

Original Article

# Synthesis and crosslinking-induced self-assembly of copolymer prodrug for tumor intracellular acid-triggered doxorubicin delivery

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## ABSTRACT

Supramolecular self-assembled copolymer-based micelles and nanoparticles show promising potential as drug delivery systems (DDSs) for tumor chemotherapy. However, the stability upon dilution and premature leakage of encapsulated drugs limit their practical application. Herein, core-crosslinked copolymer prodrug nanoparticles were developed via facile crosslinking-induced self-assembly (CISA) of doxorubicin (DOX)-conjugated poly(hydrazide oxyethyl methacrylate)-*b*-PEG copolymer prodrug through acid-labile hydrazone and imine bonds, with hexamethylene 1,6-bis(4-oxybenzaldehyde) (BFA) as crosslinker. The optimized core-crosslinked copolymer prodrug nanoparticles (PEG-cPMN-DOX) with a high DOX content of 35.72% and average hydrodynamic diameter (Dh) of 183 nm possessed better acid-triggered controlled DOX release performance than the corresponding supramolecular self-assembled copolymer prodrug nanoparticles (PEG-PMN-DOX), showing tumor intracellular acid-triggered sustained release of DOX with similar cumulative DOX release of >80% in the simulated tumor intracellular microenvironment (TME) in 72 h but much lower premature DOX leakage of 7%. Both copolymer prodrug nanoparticles can be internalized by the tumor cells and release DOX in the TME condition, showing an enhanced tumor growth inhibition than free DOX, while the core-crosslinked copolymer prodrug nanoparticles (PEG-cPMN-DOX) gave a higher anti-tumor efficiency than the corresponding supramolecular self-assembled copolymer prodrug nanoparticles (PEG-PMN-DOX), maybe due to the excellent acid-triggered sustained DOX release.

## 1. Introduction

Copolymer-based nano-self-assemblies, including micelles and nanoparticles, show promising potential as drug delivery systems (DDSs) in tumor chemotherapy, due to the outstanding advantages including molecular design, easy fabrication, stimuli-responsiveness, etc. [1]. However, such copolymer-based nano-self-assemblies are not stable upon dilution and tend to demicellize due to the weak supramolecular interaction between the copolymers. The demicellization usually expedites the premature leakage of the encapsulated drugs, causing toxic effects on the normal cells and tissues.

Core-crosslinked micelles and nanoparticles have been developed as DDSs in the last decades [2], by self-crosslinking [3-6], or crosslinking the functional copolymers with organic molecules [7-11], metal ions [12], or UV irradiation [13]. By the core-crosslinking strategy, the extracellular stability of such DDSs has been successfully improved; however, the premature leakage of the encapsulated drugs has remained a challenge.

Polymer prodrugs have been recognized as an efficient approach for controlled drug release with less premature drug leakage, by conjugating drugs via stimuli-responsive dynamic covalent bonds [14,15], although they also face the stability problem in nano-self-assemblies. So various approaches have been established for the

fabrication of core-crosslinked polymer prodrug nanoparticles to make them stable upon dilution. For example, Hennink and Lammers' group developed a crosslinking polymerization method to fabricate core-crosslinked polymer prodrug micelles with drug conjugated on the side groups of the crosslinked framework in the hydrophobic cores, by the radical surfactant-free emulsion polymerization of the drug-conjugated monomers with poly(ethylene glycol) (PEG) conjugated (PEGylated) copolymers with polymerizable side groups as both comonomer and surfactant [16,17]. The drug content was < 10% in the resultant core-crosslinked polymer prodrug micelles, due to the fabrication method.

Most recently, Yu *et al* developed a new method to fabricate core-crosslinked polymer prodrug micelles by conjugating camptothecin (CPT) onto the side groups of the PEGylated polyglutamic acid, following a crosslinking with cystamine [18]. Because both CPT conjugation and crosslinking were designed on the carboxyl side groups in the polyglutamic acid block, the conjugated drug content and the crosslinking degree could not be coordinated in the method. For a desired crosslinking degree, the CPT-conjugated copolymer with CPT content of 19.9% was used for the fabrication of the core-crosslinked polymer prodrug micelles. Thus, a low CPT content of <19.9% was achieved in the final core-crosslinked polymer prodrug micelles. Besides, the space hindrance caused by the conjugated drugs would also restrict the crosslinking.

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Here, a di-block copolymer containing hydrazide side groups was synthesized via the RAFT polymerization of 4-nitrophenoxy carbonyloxyethyl methacrylate (HEMAN) with a PEGylated chain transfer agent (mPEG-CTA), following a functionalization of the side group with *tert*-butyl carbazate (Boc-hydrazide) (Scheme 1). After conjugating DOX onto the hydrazide side groups via hydrazone linker, the core-crosslinked copolymer prodrug nanoparticles were fabricated by intermolecular crosslinking the copolymer prodrug with hexamethylene 1,6-bis(4-oxybenzaldehyde) (BFA), on both the hydrazide side groups through hydrazone linker and the amino groups on the conjugated DOX units through imine bond. As a result, the competition of the side groups for drug conjugation and crosslinking was successfully solved. And core-crosslinked copolymer prodrug nanoparticles, in which DOX was incorporated as both side group on the crosslinked framework and structural unit in the crosslinked framework, were obtained with a higher DOX content, as well as controllable crosslinking degree.

