

Comb-like Poly(*N*-(2-hydroxypropyl) methacrylamide) Doxorubicin Conjugates: The Influence of Polymer Architecture and Composition on the Biological Properties

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Electronic Supplementary Information

Abstract The synthesis and structure-property correlation of poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) conjugates with various architectures including random, block, branched or star-like structures and compositions have been thoroughly explored. However, related synthesis and structure-property data are still lacking for comb-like PHPMA. In this work, we report the synthesis of comb-like PHPMA copolymer-doxorubicin (DOX) conjugates with different backbone/side-chain lengths and location of drug moieties. Well-defined comb-like PHPMA-DOX conjugates are obtained *via* the combination of controlled radical polymerization and fractional precipitation techniques. The influences of structural factors on the biological properties such as cellular uptake, blood circulation and tumor accumulation have been investigated. Long blood circulation and efficient tumor accumulation can be achieved by proper control of the comb number, length and location of drug moieties. These facile comb-like structures possess great potentials in future theranostics for brachytherapy or surgical navigation.

Keywords Poly(*N*-(2-hydroxypropyl) methacrylamide); Comb-like copolymer; Anticancer drug delivery; Synthesis; Structure-property correlation

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INTRODUCTION

Nano-sized anticancer drug delivery systems (DDS) hold great promise for safe and effective delivery of drugs to malignant solid tumors by virtue of the enhanced permeability and retention (EPR) effect^[1–4]. Taking into account the majority, if not all, of the biological barriers that nanomedicines would encounter upon intravenous administration, DDS with characteristics like long half-life and tumor-specific delivery should be designed^[5]. Among all reported DDS, tremendous progresses of polymer-drug conjugates have been achieved, such as various poly(ethylene glycol)-drug conjugates EZN-2208 (PEGylated SN38), NKTR-102 (PEGylated irinotecan) and NKTR-105 (PEGylated docetaxel)^[6, 7]. Pharmacokinetic behaviors of polymer-drug conjugates mainly depend on the

molecular weight (polymer size), conformation, chain motility and branch degree^[8, 9]. Several studies have demonstrated that as the polymer size exceeded the renal filtration threshold (about 5 nm), the blood circulation time of polymers with identical structural and chemical characteristics extended with size, until they can be recognized by opsonin^[10–12]. In terms of molecular conformation and chain motility, polymers with more deformable chains (*e.g.*, linear polymers) can easily pass through pore structures on glomerulus surface, whereas spherical and rigid polymer do not^[13, 14]. The works of Gillies and Lim *et al.* have proven that PEG with the same molecular weight but higher branch degree presents longer blood circulation half-life^[12, 15–17]. Therefore, the preparation of highly branched polymers has become an important trend in the development of polymer-drug conjugate systems. In addition to classical dendritic or branched structure, which displays great potentials in delivering pharmaceuticals^[18, 19], comb-like structure is another potential architecture that greatly increases branch degree^[20, 21]. Müllner *et al.* have

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reported that the blood clearance half-life of PEGylated comb-like polymers can reach 29.3 h by molecular design and composition control^[22]. Besides, comb-like PEG (CPEG) presents enhanced resistance to protein adsorption during blood circulation compared with linear PEG, which benefits for tumor accumulation^[23]. CPEG also provides more reactive hydroxyl groups for further modification, such as hydrophobic moieties including cholesterol moieties or 2-dizao-1-naphthol-5-sulfonyl chloride (sc-DNQ) introduction and cross-linking^[24, 25]. In all, the design of precisely controlled comb-like polymers as drug carriers has always been an enduring topic.

Similar to PEG, PHPMA copolymers have been widely investigated in the field of nanomedicines due to its biocompatibility, non-immunogenicity and nontoxicity^[26–28], and varieties of branched HPMA-based DDSs have been reported. Compared with linear structures, high-molecular-weight (HMW) polymers based on branched PHPMA display prolonged blood circulation, enhanced tumor accumulation, and most importantly, superior anti-tumor activity^[29–34]. Nevertheless, most of current branched PHPMA are synthesized using a dendritic or hyper-branched core *via* the ‘grafting-onto’ approach, which involves complicated synthetic procedures^[34, 35]. The branch degree mainly depends on the functional group numbers on core materials and the topological structure is lack of variability. Compared with star-like conjugates, branched conjugates with a comb-like structure may involve simpler preparation process but provide comparable branch degree. However, to our knowledge, such kind of branched PHPMA conjugates have merely been investigated.

Herein, to investigate the potential of comb-like PHPMA conjugates in substituting the dendritic structures, well-defined comb-like PHPMA copolymers were synthesized by the combination of reversible addition-fragmentation chain transfer (RAFT) polymerization and atom transfer radical polymerization (ATRP). The polymerization kinetics of comb-like PHPMA were systematically explored. Gradients with distinct chemical compositions and low dispersity of molecular weight were obtained by fractional precipitation approach. After introducing model drug (DOX) into BHMAGG moieties *via* acid-labile hydrazone bonds, the influence of polymer architectures, including backbone length, side chain length and location of DOX, on the physicochemical and biological properties of comb-like PHPMA-DOX conjugates were also investigated.

EXPERIMENTAL

Materials

2-Hydroxyethyl methacrylate (HEMA, Adamas Reagent Co., Ltd.), 2-bromoisobutryl bromide (J&K), *N*-(2-hydroxypropyl) methacrylamide (HPMA, Polysciences Inc.), 2,2'-azoisobutyronitrile (AIBN, J&K), 1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane (Me₄Cyclam, Alfa Aesar), anisole (J&K), doxorubicin hydrochloride (DOX, Beijing Huafeng United Technology Co. Ltd.), and triethylamine (TEA, Sinopharm) were used as received without further purification. The other chemicals (reagent grade) in this work

were purchased from Sigma-Aldrich and used without further purification. *N*-(*tert*-butoxycarbonyl)-*N'*-(methacryloylglycylglycyl) hydrazine (BHMAGG), *N*-(3-azidopropyl)-methacrylamide (AzMA), 4-cyanopentanoic acid dithiobenzoate (CPAD) and 2-(2-bromoisobutyryloxy) ethyl methacrylate (HEMA-Br) were synthesized according to the literatures^[36–38].

Instruments

The ¹H-NMR and ¹³C-NMR spectra were recorded on a Bruker AV400 NMR instrument at 25 °C in CDCl₃ or DMSO-d₆. Gel permeation chromatography (GPC) analysis of the PHPMA copolymers was carried out on a Waters 1515-2414 GPC system using dimethylformamide or tetrahydrofuran as a mobile phase at a flow rate of 1 mL/min. A column set of Styragel HT2, Styragel HT4 and Styragel HT6 or a set of HSPgel HT 4 was used. The number-average (*M_n*) and weight-average (*M_w*) molecular weights of samples were determined with polystyrene (PS) standards and calibrated with PHPMA fractions. The sizes and zeta potentials of micelles were determined at 25 °C by dynamic light scattering (DLS) using a Zetasizer equipped with 633 nm laser (Malvern, Zeta-Sizer ZS90, UK). The transmission electron microscopy (TEM) analysis was carried out on a JEM-2100F apparatus (JEOL, Japan) with 200 kV accelerating voltage.

