring for 48 h at 40 °C, the resultant PDOX was collected by precipitation with ethanol and centrifugation (1.0×10⁴ r/min for 5 min), purified by three DMF dissolution/ethanol precipitations, and dried under vacuum at room temperature.

The nanomedicines were fabricated by dripping 1.0 mL of the DMF solution of PDOX with different concentrations into

10 mL of water under stirring at a rate of 1 drop per 10 s (PDOX-NPs 1), or by dripping 10 mL of water into 1.0 mL of the DMF solution of PDOX at the same rate (PDOX-NPs 2). After dialysis against water (MWCO 1000) for 24 h, the nanomedicines were lyophilized.

RESULTS AND DISCUSSION

The pH/GSH co-triggered degradable polyprodrug PDOX was designed with alternate pH/GSH dual-triggered disulfide/ α -amide linker and acid-labile ketal linker between the DOX structural units for tumor-specific pH/GSH co-triggered DOX release by the polymerization of DDOX_{ss} and DMP. Its successful synthesis was confirmed by ¹H nuclear magnetic resonance spectroscopy (¹H-NMR) and gel permeation chromatography (GPC)



Scheme 1 Synthetic route to the pH/GSH co-triggered degradable polyprodrug PDOX.

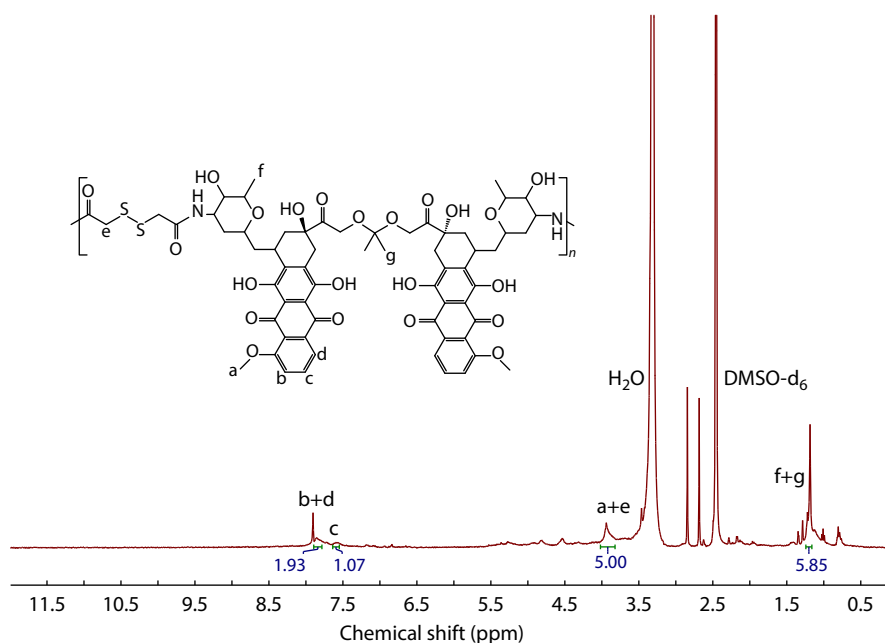


Fig. 1 ¹H-NMR spectrum of PDOX (DMSO-d₆, 20 °C).

analyses. The characteristic proton signals on DOX and the disulfide/ α -amide and ketal linkers could be seen in the ^1H -NMR spectrum (Fig. 1), such as $\text{H}_{\text{b+d}}$ ($\delta=7.78\text{--}7.89$, 1.93H), H_{c} ($\delta=7.55\text{--}7.64$, 1.07H), $\text{H}_{\text{a+e}}$ ($\delta=3.82\text{--}4.02$, 5.00H) and $\text{H}_{\text{f+g}}$ ($\delta=1.06\text{--}1.24$, 5.85H). The DP was calculated as 15 from the integral area ratio of $\text{H}_{\text{a+e}}$ to $\text{H}_{\text{f+g}}$. This is close to the relative number-averaged molecular weight of 1.55×10^4 , measured by the GPC technique with PMMA as the standard (Fig. S1 in the electronic supplementary information, ESI).