## 2. Materials and Methods

### 2.1. Synthesis of hexamethylene 1,6-bis(4-oxybenzaldehyde) (BFA)

BFA was synthesized by coupling 4-hydroxybenzaldehyde (5.15 g, 42 mmol) with 1,6-dibromohexane (4.88 g, 20 mmol) with potassium carbonate (2.77 g, 20 mmol) as a catalyst [19]. The product was washed and recrystallized from ethanol. The pure BFA was characterized by thin-layer chromatography (TLC) and nuclear magnetic resonance ( $^1\text{H}$  NMR) analysis, with a yield of 90%.  $^1\text{H}$  NMR (400 MHz in  $\text{CDCl}_3$ ):  $\delta$  (ppm): 9.8-10 (s, 2H), 7.7-7.9 (d, 4H), 6.9-7.1 (d, 4H), 4-4.1 (t, 4H), 1.7-1.9 (m, 4H), 1.50-1.65 (m, 4H) (Figure S1).

### 2.2. Synthesis of 4-nitrophenoxy carbonyloxyethyl methacrylate (HEMAN)

The functional monomer, 4-nitrophenoxy carbonyloxyethyl methacrylate (HEMAN), was synthesized by the acylation reaction of

2-hydroxyethyl methacrylate and 4-nitrophenyl chloroformate [20]. The pure HEMAN was characterized by TLC and  $^1\text{H}$  NMR analysis, with a yield of 50%.  $^1\text{H}$  NMR (400 MHz in  $\text{CDCl}_3$ ):  $\delta$  (ppm) 8.28 (d, 2H), 7.38 (d, 2H), 6.17 (s, 1H), 5.63 (s, 1H), 4.40-4.48 (m, 2H), 4.48-4.56 (m, 2H) (Figure S2).

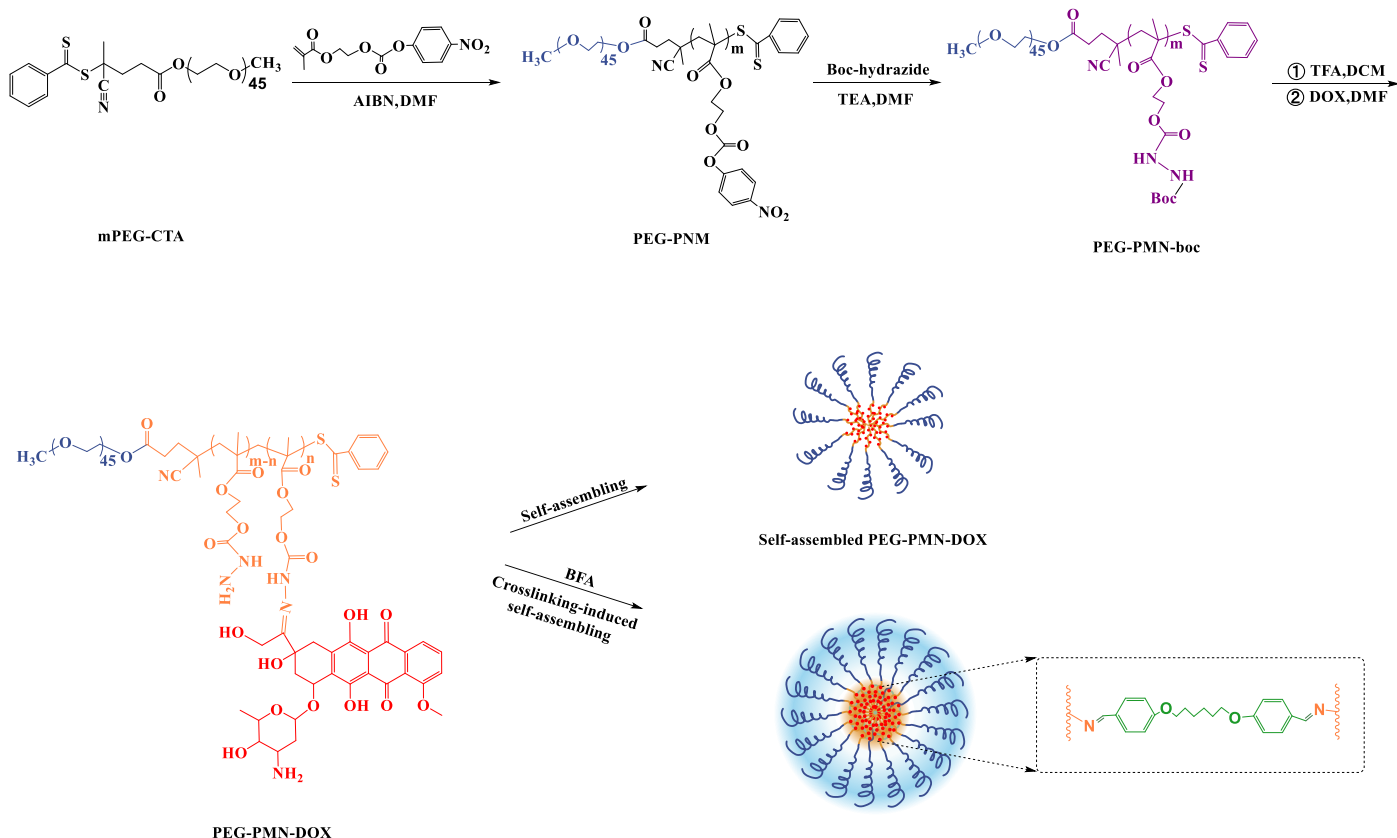
### 2.3. Synthesis of di-block copolymer (PEG-PNM)