Solvolysis of Comb-like PHPMA Copolymers

A comb-like PHPMA copolymer (0.1 g) was dissolved in 0.125 mmol/L sodium hydroxide (NaOH) solution (2 mL) in a 20 mL vial. The capped vial was then heated at 50 °C for 5 h. After this time, the solution was cooled and dialyzed in water. After lyophilization, the products were collected and analyzed by GPC.

Conjugation of DOX and Labeling of Cy7

The model drug DOX was conjugated on PBHMAGG block *via* acid-sensitive hydrazone bond. Typically, C15-H₇₀(B₃) (50 mg, 0.02 mmol/L) was dissolved in 3 mL of trifluoroacetic acid (TFA) and stirred for 30 min to deprotect the Boc groups. TFA was then removed by rotary evaporation and the polymer was redissolved in DMSO. After the addition of DOX (33.7 mg, 5.6 mmol/L), the reaction was conducted in dark at 37 °C for 72 h with a catalytic amount of acetic acid. The resulting solution was purified by dialysis (MW = 3500 Da) to remove free DOX. The DOX-conjugated polymers were obtained by lyophilization as a red solid and stored under –20 °C for use. The labeling of Cy7 *via* click chemistry between AzMA moieties and alkynyl-modified Cy7 was carried out according to our previous procedures^[39].

Fabrication and Characterization of Polymeric Micelles

The polymeric micelles were prepared using the dialysis method. Typically, 1 mL of 10 mg/mL C15-H₇₀(B₃)-DOX conjugates in DMSO was injected into 10 mL of PBS (pH = 7.4) by an injection pump. After stirring for 3 h, the solution was dialyzed against PBS to remove DMSO. The size and zeta potentials of micelles were determined by DLS and TEM. Micelles at the concentration of 1 mg/mL were used for DLS measurement, and the experiments were conducted

at 25 °C using a Zeta-Sizer equipped with a 633 nm laser. TEM analysis was carried out on JEM-2100F with 200 kV accelerating voltage.

Cellular Uptake Experiment

4T1 cells were seeded onto glass-bottom petri dishes at a density of 1×10^5 cells per dish, and cultured overnight. Then, fresh medium with free DOX and DOX-conjugates was added at the concentration of 10 µg/mL DOX equivalent. After co-incubated for 1, 3, and 6 h, the cells were washed with ice-cold PBS, followed by nuclei and lysosomes staining with DAPI (0.1 mg/mL) and LysoTracker Blue (DND-22, 200 nmol/L), respectively, according to the standard procedures. Finally, the cells were subjected to CLSM analysis (Leica, TCS SP8, Germany).

Cytotoxicity Study

In vitro cytotoxicity of free DOX and DOX-loaded conjugates against 4T1 cells was determined by CCK-8 assay. Briefly, cells were seeded onto a 96-well plate at a density of 5×10^3 cells per well. After the cell attachment, the medium was substituted by free DOX and DOX-conjugates with concentration ranging from 0.001 µg/mL to 100 µg/mL (DOX equivalent) and 48 h incubation. For the viability assay, CCK-8 was added into each well and incubated for another 2 h according to the standard procedures. Then the 96-well plate was subjected to a microplate reader at a wavelength of 450 nm.

Blood Clearance and Biodistribution Study

For *in vivo* studies, IVIS spectrum imaging system (PerkinElmer, USA) was applied to monitor the fluorescence of Cy7. The exposure time, F/stop and binning value were set as 15 s, 1 and 2, respectively. The quantitative analysis was carried on by ROI measurements. Blood clearance analysis was conducted by a retro-orbital bleeding technique with 3 mice in each group at 1, 3, 6, 12, 24, and 48 h post injection.

Determination of DOX Content in Tumor Tissue

The DOX content in tumor tissue was determined according to previously reported procedures^[40]. Briefly, tumor tissues were collected at 48 h after the injection of various conjugates and homogenized to known volumes in Tris-phosphate buffer (0.2 mol/L, pH 9.5). These samples were then exposed to HCl (final concentration 1.0 mol/L) at 85 °C for 20 min. An equal volume of tris-phosphate buffer (1.0 mol/L, pH 9.5) was then added, followed by NaOH (2.0 mol/L, volume adjusted to neutralize the acid). These samples were diluted with the same buffer to a total volume of 1.0 mL. Daunomycin (100 or 200 ng) was added to each tube as an internal standard, vortex-mixed, and then silver nitrate (20% V:V of a 0.33 g/mL solution) was added. The subsequent separation process was consistent with the literature. The extracted DOX was quantified by high performance liquid chromatography (LC-20A, Shimadzu, Japan) with Waters µBondapak C18 column and eluted at a flow rate of 1.0 mL/min with a mobile phase of aqueous propan-2-ol (29%) adjusted to pH 3.2 with orthophosphoric acid. Elution was isocratic and detection fluorimetric (RF-10A, excitation wavelength 480 nm, emission wavelength 560 nm).

Statistical Analysis

All data were reported as averages plus/minus the standard deviation (Mean ± SD). Statistical significance was assessed by one-way ANOVA with Bonferroni post-tests using GraphPad Prism 5.0 software.