PDOX-NPs1 and PDOX-NPs2 nanomedicines were fabricated using fast precipitation and slow self-assembly approaches at different concentrations, respectively. The hydrodynamic diameters increased with increasing the PDOX concentration in DMF solution from 0.10 mg/mL to 0.40 mg/mL (Fig. 2 and Table S1 in ESI). The nanomedicines fabricated with a concentration of 0.20 mg/mL give similar mean hydrodynamic diameters (D_{h}) of 172 and 168 nm respectively, as well as a similar diameter distribution. Therefore, they were selected for further *in vitro* investigation.

The *in vitro* drug release performance of the PDOX-NPs1 and PDOX-NPs2 nanomedicines was investigated in different simulated media: pH 7.4/10 $\mu\text{mol/L}$ GSH, pH 6.5/10 $\mu\text{mol/L}$ GSH, pH 5.0/2 mmol/L GSH, and pH 5.0/10 mmol/L GSH, mimicking normal physiological media such as the blood, extra-

cellular environment, and intracellular microenvironment of normal and tumor cells, respectively. Both nanomedicines exhibited a low premature drug leakage of $< 4.5\%$ in simulated blood and extracellular environments (Fig. 3). The PDOX-NPs1 and PDOX-NPs2 nanomedicines possessed much higher cumulative DOX release of 25.2% and 21.4% in the simulated intracellular microenvironment of the tumor cells respectively, despite the premature drug leakage of approximately 5.5% in the simulated intracellular microenvironment of the normal cells, indicating the pH/GSH co-triggered degradation and DOX release (Scheme 2). The results demonstrate excellent tumor-specific DOX release with less premature leakage in the blood and extracellular environments during transportation and less mis-release in normal cells.

The effect of the DP of the polyprodrug was also assessed by synthesis of the oligomers of PDOX₅ and PDOX₃ by altering the DMP amount to 2 μL (0.016 mmol, 0.80 equiv.) and 1.25 μL (0.010 mmol, 0.50 equiv.) in the polymerization respectively, as revealed by ^1H -NMR analysis (Fig. S2 in ESI). As shown in Fig. 4, both the cumulative DOX release in the simulated tumor intracellular microenvironment and the premature leakage in the simulated normal physiological medium increased by decreasing the DP of the polyprodrug owing to the faster degradation and DOX-releasing. However, the drug

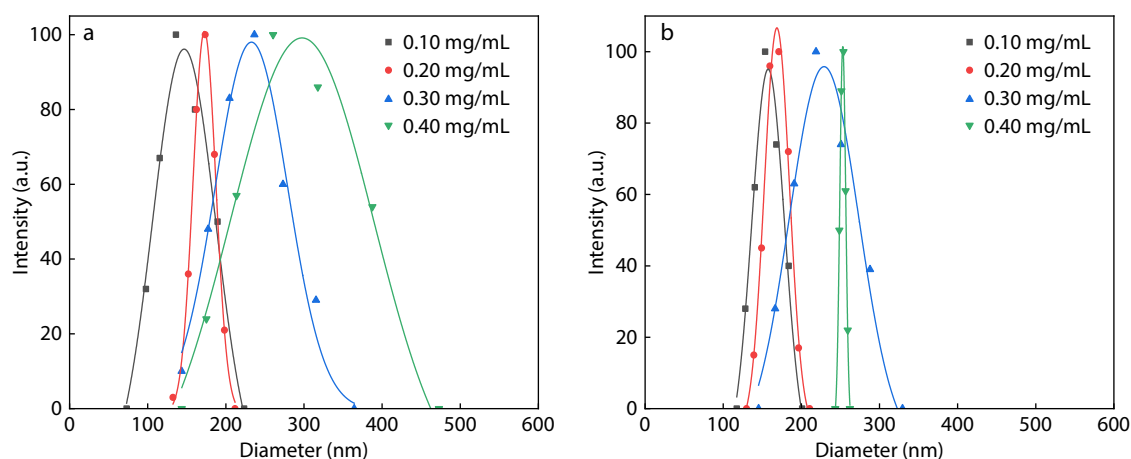


Fig. 2 Typical hydrodynamic diameters and distributions of the PDOX-NPs1 (a) and PDOX NPs2 (b) nanomedicines.