The PEGylated chain transfer agent (mPEG-CTA) was synthesized as reported in previous work [21]. Then the diblock copolymer (PEG PNM) was synthesized by a RAFT polymerization method, as shown in Scheme 1. Briefly, 2.43 g of HEMAN (55 eq), 0.31 g mPEG-CTA (1 eq), and AIBN (0.167 eq) were dissolved in 5 mL of DMF in a Schlenk tube. The flask was sealed under dry argon, and the polymerization was conducted at 70°C for 24 h. The di-block copolymer was collected by precipitation with cold diethyl ether and drying under vacuum at room temperature.

### 2.4. Preparation of copolymer prodrug (PEG-PMN-DOX)

The PEG-PMN-boc was synthesized by modification of PEG-PMN with excessive *tert*-butyl carbazate (1:5 eq) for 48 h in nitrogen [22]. The resultant product was obtained by participation in cold ether twice and drying under vacuum.

For the BOC deprotection, 2 mL of trifluoroacetic acid was charged in 5 mL of dichloromethane (DCM) solution containing 200 mg of PEG-PMN-boc. After stirring for 3 h at room temperature, the solvent was removed by rotary evaporation, and the residue was dissolved in 10 mL of DMF/methanol (volume ratio of 1:3); 419.9 mg of DOX and a catalytic amount of acetic acid were added. The reaction was conducted in the dark at 40°C for 48 h. The resulting copolymer prodrug was isolated by dialysis against DMF and anhydrous methanol (MWCO of 3500), respectively. Then the copolymer prodrug solution in the dialysis bag was concentrated and precipitated by adding diethyl ether dropwise with stirring. Finally, the copolymer prodrug PEG-PMN-DOX



Scheme 1. Fabricating core-crosslinked copolymer prodrug nanoparticles (PEG-cPMN-DOX) with the crosslinking-induced self-assembly (CISA) approach.

was obtained by centrifugation (8000 rpm for 5 min), washing with diethyl ether, and drying under vacuum.

### 2.5. Fabrication of core-crosslinked copolymer prodrug nanoparticles (PEG-cPMN-DOX)

The core-crosslinked copolymer prodrug nanoparticles (PEG-cPMN-DOX) were fabricated via CISA of copolymer prodrug PEG-PMN-DOX with BFA as crosslinker via acid-labile hydrazone and imine bonds (Scheme 1). The feeding molar ratio of the  $-NH_2$  on the PEG-PMN-DOX (including the residual hydrazide side groups and the amino group on the conjugated DOX molecules) to BFA was set as 2:1, 3:1, or 4:1, respectively. With the molar ratio of 4:1 as an example, PEG-PMN-DOX (20 mg) and BFA (3.6 mg) were dissolved in DMF, and the mixture was stirred at room temperature in a  $N_2$  atmosphere in the dark for 48 h. The resultant core-crosslinked copolymer prodrug nanoparticles (PEG-cPMN-DOX) were collected by centrifugation (10000 rpm/5 min), washing with DMF and ethanol in turn, and finally drying in a vacuum oven overnight at room temperature.

For comparison, the supramolecular PEG-PMN-DOX micelles were also fabricated via dialysis (MWCO of 3500) in their DMSO solution against pH 7.4 PBS [23].

## 3. Results and Discussion

### 3.1. Synthesis of copolymer prodrug PEG-PMN-DOX

To solve the competition of the side groups for drug conjugation and crosslinking in the fabrication of desired core-crosslinked copolymer prodrug nanoparticles for high-performance tumor chemotherapy, namely higher drug content and appropriate crosslinking degree, an amphiphilic di-block copolymer containing hydrazide side groups was designed via a facile RFAT polymerization with PEGylated chain transfer agent (mPEG-CTA) for tumor intracellular acid-triggered DOX delivery, as illustrated in Scheme 1. In the proposed strategy, DOX was conjugated to the hydrazide side groups in the copolymer through an acid-labile hydrazone bond. In the designed copolymer prodrug PEG-PMN-DOX, both the amino group on the conjugated DOX and the residual hydrazide side groups in the copolymer could be used for core-crosslinking. Thus, the crosslinking degree of the crosslinked hydrophobic core could be efficiently controlled by adjusting the feeding ratio of crosslinking, for a better on-demand DOX release.