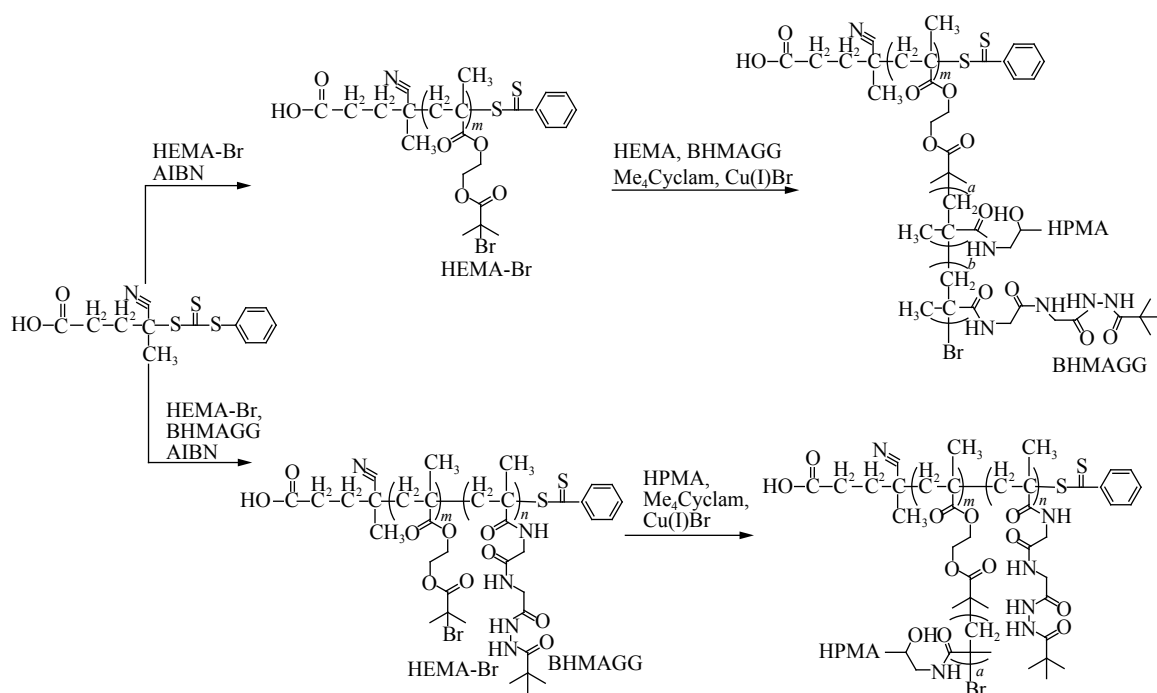
RESULTS AND DISCUSSION

Polymerization Kinetics of Me₄Cyclam-based ATRP of Comb-like PHPMA

Since polymer architectures greatly influenced the pharmacokinetics of nanomedicines, the design and synthesis of polymers with distinct architectures for drug delivery have been widely explored. Both star-like and comb-like polymers based on PEG have been thoroughly studied^[41–44], however, the comb-like PHPMA copolymers have been merely investigated. Generally, there are three approaches to synthesize comb-graft copolymers: (1) ‘grafting-onto’ approach, by which the side chains with reactive functional end-groups are synthesized and then attached to a backbone containing ‘complementary’ functional groups; (2) ‘grafting-through’ approach, by which a polymeric macromonomer is (co)polymerized with a backbone-forming monomer; and (3) ‘grafting-from’ approach, by which the side chains are grown from a backbone containing multiple initiating sites^[45]. Steric issues caused by the gradual growth of side chains are major limitations of the ‘grafting onto’ and ‘grafting through’ strategies. In this work, we explored the synthesis of comb-like PHPMA copolymers *via* the ‘grafting from’ approach. As shown in Scheme 1, the bromine-containing inimer (HEMA-Br) (Fig. S1, in electronic supplementary information, ESI) was applied to homopolymerize or copolymerize with BHMAGG monomer by RAFT polymerization to obtain macro-initiators P(HEMA-Br) and P(HEMA-Br-*co*-BHMAGG), respectively. Then comb-polymers P(HEMA-Br)-*g*-P(HPMA-*co*-BHMAGG) and P(HEMA-Br-*co*-BHMAGG)-*g*-PHPMA were synthesized by ATRP *via* grafting side chains from the macro-initiator backbone (Fig. 1A).

A series of narrow distributed P(HEMA-Br) macro-initiators with varied repeating units (C15, C50, C150 and C300) were synthesized and used to graft PHPMA side chains (Table S1, in ESI). The structure of P(HEMA-Br) was characterized by GPC and ¹H-NMR (Figs. 1B and 1C). It was found that well-defined structures (PDI < 1.2) could be obtained by the RAFT polymerization of HEMA-Br inimer with CPAD and AIBN in DMSO at 70 °C for 24 h^[38].

We found that gelation occurred easily during polymerization of comb-like PHPMA. Therefore, before the kinetic study of comb-like PHPMA, a series of pre-experiments were carried out to determine the experimental parameters, such as initiator concentration ([I]₀), monomer concentration ([M]₀), ligand concentration [ligand]₀, [Cu(I)Br]₀ and reaction temperature (*T*). As illustrated in Table S2 (in ESI), Me₆TREN led to no monomer conversion. However, with Me₄Cyclam, gelation frequently occurred under high [M]₀ or macro-initiators containing more than 150 initiating sites. The gelation phenomenon might be caused by either the ultra-high molecular weight which



Scheme 1 Synthetic routes of comb-like PHPMA *via* the combination of RAFT and ATRP

causes low solubility or the intramolecular and intermolecular termination which lead to pendant macrocycles and macroscopic gelation^[46]. Gelation became more serious with the increase of temperature and number of initiating sites. After screening a library of 30 reactions, the reaction temperature was selected as 30 and 50 °C, at which gelation merely happened. Moreover, different $[I]_0$: $[Me_4Cyclam]_0$: $[Cu(I)Br]_0$ ratios such as 1:1:1, 1:0.5:1 and 1:1:3 were tested (data not given). The final $[I]_0$: $[Me_4Cyclam]_0$: $[Cu(I)Br]_0$ ratio was set as 1:1:1. Furthermore, to prevent the occurrence of gelation, macro-initiators (C15 and C50) with fewer initiating sites were used in the subsequent studies (Fig. 1B).

The kinetic study of comb-like PHPMA *via* ATRP was carried out at 30 and 50 °C, with C15 as the macro-initiator and $[M]_0$: $[I]_0$ of 150:1. $[M]_0$ was set as 0.7 mol/L ($[M]_0$:solvents = 1:10, *W*:*V*), 0.46 mol/L ($[M]_0$:solvents = 1:20 *W*:*V*) and 0.23 mol/L ($[M]_0$:solvents = 1:30 *W*:*V*), respectively. The GPC elution curves at different time points under various condition are shown in Fig. 1(D) and Fig. S2 (in ESI). The gradual chain extension process could only be observed in the initial 6 min at 30 °C in low $[M]_0$ groups, *i.e.* $[M]_0$:solvents = 1:30 and 1:20 *W*:*V*. The average side chain molecular weight increased from 1.8 kDa to 9.4 kDa in 1:30 group and from 1.9 kDa to 10.8 kDa in 1:20 group within the initial 6 min (Fig. 1E). Meanwhile, the PDI of the correspondent copolymer sharply increased from 1.34 to 2.46 and 1.46 to 2.66, respectively (Fig. 1F). With the extension of reaction time after 6 min, the molecular weight did not undergo further increase, but the molecular weight distribution got very wide (up to 4.0). When increasing $[M]_0$:solvents to 1:10 *W*:*V* or reaction temperature to 50 °C, the polymerization became uncontrollable, with wide distributed high molecular weight polymers being observed

at the beginning of polymerization. Therefore, the reaction time and reaction temperature were set as 4–6 min and 30 °C respectively in further reactions.