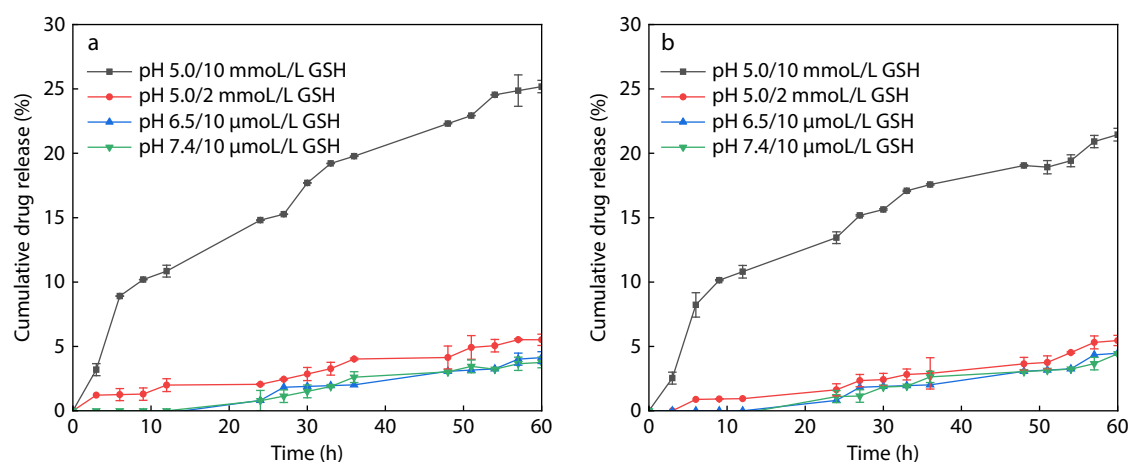
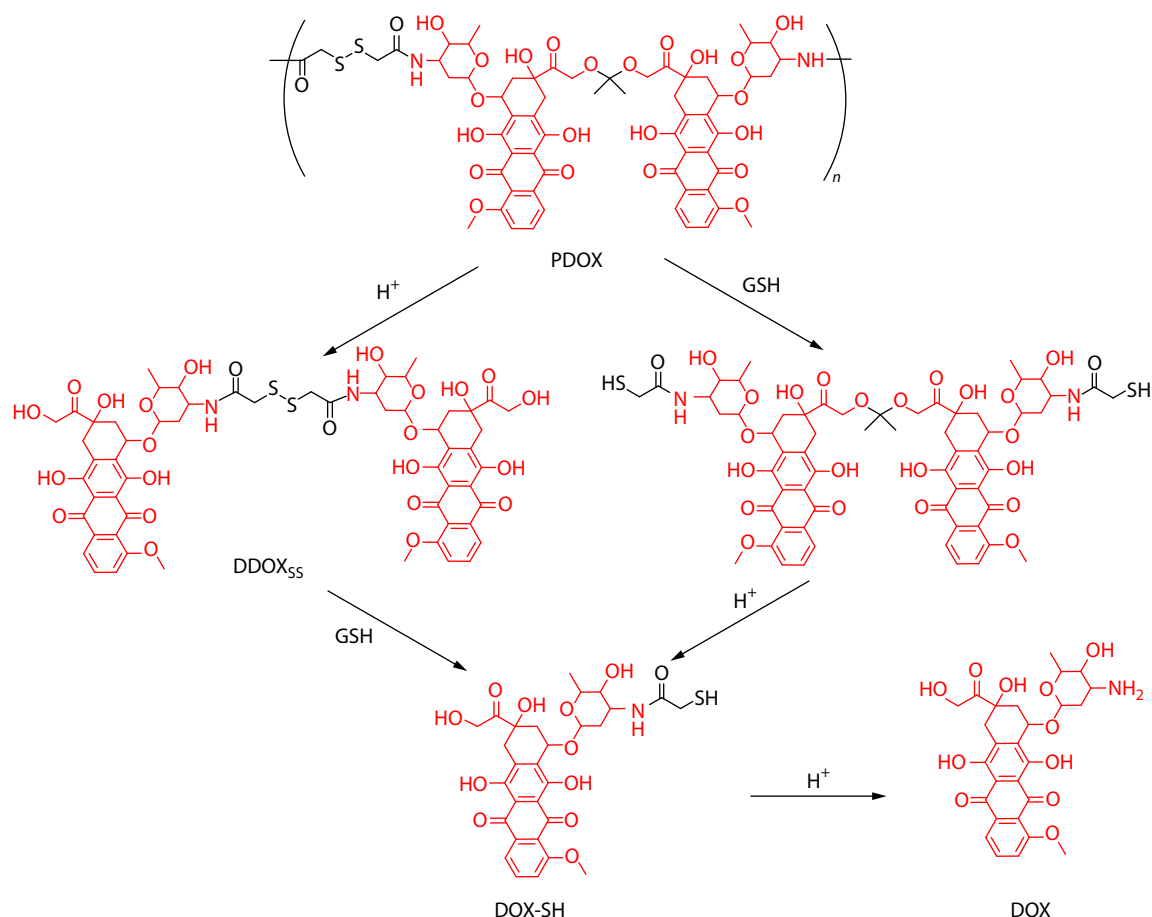