Firstly, the di-block copolymer PEG-PNM was synthesized via the RAFT polymerization of HEMAN with mPEG-CTA. Comparing the integral areas of the proton signals on the benzene ring in the HEMAN units ( $\delta = 8.35\text{--}7.33$  ppm) and the methylene group in the PEG blocks ( $\delta = 3.65$  ppm) (Figure 1a), the degree of polymerization (DP) of HEMAN units was established as 52. The relative number-averaged molecular weight of the PEG-PNM was measured as  $1.25 \times 10^4$  with the GPC technique with polystyrene standards, with a PDI of 1.27 (Figure S3). It was very near to the theoretical value of PEG-PNM of  $1.21 \times 10^4$  with the DP of HEMAN units of 52.

Secondly, the PEG-PMN-boc diblock copolymer was prepared by post-polymerization modification with tert-butyl carbazate. Comparison with the  $^1H$  NMR spectrum of PEG-PNM, the benzene ring proton signal at 8.35-7.33 ppm disappeared, while a new proton peak emerged at 1.5 ppm, ascribed to the methyl signal in boc. Furthermore, by comparing the integral areas of the proton signals on methyl in boc ( $\delta = 1.5$  ppm) and the methylene group in the PEG blocks ( $\delta = 3.65$  ppm), the degree of substitution of carbazate was found to be 52, demonstrating the complete conversion of the NM units (Figure 1a).

Finally, the copolymer prodrug PEG-PMN-DOX was synthesized by conjugating DOX onto PEG-PMN through a pH-sensitive hydrazone bond, after the BOC deprotection of PEG-PMN-boc. The chemical shift of  $-OCH_3$  on DOX could be seen at  $\delta = 3.94$  ppm in the  $^1H$  NMR spectrum (Figure 1b), revealing the successful conjugation of DOX. Moreover, the DOX conjugating degree was calculated as 16 among 52 hydrazide side groups in a PEG-PMN molecule by comparing the integral areas of the characteristic signals at 3.94 ppm (48H,  $-OCH_3$  on DOX) and 3.61–3.45 ppm (180H,  $-CH_2-$  on PEG) (Figure 1c), meaning a DOX content of 41.92% in the proposed copolymer prodrug. About one-third of the

hydrazide side groups on the PEG-PMN were conjugated with DOX, because of the space hindrance as reported previously [21,23].

To reveal the drug content, the absorption of the copolymer prodrug PEG-PMN-DOX solution in DMSO was measured at 480 nm. The DOX content in the copolymer prodrug PEG-PMN-DOX was measured as 40.52%. The result was slightly lower than the  $^1H$  NMR result, which might be due to the  $\pi$ - $\pi$  stacking interactions between the conjugated DOX [24,25].

### 3.2. Fabrication of core-crosslinked copolymer prodrug nanoparticles via CISA

The core-crosslinked copolymer prodrug nanoparticles were fabricated via a facile CISA strategy, by crosslinking the hydrazide side groups ( $NH_2NH-$ ) on the copolymer and the amino group ( $NH_2-$ ) on the conjugated DOX. The crosslinking degrees of the designed core-crosslinked copolymer prodrug nanoparticles were controlled by altering the feeding ratios between the crosslinker BFA and the total of the hydrazide side groups ( $NH_2NH-$ ) on the copolymer and the amino group ( $NH_2-$ ) on the conjugated DOX from 1:2, 1:3, and 1:4, respectively.

After the crosslinking with BFA at different feeding ratios, the hydrodynamic diameter of the resultant core-crosslinked nanoparticles was measured with the dynamic light scattering (DLS) technique. As shown in Figure 2(a), the mean hydrodynamic diameter (Dh) decreased from 250 nm to 206 nm and 182 nm in DMF, with decreasing the feeding ratio of BFA from 1:2 to 1:3 and 1:4, respectively. All the data were much higher than the molecular size of a copolymer with Mn about 20,000 (PEG-PMN<sub>52</sub>-DOX<sub>16</sub>), demonstrating the successful crosslinking of the copolymer prodrug. For a smaller diameter for the electron paramagnetic resonance (EPR) effect and a higher DOX content, the core-crosslinked copolymer prodrug nanoparticles fabricated with the BFA feeding ratio of 1:4 were optimized for further investigation.

To further reveal the core-crosslinking of the copolymer prodrug, the hydrodynamic diameters of the core-crosslinked PEG-cPMN-DOX nanoparticles fabricated with the BFA feeding ratio of 1:4 were determined in water (bad solvent for PEG-PMN-DOX) and DMF (good solvent for PEG-PMN-DOX), respectively, in comparison with the self-assembled micelles before core-crosslinking. The core-crosslinked PEG-cPMN-DOX nanoparticles showed a slightly bigger Dh than the self-assembled PEG-PMN-DOX micelles in water (Figure 2b), due to the incorporation of the BFA crosslinker. Similar to the DLS results, the core-crosslinked PEG-cPMN-DOX nanoparticles showed a slightly bigger particle size (148 nm) than the self-assembled PEG-PMN-DOX micelles (120 nm) in the TEM observation, despite a similar near spherical shape (Figure S4).