The large PDI values might be attributed to two possible reasons. Firstly, it might be due to the fast reaction rate, which caused rapid increase of viscosity and prevented the monomers from free diffusion. Moreover, considering the brush-like structure and high initiator density, this might also be caused by the intermolecular crosslinking at the end of the polymerization so that dimer or larger cross-linked polymers were formed^[46]. To unveil the reason for this phenomenon and ensure the well control of graft polymerization, solvolysis procedure (0.125 mol/L NaOH, 50 °C, 5 h) was introduced to detach the side chains. The GPC curves of comb-like copolymers (obtained at 6 min under the condition of 30 °C and $[M]_0$:solvent of 1:30) before and after solvolysis were compared with the linear polymers obtained under the same condition (including $[M]_0$, feeding ratios of reactants and polymerization conditions). As shown in Fig. S3 (in ESI), it could be found that the retention time of the detached side chains well coincided with the linear polymers, of which the well-controlled ATRP polymerization has been reported in our previous work. The delayed shoulder peak could be attributed to the macro-initiator C15. Therefore, it could be concluded that during the initiate 6 min, the graft polymerization was a well-controlled process and identical side chain without intra- or inter-molecular crosslinking could be obtained. Moreover, since GPC curves in Fig. S3 (in ESI) revealed 100% scission of ester bonds, the grafting efficiency could also be determined by calculating the molecular weights of copolymers before and after solvolysis^[47]. As a result, we estimated the grafting efficiency of PHPMA side chains from P(PHPMA-Br) to be 85.7%, which was much higher

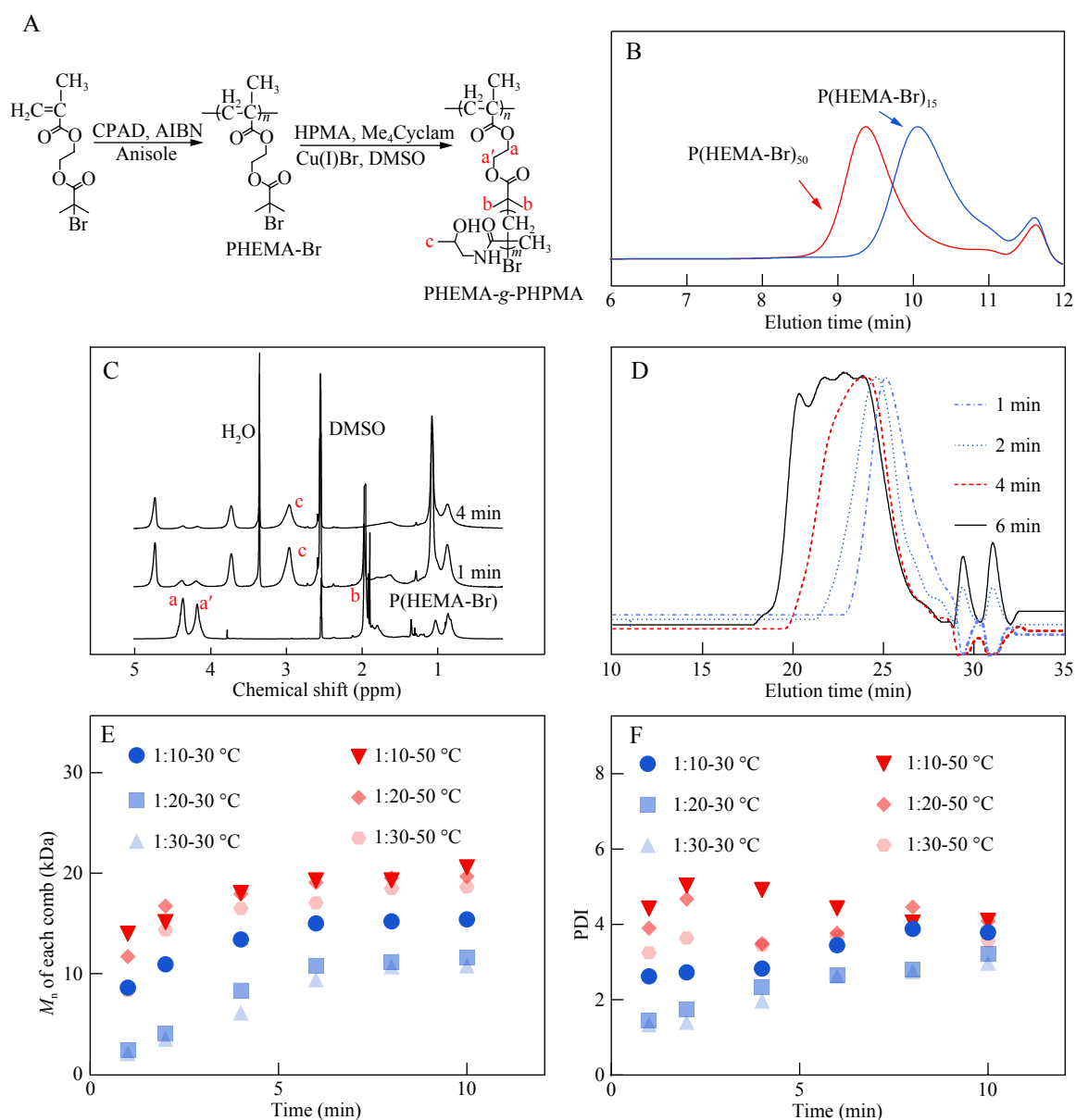


Fig. 1 (A) Synthetic routes of macro-initiators P(HEMA-Br) and comb-like PHPMA; (B) GPC elution curves of P(HEMA-Br)₁₅ and P(HEMA-Br)₅₀; (C) Representative ¹H-NMR spectra in DMSO-d₆ and (D) GPC curves of comb PHPMA at different time point (0, 1, and 4 min) of the polymerization initiated by P(HEMA-Br)₁₅ (Polymerization conditions: [I]₀: [Me₄Cyclam]₀: [Cu(I)Br]₀ = 1:1:1, [M]₀:solvents = 1:30 (*W/V*), *T* = 30 °C); (E) *M_n* of each side chain and (F) PDI changes of comb-PHPMA as a function of time ranging from 1 min to 10 min under different polymerization conditions

than the data in reported literatures (50%)^[22].