Fig. 3 Drug release profiles of the PDOX-NPs1 (a) and PDOX-NPs2 (b) nanomedicines at different simulated media.



Scheme 2 pH/GSH co-triggered degradation and DOX release from PDOX.

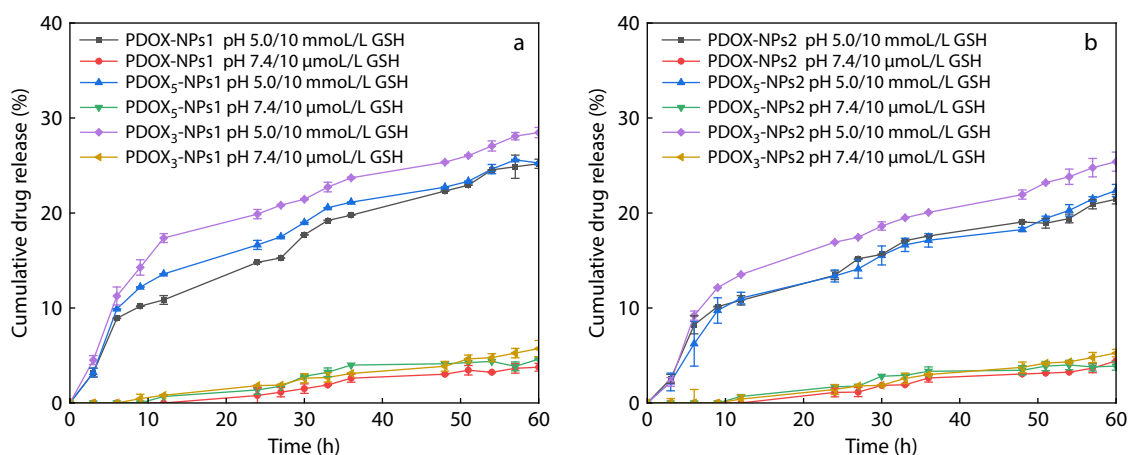


Fig. 4 *In vitro* DOX release profiles of the nanomedicines with different DPs and fabricated *via* the two approaches: fast precipitation (a) and slow self-assembly (b).

release from the PDOX₅-NPs1 and PDOX₃-NPs1 fabricated *via* fast precipitation was faster than those from PDOX₅-NPs2 and PDOX₃-NPs2 *via* slow self-assembly, similar to the PDOX with a DP of 15.