While in the DMF system, the self-assembled PEG-PMN-DOX micelles were dissolved, showing a Dh around 10 nm (Figure 2c). And the core-crosslinked PEG-cPMN-DOX nanoparticles gave a Dh of 182 nm, significantly higher than the Dh in water of 162 nm, because of the swelling of the crosslinked hydrophobic core in DMF.

The DOX content in the resultant PEG-cPMN-DOX nanoparticles was measured as 35.72% by the UV-vis technique after the complete DOX release under extreme conditions [26]. The decrease in DOX content in the PEG-cPMN-DOX nanoparticles in comparison with that of the copolymer prodrug PEG-PMN-DOX resulted from the incorporation of the BFA crosslinker. So the actual BFA content in the proposed PEG-cPMN-DOX nanoparticles fabricated with a BFA feeding ratio of 1:4 was calculated as 12:1 of BFA/PEG-PMN-DOX.

### 3.3. In vitro acid-triggered drug release

The tumor intracellular acid-triggered DOX release from the core-crosslinked PEG-cPMN-DOX nanoparticles was assessed *in vitro* in the simulated media of pH 5.0 ABS and pH 7.4 PBS, mimicking the tumor intracellular microenvironment and the normal physiological medium, such as blood circulation. As shown in Figure 3, both nanoparticles exhibited acid-triggered drug-releasing characteristics with the cumulative DOX release of > 80% in 72 h, owing to the acid-labile crosslinking and drug conjugation. However, an acid-triggered DOX sustained release was achieved with the proposed core-crosslinked