Based on the aforementioned ¹H-NMR and GPC results, successful synthesis of comb-like PHPMA was achieved *via* Me₄Cyclam-catalyzed ATRP. However, the possible existence of large molecular weight gradients was not conducive to the further structure-property studies. Thus, further purification of the resultant products was needed to obtain polymer gradients with much narrower PDI. To this end, the fractional precipitation method was applied to remove the high molecular weight components resulting from possible intra- or inter-molecular crosslinking^[48]. As shown in Fig. S4 (in ESI), by employing fractional precipitation approach, three portions with distinct molecular

weight initiated from C15 were obtained, *i.e.* portion 1: *M_n* = 289.5 kDa, PDI = 1.21; portion 2: *M_n* = 206.5 kDa, PDI = 1.18; portion 3: *M_n* = 138.5 kDa, PDI = 1.15. In further studies, we divided the resultant polymers into two portions by fractional precipitation, and the higher molecular weight portion (5%–15%) which may contain cross-linked polymers was discarded.

Synthesis and Physicochemical Characterization of Comb-like PHPMA-DOX Conjugates

To investigate the influences of comb number and comb length on their physicochemical and biological properties, macro-initiators C15 and C50 were used to initiate the copolymerization of HPMA with BHMAGG (Fig. 2a),

another functional monomer commonly used for conjugating anti-cancer drug DOX *via* an acid-sensitive hydrazone bond^[36, 49]. The reaction was accomplished under 30 °C and [M]₀:solvent of 1:30 with conversion of 40%–50% for all copolymers. After employing fractional precipitation methods, copolymers C15-*g*-P(HPMA_{70-co}-BHMAGG₃) (C15-H₇₀(B₃)), C15-*g*-P(HPMA_{130-co}-BHMAGG₅) (C15-H₁₃₀(B₅)), and C50-*g*-P(HPMA_{70-co}-BHMAGG₃) (C50-H₇₀(B₃)) were obtained after removing the high molecular weight portions which may contain cross-linked polymers. The molecular weight and chemical composition are summarized in Table 1. The PDI values of C15-H₇₀(B₃), C15-H₁₃₀(B₅) and C50-H₇₀(B₃) were 1.29, 1.27 and 1.24, respectively, indicating the well-defined architectures (Fig. 2b and Fig. S5 in ESI).

Furthermore, to explore the influences of drugs locations (on backbones or side chains) on their physicochemical and biological properties, macro-initiators C20(B₅) and C20(B₁₀) consisting of HEMA-Br and BHMAGG were prepared (Fig. 3a). The GPC curves of C20(B₅) and C20(B₁₀) are shown in Fig. S6 (in ESI), and chemical structures of macro-initiators were determined by ¹H-NMR and ¹³C-NMR

(Figs. S7 and S8, in ESI). According to the ¹H-NMR results, the number of repeating units of BHMAGG in backbones was 6 and 11 for C20(B₅) and C20(B₁₀), respectively. The random structure of the macro-initiators was proven by the appearance of new peaks at $\delta = 29.73$ ppm and $\delta = 25.74$ ppm in ¹³C-NMR spectra of the copolymer compared with that of homopolymers PBHMAGG and P(HEMA-Br). C20(B₅) and C20(B₁₀) were then employed for the ATRP of HPMA to obtain C20(B₅)-H₇₅ (PDI = 1.24) and C20(B₁₀)-H₇₅ (PDI = 1.25) (Table 1, Fig. 3b and Fig. S9, in ESI).

The model drug DOX was conjugated to either the side chains or backbones *via* hydrazone bond after removing Boc groups. The solution properties of the obtained conjugates were investigated with DLS and TEM. As shown in Figs. 2(c) and 2(f), the average size by number was only about 3 nm for C15-H₇₀(B₃)-DOX conjugates. As reported in the literature, the fully stretched length of each monomeric unit was about 0.24 nm^[50]. Therefore, under the fully stretched conditions, the axial length and contour length of as-prepared conjugates should be 37.2 and 33.6 nm, respectively, which were much higher than the observed values. The small size indicated the formation of uni-