To explain the faster drug release from the PDOX-NPs1 nanomedicine compared to the PDOX-NPs2 nanomedicine, the microstructure of the nanomedicines was observed using

TEM technique. Both nanomedicines showed a near-spherical shape with an obvious porous structure (Fig. 5), similar to DOX-based dimeric prodrug nanoparticles conjugated with a ketal bond (DDOX_{ketal})^[10] which is the first report of porous dimeric prodrug nanoparticles. The porous microstructure was likely caused by the rigid fragment of DDOX_{ketal} with a very short flexible linker between the two DOX units.^[10,11] The

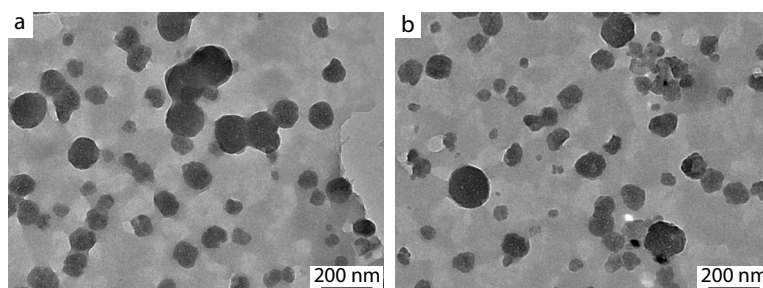


Fig. 5 Typical TEM images of the PDOX-NPs1 (a) and PDOX-NPs2 (b).

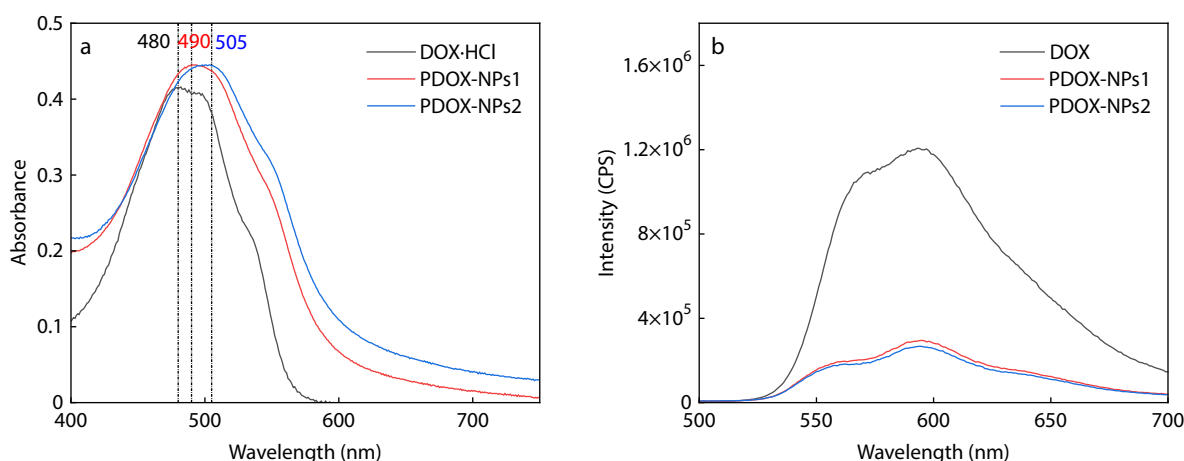
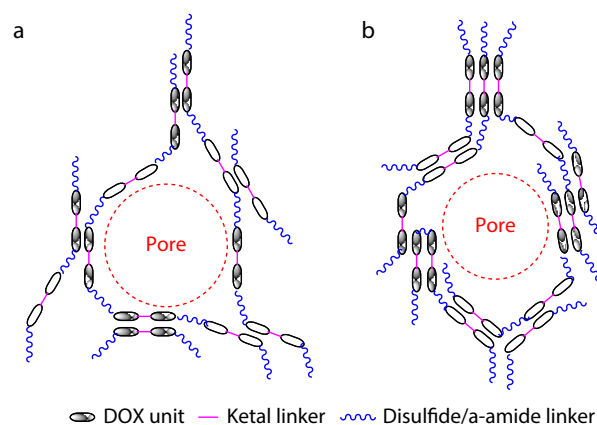


Fig. 6 UV-Vis absorption (a) and fluorescence spectra (b) of free DOX and the PDOX nanomedicines in water at a DOX-equivalent concentration of 40 $\mu\text{g/mL}$.

PDOX-NPs2 nanomedicines exhibited a more significant red-shift in UV-Vis absorption (Fig. 6a) and more complete fluorescence quenching in fluorescence analysis (Fig. 6b). Such differences may result from the various degrees of intramolecular and intermolecular π - π stacking of the DOX units,^[12–15] similar to those in dimeric prodrugs^[9–11,16] and analogs^[17].