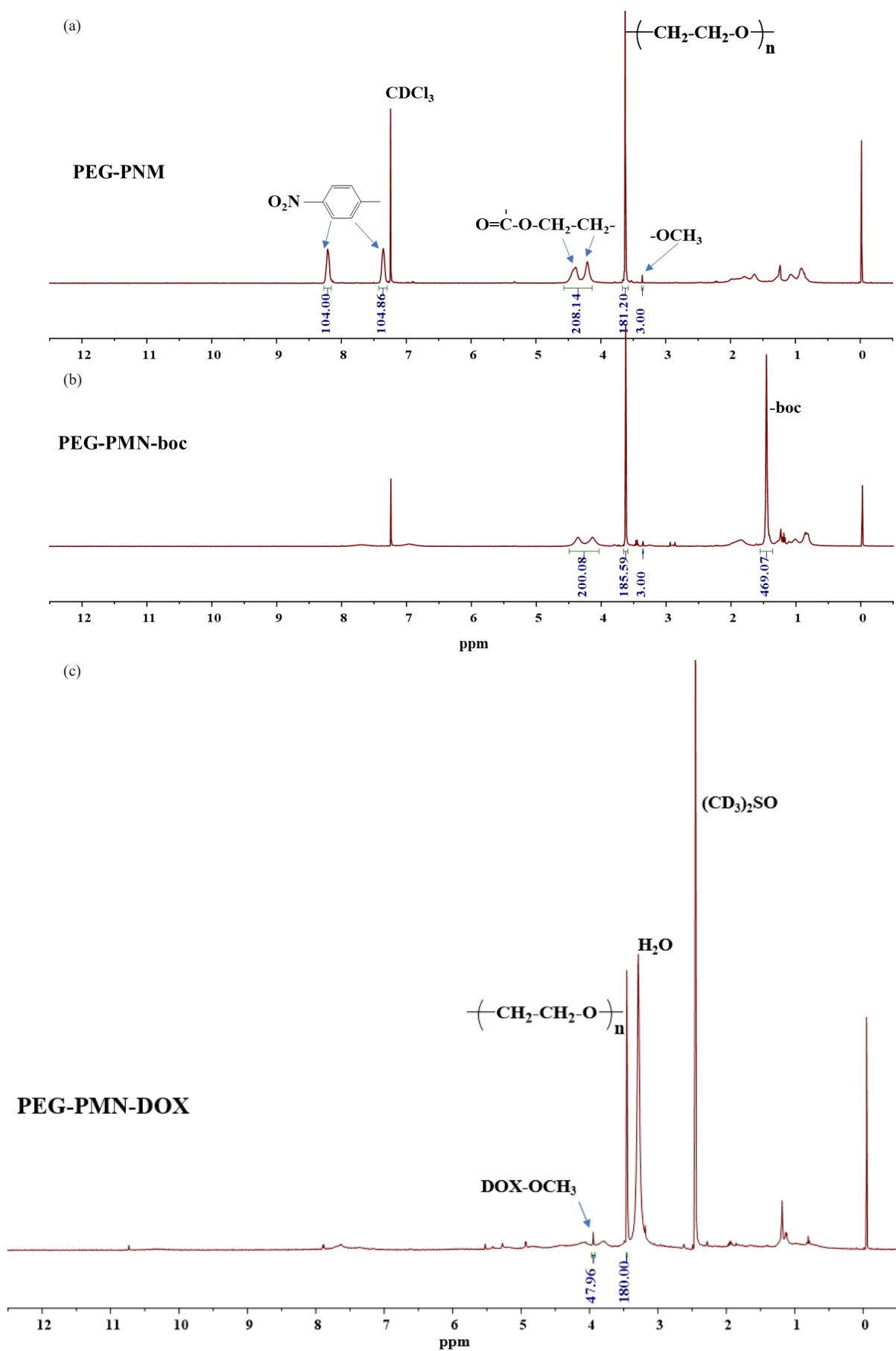


Figure 1. <sup>1</sup>H NMR of (a) PEG-PNM and (b) PEG-PMN-boc in  $\text{CDCl}_3$ , (c) <sup>1</sup>H NMR of PEG-PMN-DOX in  $\text{DMSO}-d_6$ .

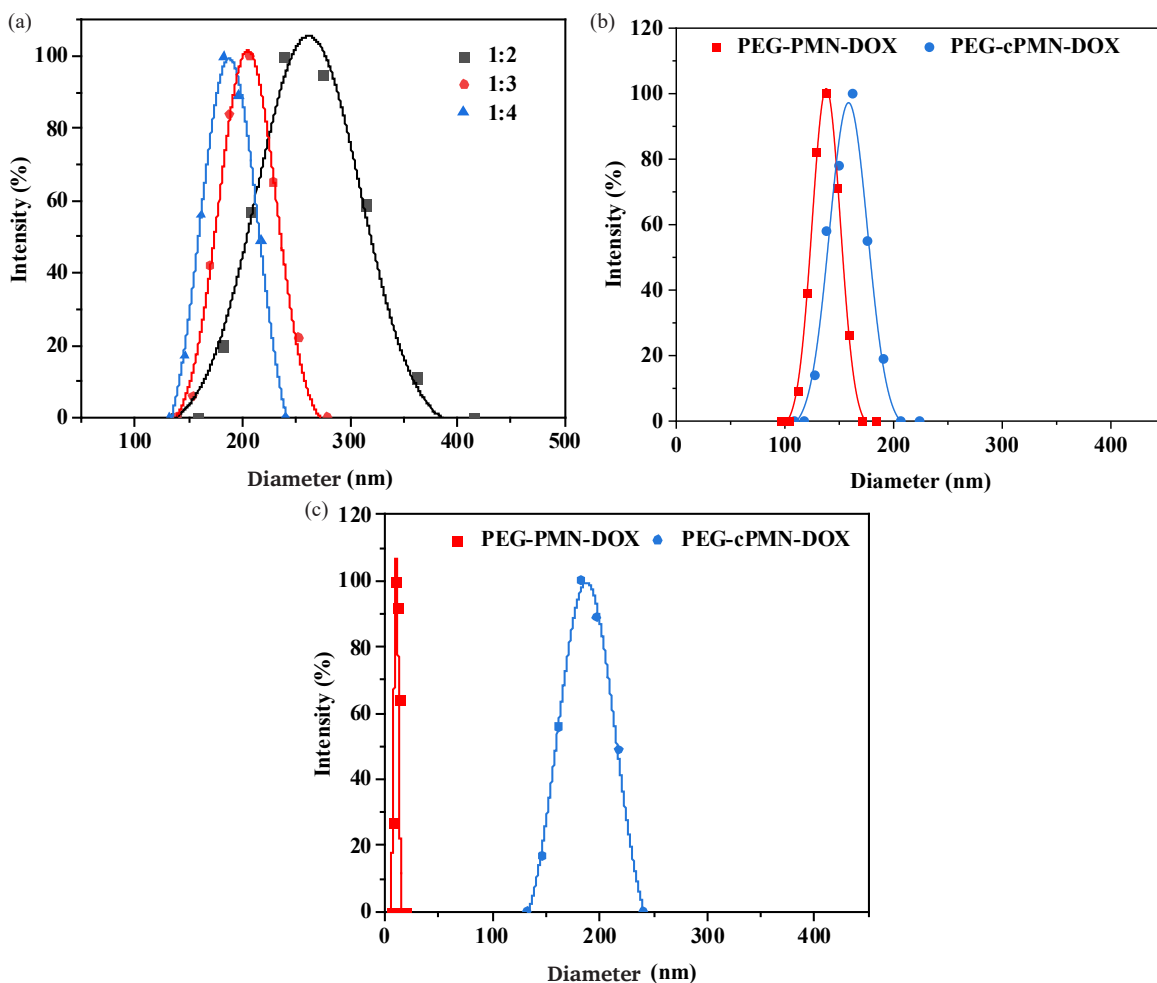


Figure 2. Hydrodynamic diameters of the PEG-cPMN-DOX nanoparticles fabricated at different ratios in DMF (1:2, 1:3, and 1:4) (a), self-assembled PEG-PMN-DOX nanoparticles and core-crosslinked PEG-cPMN-DOX nanoparticles (b) in water and (c) in DMF.