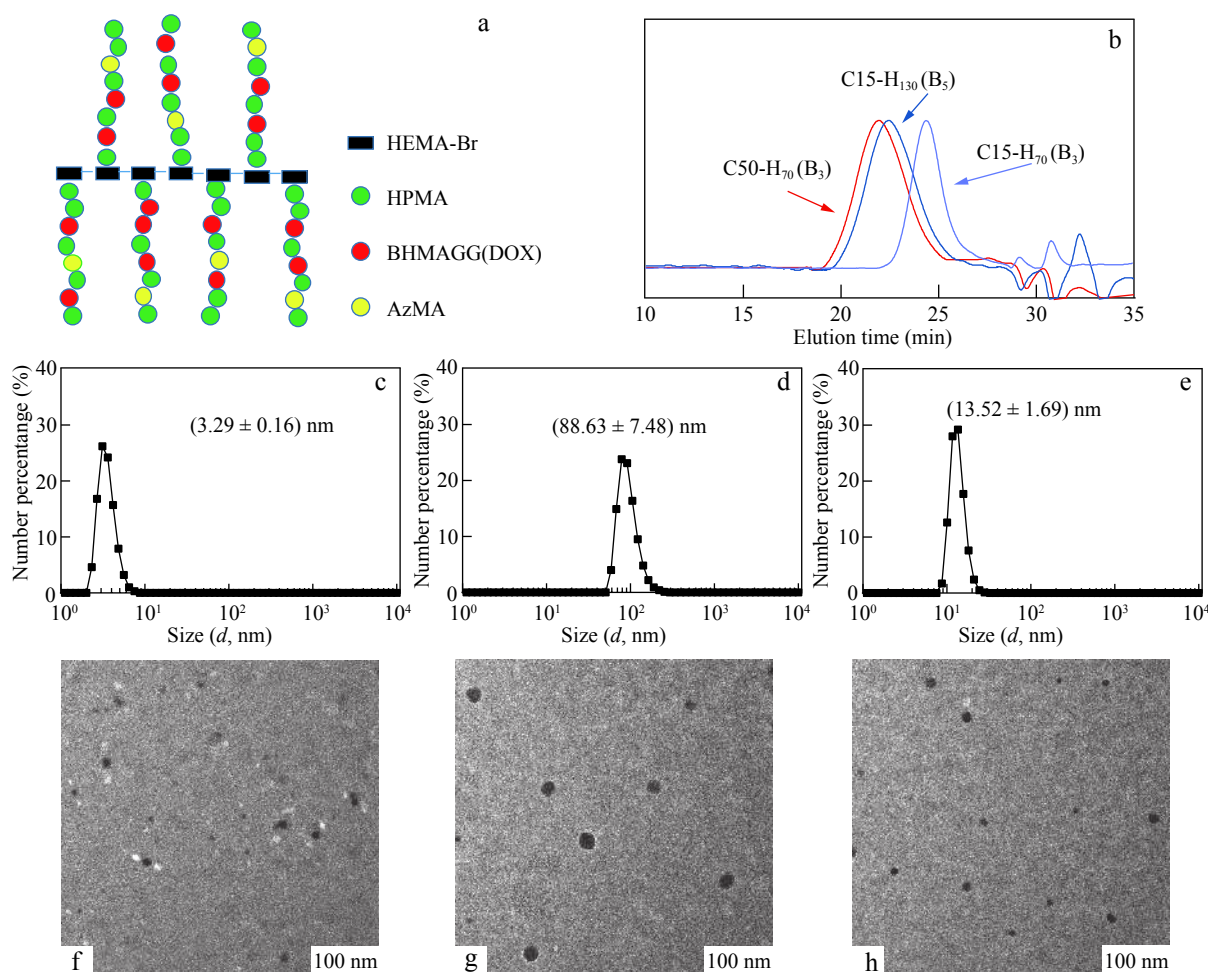


Fig. 2 (a) Schematic illustration of comb-like PHMA containing BHMAGG(DOX) moieties on the side chains; (b) GPC elution curves of C15-H₇₀(B₃), C15-H₁₃₀(B₅) and C50-H₇₀(B₃); (c–e) Micellar size distribution by number of C15-H₇₀(B₃), C15-H₁₃₀(B₅) and C50-H₇₀(B₃) DOX conjugates in PBS (10 mmol/L); (f–h) TEM images of DOX conjugates of C15-H₇₀(B₃), C15-H₁₃₀(B₅) and C50-H₇₀(B₃), respectively.

Table 1 Molecular weight and chemical composition of different comb-like PHPMA-DOX conjugates

Entry ^a	Comb number ^b	Feeding ratio [H] ₀ : [B] ₀ : [I] ₀ ^c	H ^d (kDa)	B ^d (kDa)	M _n ^d (kDa)	M _w /M _n ^e	DLE ^f (%)	D _h ^g (nm)	ζ Potential ^g (mV)
C15-H ₇₀ (B ₃)	15	80:7:1	150.15	14.85	169.17	1.29	6.7	3.2 ± 0.2	−1.0
C15-H ₁₃₀ (B ₅)	15	150:12:1	278.85	24.75	307.77	1.27	6.4	88.6 ± 7.4	−2.1
C50-H ₇₀ (B ₃)	54	75:7:1	500.50	50.49	566.00	1.24	7.2	13.5 ± 1.6	−1.2
C20(B ₅)-H ₇₅	20	70:1	214.50	33.00	253.06	1.24	0.83	19.1 ± 2.2	−0.8
C20(B ₁₀)-H ₇₅	20	70:1	214.50	39.60	259.66	1.25	0.98	65.2 ± 6.8	−1.4

^a C, H and B stand for the comb-like backbone, HPMA and BHMAGG, respectively. The numbers following C/H/B represented the repeating units of HEMA-Br on the backbone and the repeating units of HPMA and BHMAGG on each side chain; ^b Comb number of the polymers was recorded according to the repeating units of HEMA-Br, which was calculated using 85.7% grafting efficiency from P(HEMA-Br); ^c For C20(B₅)-H₇₅ and C20(B₁₀)-H₇₅, the displayed data were feeding [H]₀: [I]₀ ratios; ^d The chemical composition and molecular weight of the comb polymers were determined by ¹H-NMR; ^e The M_w/M_n (PDI) was determined by GPC; ^f The drug-loading efficiency (DLE) was determined by UV-Vis spectrometry, representing the weight percentage of DOX, and DOX contents were absent from abbreviations; ^g The hydrodynamic diameter (D_h) and ζ potential values were determined by DLS measurement.

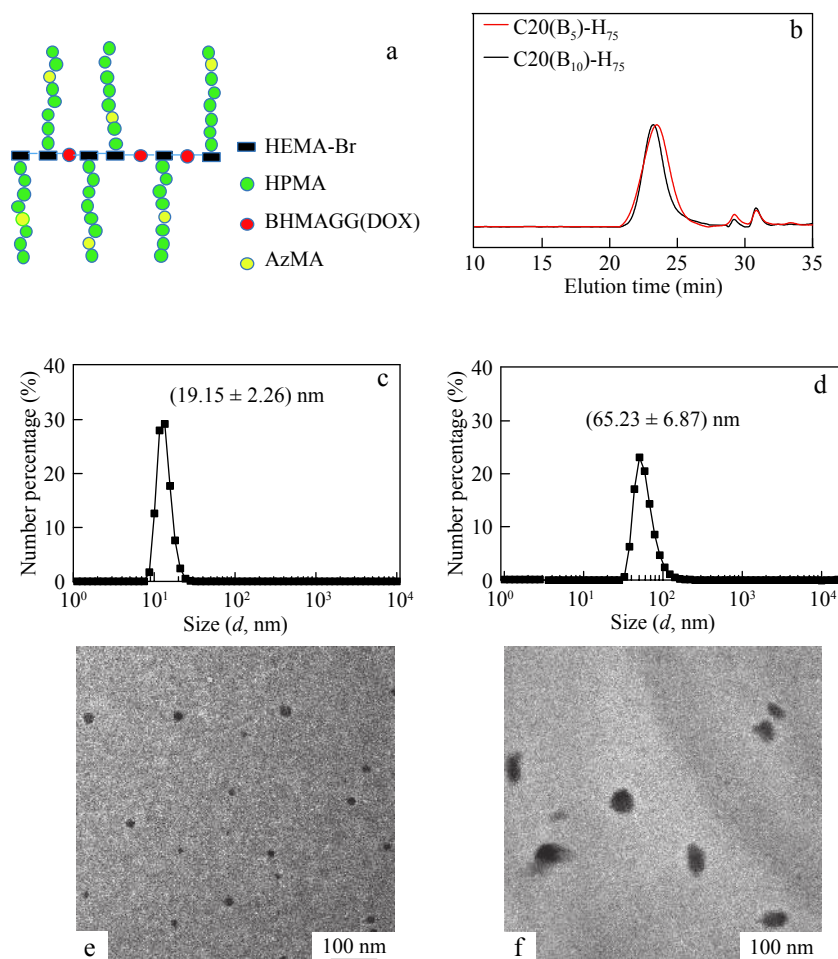


Fig. 3 (a) Schematic illustration of comb-like PHPMA copolymers containing BHMAGG(DOX) moieties on the backbone; (b) GPC elution curves of C20(B₅)-H₇₅ and C20(B₁₀)-H₇₅; (c, d) Micellar size distribution by number of C20(B₅)-H₇₅ and C20(B₁₀)-H₇₅ DOX conjugates in PBS (10 mmol/L), respectively; (e, f) TEM images of DOX conjugates of C20(B₅)-H₇₅ and C20(B₁₀)-H₇₅, respectively.

molecular micelles by the shrinkage of coiled side chains due to the existence of partially hydrophobic DOX. The size distribution by intensity (Fig. S10, in ESI) revealed the existence of multi-micelle aggregates (80 nm) with much larger size than that of fully stretched polymers, possibly caused by the secondary aggregation of small micelles (size smaller than 10 nm in Fig. S10A, in ESI). Therefore, unlike fully soluble polymers, structure-property correlations of

polymer-drug conjugates might not be simply interpreted by the influence of the architecture and composition due to the existence of hydrophobic drugs.