Both the disulfide/ α -amide-bridged dimeric prodrug (DDOX_{SS}) units and ketal-bridged dimeric prodrug (DDOX_{ketal}) units were incorporated simultaneously as repeating units in the proposed polyprodrug PDOX. Because of the ketal bond as a short flexible linker between the rigid conjugated anthraquinone rings on DOX, the DDOX_{ketal} repeating units could only adopt the rod-like topological structure, restricting compact stacking, resulting in a porous microstructure in the nanomedicines even *via* slow self-assembly. Compact stacking *via* self-assembly is almost prohibited in PDOX-NPs1 *via* fast precipitation. Thus, the various degrees of the red-shift in UV-Vis absorption and fluorescence quenching in fluorescent analysis between the two nanomedicines should be caused by the different aggregation states of the polyprodrug in the various fabrication approaches, as shown in Scheme 3.

The *in vitro* cellular uptake and cytotoxicity of PDOX nanomedicines were evaluated using HepG2 cells as a tumor cell model. After co-incubation for 24 h, the red fluorescence of DOX was observed, mainly overlapping with the blue fluorescence of the nuclei stained with DAPI (Fig. 7), indicating that both nanomedicines could be internalized by the HepG2 cells, and then degraded to release DOX in response to the



Scheme 3 Schematic illustration of the loose π - π stacking in the PDOX-NPs1 (a) and the more ordered π - π stacking in the PDOX-NPs2 (b).

higher acidity and GSH level in the tumor cells.^[18] Moreover, the red fluorescence was stronger in HepG2 cells co-incubated with PDOX-NPs1 than with PDOX-NPs2, due to their faster degradation and DOX release, as revealed by the *in vitro* drug release profiles (Fig. 3).

In the MTT assays, neither nanomedicine showed obvious cytotoxicity on the normal human liver cell line (L02 cells), which is distinctly different from the high dose-dependent cytotoxicity of free DOX (Fig. 8a). The results demonstrated the safety of the proposed nanomedicines on normal cells due to their very slow degradation and drug release triggered by the

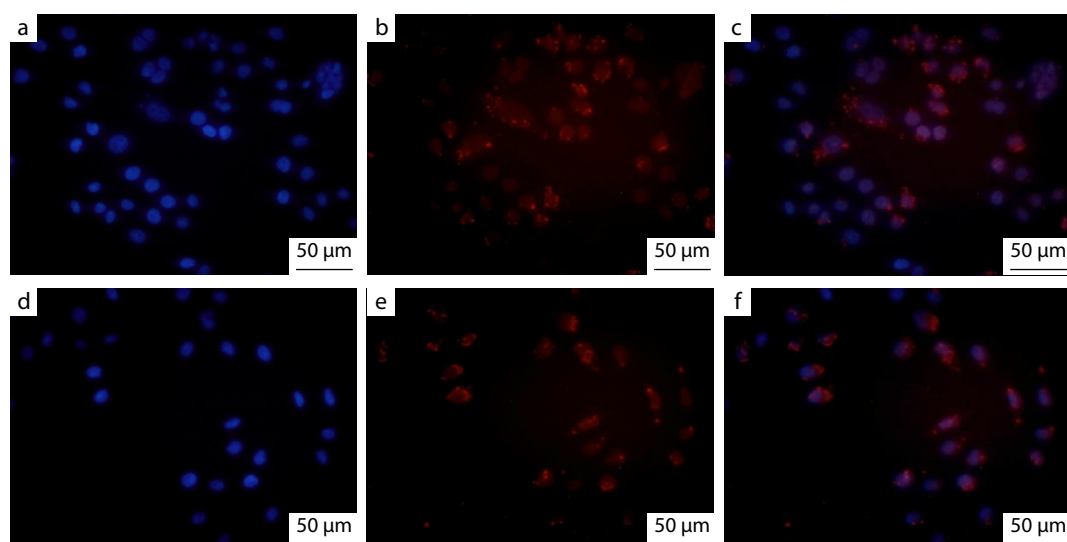


Fig. 7 Fluorescence microscope images of the HepG2 cells after incubation with the PDOX-NPs1 (a–c) and PDOX-NPs2 (d–f) for 24 h at a DOX-equivalent dose of 15 $\mu\text{g}/\text{mL}$: DAPI (a, d), DOX (b, e), and merged (c, f).