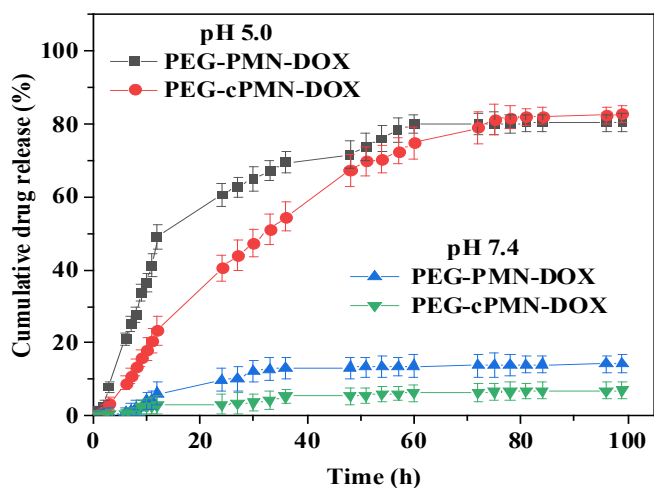


Figure 3. *In vitro* drug release from the self-assembled PEG-PMN-DOX nanoparticles and the core-crosslinked PEG-cPMN-DOX nanoparticles at a dose of 1.00 mg in 120 mL of pH 7.4 PBS and pH 5.0 ABS. Error bars were based on three repetitions at each time point.

copolymer prodrug nanoparticles PEG-cPMN-DOX in the simulated TME in 72 h, in comparison with the fast DOX release from the self-assembled PEG-PMN-DOX nanoparticles in the first 12 h. Furthermore, a much lower premature drug leakage of 7% was obtained with the proposed core-crosslinked copolymer prodrug nanoparticles PEG-

Table 1. DOX content, leakage, and release of the pH-responsive polymer prodrug-based crosslinked nanomedicines.

DDSs	DOX content	Leakage	Release	Refs.
Core-crosslinked micelles with covalently linked DOX	<10%	~5% at pH 7.4 in 24 h	~100% at pH 5.0 in 24 h	[16]
PEGylated particles	8%	~30% at pH 7.4 in 72 h	~80% at pH 5.0 in 72 h	[27]
piCNAs	26.4±1.4%	~40% at pH 7.4 in 24 h	~70% at pH 6.0 in 24 h	[28]
PPEGMA <sub>42</sub> - <i>b</i> -PFPPMA <sub>122</sub> -(CDs)-DOX	34.92%	15% at pH 7.4 in 36 h	≥98% at pH 5.0 in 36 h	[26]
CDP5/DOX3	12.8%	10.7% at pH 7.4 in 74 h	~80% at pH 5.1 in 74 h	[29]
PEG-cPMN-DOX	35.72%	7% at pH 7.4 in 72 h	>80% at pH 5.0 in 72 h	This work

cPMN-DOX in the simulated normal physiological medium, compared with the self-assembled PEG-PMN-DOX nanoparticles of 14%. The results demonstrated that the core-crosslinking strategy could not only enhance the stability of the copolymer prodrug nanoparticles, but also improve the acid-triggered drug release performance in a more desired manner, which is expected to inhibit the P-gp efflux of the quickly released DOX out of the tumor cells and avoid the toxic effect on the normal cells and tissues.

Compared with the reported pH-responsive polymer prodrug-based crosslinked nanomedicines (Table 1), the proposed PEG-cPMN-DOX nanoparticles in the present work possess higher drug content and better

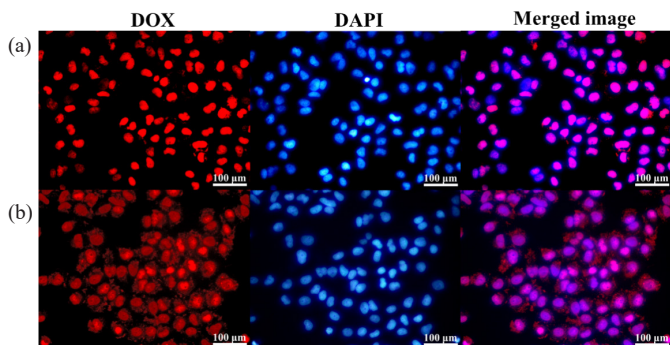


Figure 4. Fluorescence images of the HepG2 cells after incubation with the (a) PEG-cPMN-DOX and (b) PEG-PMN-DOX (15  $\mu\text{g/mL}$ ) for 48 h.