Compared with C15-H₇₀(B₃)-DOX conjugates, C50-H₇₀(B₃)-DOX owned longer backbone length, resulting in an increased micelle size of 14 nm. Meanwhile, the secondary aggregation with a smaller size (49 nm) could also be observed (Figs. 2e and 2h, and Fig. S10C, in ESI). The

increased micelle size of C50-H₇₀(B₃)-DOX conjugates might be attributed to the longer backbone length and larger molecular weight. However, the reason for the different tendencies of aggregate size change is still unclear. Even though polymers with long hydrophilic block tend to form small uni-molecular micelles in diluted solution, large aggregates were easily formed in concentrated solution^[39]. As a result, when the number of repeating units of HPMA on side chains increased from 70 to 130, the average size by number of C15-H₁₃₀(B₅)-DOX conjugates increased greatly from 3 nm to 88 nm and single distribution peaks were observed in both number and intensity distribution curves (Figs. 2d and 2g, and Fig. S10B, in ESI).

Compared with C15-H₇₀(B₃)-DOX conjugates, the location changes of BHMAGG(DOX) from side chains to the backbones of the comb-like copolymers led to larger micelle size, *i.e.* C20(B₅)-H₇₅-DOX conjugates (19 nm) (Figs. 3c and 3e). Meanwhile, secondary aggregation could also be observed in the size distribution by intensity (Fig. S10D, in ESI). Since block copolymers with longer continuous hydrophilic segments are more likely to form aggregates than their random counterparts^[39], the larger size of C20(B₅)-H₇₅ than that of C15-H₇₀(B₃) could be attributed to the existence of more deformable hydrophilic side chains in C20(B₅)-H₇₅ conjugates. With the increase of BHMAGG(DOX) content in the backbone, as for C20(B₁₀)-H₇₅-DOX conjugates, larger micelle size was observed (Figs. 3d and 3f). This should be either due to the increased hydrophobicity of the backbone which required more conjugates to form stable micelles, or due to the decreased side chain density which made the polymer chains more deformable. Moreover, considering the long side chain length (70–140 repeating units) and the relatively short backbone length (15–50 repeating units) of all conjugates, as-prepared micelles were not able to form the cylindrical structures^[46]; instead, globular micelles were observed in TEM graphs. As observed in Fig. S11 (in ESI), all micelles exhibited excellent stability within 48 h after incubation with 50% FBS.

The Influences of Architecture and Composition on Interactions between the Conjugates and Cells

To investigate the influences of polymer architectures and chemical compositions on the interaction between comb-like PHPMA-DOX conjugates and cancer cells, the cellular internalization of various DOX-conjugates by 4T1 cells was studied by CLSM and flow cytometry. The qualitative and quantitative analyses are presented in Figs. 4(a) and 4(b). Consistent with previous observations^[51, 52], C15-H₇₀(B₃)-DOX with the lowest M_n and the smallest micelle size showed the highest endocytosis. Compared with C15-H₇₀(B₃)-DOX, both the increase of comb length (C15-H₁₃₀(B₅)-DOX) and comb number (C50-H₇₀(B₃)-DOX) led to lower cellular uptake, possibly due to their increased micelle size. Long continuous hydrophilic side chains of polymers would lead to significant reduction in aspect of protein adsorption, complement activation or cellular uptake^[53, 54]. Thus, the change of drug location from side

chains to backbones significantly decreased cellular uptake levels. However, the different drug loading contents of C20(B₅)-H₇₅ and C20(B₁₀)-H₇₅ had almost no influence on their cellular uptake.

Since the pro-apoptotic effect of DOX is mainly realized by topoisomerase-II inhibition and doxorubicin-DNA adducts formation^[55], the entrance of DOX into cell nucleus is of great importance for the cell killing effect. As observed, free DOX rapidly entered the cells and localized in the nuclei in the initial 1 h. By contrast, the conjugates were mainly trapped in cytoplasm as indicated by the red fluorescence in Fig. 4(a). While free DOX can rapidly enter cells *via* free diffusion, the inefficient nucleus entrance of DOX in conjugates reflected the slow intracellular release. Considering the pH-responsive drug release properties (Fig. S12, in ESI), the slow nucleus entrance might be influenced by both the endocytic pathways and the subsequent intracellular trafficking of micelles^[56].

For endocytic pathway studies, cells were pretreated with methyl- β -cyclodextrin (M β CD), chlorpromazine (CPZ) and amiloride for caveolae-mediated endocytosis, clathrin-mediated endocytosis, and macropinocytosis inhibition, respectively^[57–60]. Cells were also pre-incubated at 4 °C to determine the energy-dependency of the endocytosis. As indicated in Fig. 4(c), all micelles shared similar endocytic pathways, in spite of their different architectures and compositions. Nearly 60% endocytosis of micelles was inhibited by low temperature. In addition, M β CD, CPZ and amiloride inhibited the endocytosis by about 30%, 20%, and 10%, respectively. These results indicated that the endocytosis of comb-like PHMA was an energy-dependent process and mainly through caveolae- and clathrin-mediated endocytosis. Compared with the linear PHPMA conjugates which merely involved caveolae-mediated endocytosis^[53], the comb-like topology somehow changed the endocytic pathway of PHPMA-based polymers.

The sub-cellular distribution and intracellular drug release of the conjugates were explored by co-localization studies of conjugates and lysosomes (the major acidic organelle inside cells) at different time points. As observed in Fig. 5(a) and Fig. S13 (in ESI), partial co-localization between lysosomes (blue) and conjugates (red) could be observed. The degree of co-localization was limited, as indicated by the low Pearson correlation coefficient (< 0.46) for all micelles (Fig. 5b)^[61–63]. The partial co-localization between conjugates and lysosomes coincided with the multiple pathways involved in the endocytosis of the conjugates. Moreover, since DOX release behaviors of all polymer-DOX conjugates were proved to be pH-dependent, the slow intracellular release of DOX caused by the bypass of lysosomal pathway also explained the slow nucleus entrance of loaded DOX.