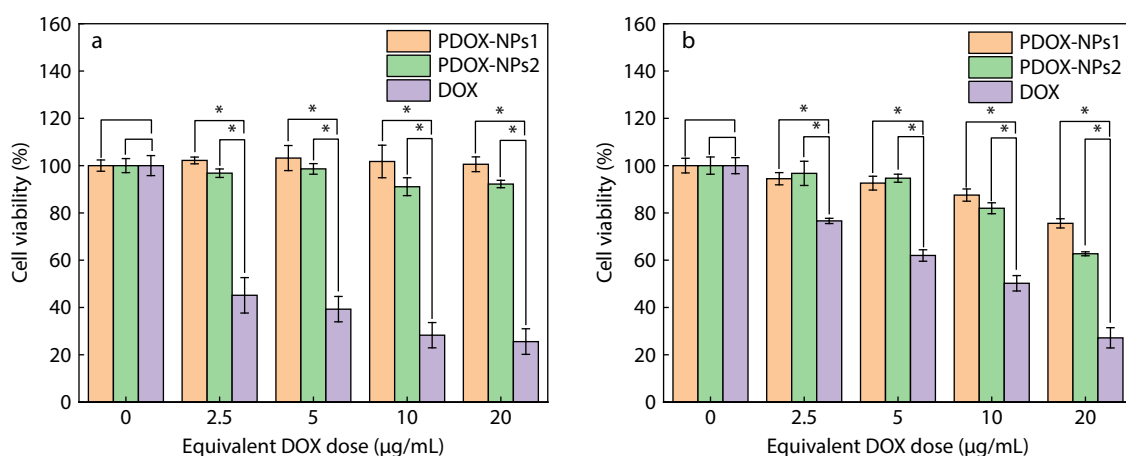


Fig. 8 *In vitro* cytotoxicity of the free DOX and PDOX nanomedicines on the L02 cells (a) and HepG2 cells (b) after incubation for 48 h (* $p < 0.05$).

lower GSH level therein. As for HepG2 cells, both nanomedicines exhibited dose-dependent cytotoxicity as DOX, while the cell viability was higher than that of free DOX because of their slow degradation and DOX release (Fig. 8b). PDOX-NPs1 showed higher *in vitro* cytotoxicity at lower doses ($\leq 5 \mu\text{g}/\text{mL}$) than PDOX-NPs2, but lower *in vitro* cytotoxicity at higher doses ($\geq 10 \mu\text{g}/\text{mL}$). The half-maximal inhibitory concentration (IC_{50}) was calculated as 478.12 and 54.95 $\mu\text{g}/\text{mL}$ the PDOX-NPs1 and PDOX-NPs2, respectively. The lower *in vitro* antitumor efficiency of PDOX-NPs1 might be caused by the higher DOX concentration in the cytoplasm due to faster drug release (as seen in Fig. 6), which might be pumped out of the HepG2 cells *via* P-gp mediated efflux.^[19]

CONCLUSIONS

In summary, the effect of the aggregation states of the polyprodrug was explored by comparing the *in vitro* drug release and cytotoxicity in the present work by altering the aggregation states of the polyprodrug nanomedicines using different fabrication approaches, namely precipitation and self-assembly. A faster drug release rate was achieved with PDOX-NPs1 *via* fast

precipitation than PDOX-NPs2 *via* slow self-assembly, regardless of the DP of the polyprodrug. The results could be explained by the resultant aggregation states at different ordering degrees *via* the two approaches, as revealed by TEM, UV-Vis, and fluorescence analyses. This finding demonstrates that the aggregation state of polyprodrugs could be used as a secondary strategy for improved on-demand drug delivery in tumor chemotherapy, in addition to their molecular structures.

Conflict of Interests

The authors declare no interest conflict.



Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-025-3340-8>.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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