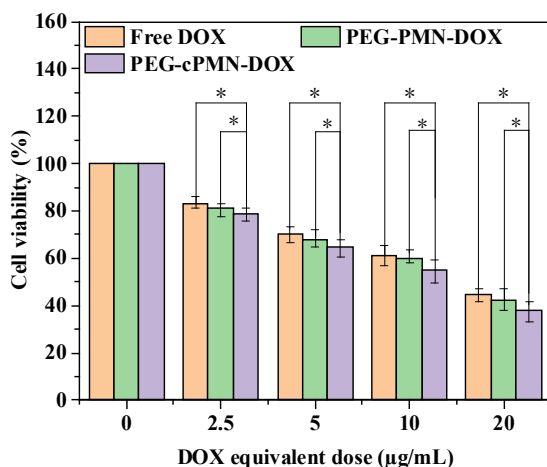


Figure 5. Cell viability assay of the HepG2 cells after incubation with free DOX, self-assembled PEG-PMN-DOX, and core-crosslinked PEG-cPMN-DOX for 48 h. Values are expressed as mean  $\pm$  SD ( $n = 6$ ) (\* denotes the significant difference,  $p < 0.05$ ).

controlled drug release with lower premature drug leakage and faster drug release in the simulated tumor intracellular microenvironment, because the competition of the side groups for drug conjugation and crosslinking was successfully solved [16,26-29].

### 3.4. *In vitro* cellular uptake and cytotoxicity

The *in vitro* cellular internalization performance of the core-crosslinked PEG-cPMN-DOX nanoparticles and the self-assembled PEG-PMN-DOX nanoparticles was assessed with the DMI4000 B Automated Inverted Microscope, after incubating the HepG2 cells in the presence of the nanoparticles at a concentration of 15  $\mu\text{g mL}^{-1}$  for 48 h. Clearly, the red DOX fluorescence was completely overlapped with the blue DAPI fluorescence (Figure 4a), meaning the proposed core-crosslinked copolymer prodrug nanoparticles PEG-cPMN-DOX had been successfully internalized by the HepG2 cells, and the released DOX was mainly accumulated into the cellular nuclei [30].

As for the self-assembled PEG-PMN-DOX nanoparticles, the released DOX was found in both cellular nuclei and cytoplasm (Figure 4b), maybe due to the faster DOX release from the self-assembled PEG-PMN-DOX nanoparticles in the first 48 h. Moreover, the drug in the cytoplasm might be bumped out of the tumor cells via the P-gp efflux, causing a declined in anti-tumor efficiency and possible multi-drug resistance [31].

Finally, the *in vitro* cytotoxicity of the core-crosslinked PEG-cPMN-DOX nanoparticles and the self-assembled PEG-PMN-DOX nanoparticles was evaluated with the MTT assays. All cases showed the dose-dependent cytotoxicity on the HepG2 cells (Figure 5). The two copolymer prodrug nanoparticles exhibited lower cell viability than the free DOX at the same DOX equivalent doses, due to the suppression of the P-gp efflux [32]. The half maximal inhibitory concentration (IC<sub>50</sub>)

was calculated as 15.98, 14.31, and 11.58  $\mu\text{g mL}^{-1}$  for the free DOX, self-assembled PEG-PMN-DOX nanoparticles, and core-crosslinked PEG-cPMN-DOX nanoparticles, respectively. Compared with the self-assembled PEG-PMN-DOX nanoparticles, the higher tumor growth inhibition effect was obtained with the proposed core-crosslinked PEG-cPMN-DOX nanoparticles, owing to their tumor intracellular acid-triggered DOX sustained release [33].

## 4. Conclusions

In summary, a novel approach was developed to coordinate the functional groups for drug conjugation and crosslinking in the fabrication of core-crosslinked copolymer prodrug nanoparticles. With DOX as a model, a di-block copolymer containing hydrazide side groups was designed. After conjugating DOX onto the hydrazide side groups through an acid-labile hydrazone bond from its carboxyl group, the amino group on the conjugated DOX could also be used for the core-crosslinking of the copolymer prodrug, as well as the residual hydrazide side groups. Therefore, the competition of the side groups for drug conjugation and crosslinking was successfully solved with a high DOX content and controllable crosslinking degree. The *in vitro* DOX release profiles demonstrated that the proposed core-crosslinking strategy could not only enhance the stability of the copolymer prodrug nanoparticles, but also improve the acid-triggered drug release performance into a more on-demand manner. Thus, a higher tumor growth inhibition effect was achieved in the MTT assays, indicating the promising application for future tumor chemotherapy.

### CRedit authorship contribution statement

**Jimin Xue:** Investigation, visualization, data curation. **Peng Liu:** Writing – original draft, writing – review & editing, supervision, methodology, resources, investigation, conceptualization.

### Declaration of competing interest

There are no conflicts of interest.

### Data availability

Data will be made available on request.

### Declaration of generative AI and AI-assisted technologies in the writing process

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

### Supplementary data

Supplementary material to this article can be found online at [https://dx.doi.org/10.25259/AJC\\_570\\_2025](https://dx.doi.org/10.25259/AJC_570_2025)

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