To investigate whether the inefficient nucleus entrance would influence the *in vitro* anticancer effect of the conjugates, cell proliferation study was carried out with 4T1 cells. As presented in Fig. 5(c), all conjugates exhibited much lower cytotoxicity than that of free DOX (IC₅₀ =

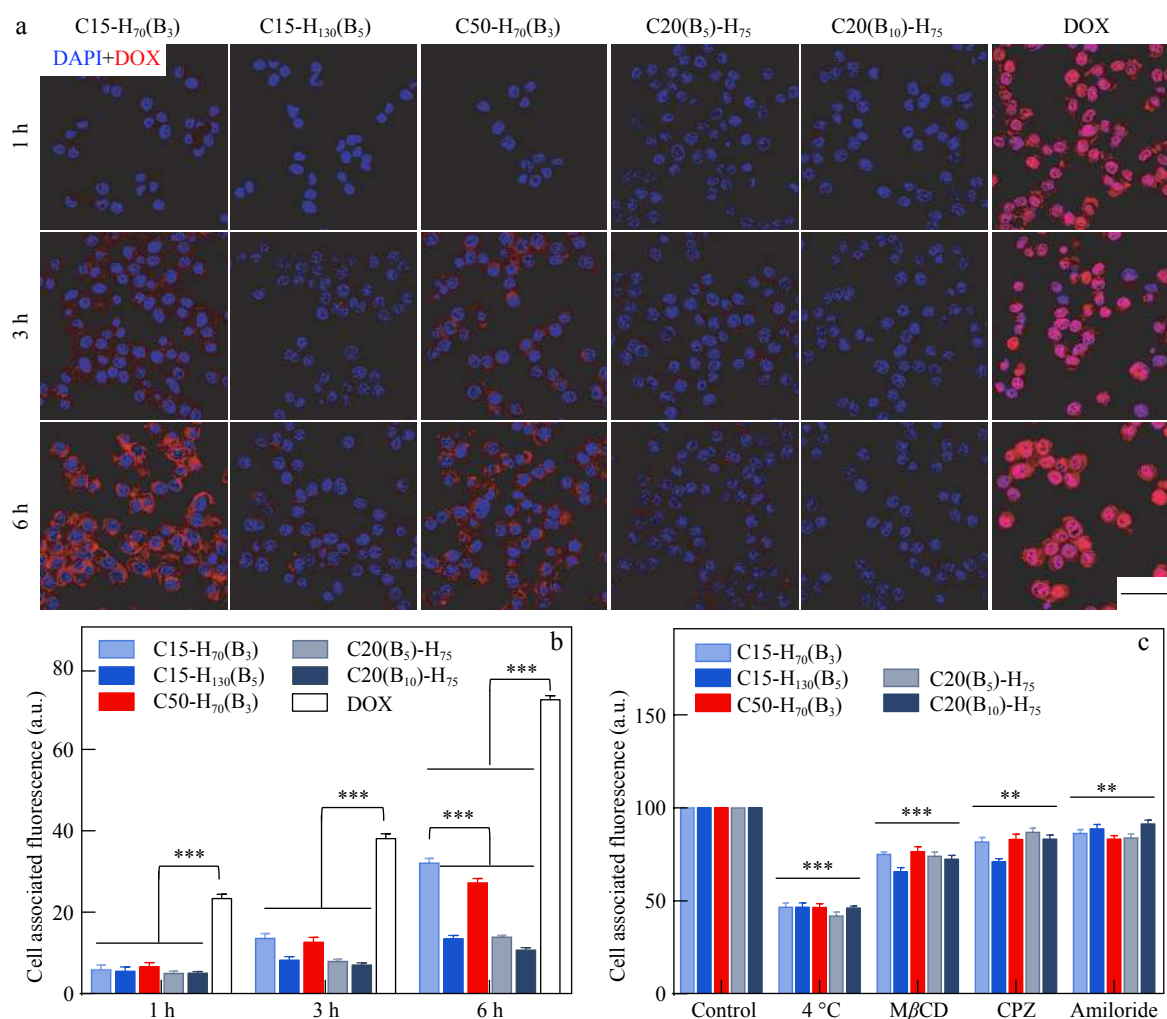


Fig. 4 (a) CLSM images of time-dependent cellular uptake of DOX conjugates of C15-H₇₀(B₃), C15-H₁₃₀(B₅), C50-H₇₀(B₃), C20(B₅)-H₇₅, C20(B₁₀)-H₇₅ and free DOX in 4T1 cells. The nuclei were stained by DAPI (blue), and the red fluorescence was indicative of DOX. The scale bar represents 100 μm; (b) Quantitative analysis of cellular uptake of comb-like PHPMA-DOX conjugates by flow cytometry, ****p* < 0.001; (c) Relative cellular uptake of comb-like PHPMA-DOX conjugates by 4T1 cells after pretreatment with different conditions (MβCD, CPZ, amiloride, and 4 °C). ***p* < 0.01, ****p* < 0.001 compared with control group.

1.2 μg/mL) and 75% viable cells could be observed at up to 100 μg/mL DOX equivalent. This might be explained either by the low uptake level or by the inefficient intracellular drug release due to the bypass of lysosomal trafficking pathways.

Biodistribution and Blood Clearance of Comb-like PHPMA-DOX Conjugates

Prolonged blood circulation, tumor-specific accumulation and retention are major advantages of nanomedicines compared with free drugs. For linear and star-like PHPMA-drug conjugates, the plasma half-life and tumor accumulation increased with molecular weight and arm numbers^[34, 48]. However, for brush-like PHPMA, studies on the influences of backbone and side chain length on their pharmacokinetics were lacking. Therefore, we evaluated the pharmacokinetics and biodistribution of comb-like PHPMA-DOX conjugates containing distinct backbone and side chain length with a 4T1 tumor model using IVIS fluorescent imaging system.

For NIRF imaging, the conjugates were firstly labeled with Cy7 via copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC)^[34]. The biodistribution and pharmacokinetics within 48 h after tail vein injection of the conjugates are shown in Figs. 6(a)–6(c) and Fig. S14 (in ESI). The plasma half-lives (*t*_{1/2}) of C15-H₇₀(B₃), C15-H₁₃₀(B₅), C50-H₇₀(B₃), C20(B₅)-H₇₅, and C20(B₁₀)-H₇₅ conjugates were 3.03, 8.35, 3.41, 3.27 and 2.15 h, respectively. Moreover, the area under curves (AUC) of C15-H₇₀(B₃), C15-H₁₃₀(B₅), C50-H₇₀(B₃), C20(B₅)-H₇₅, and C20(B₁₀)-H₇₅ conjugates were 226.2, 354.7 and 225.2, 205.9 and 224.8, respectively. The quantitative analysis of the fluorescence intensity differences (FID) between tumor site and normal site (marked with red circles in Fig. 6a) at different time points, which reflected the real-time tumor accumulation of various conjugates, could be found in Fig. 6(e). Obviously, C15-H₁₃₀(B₅)-DOX conjugates exhibited the longest blood circulation and the most efficient tumor accumulation among all conjugates. Except for the continuous increase of tumor accumulation

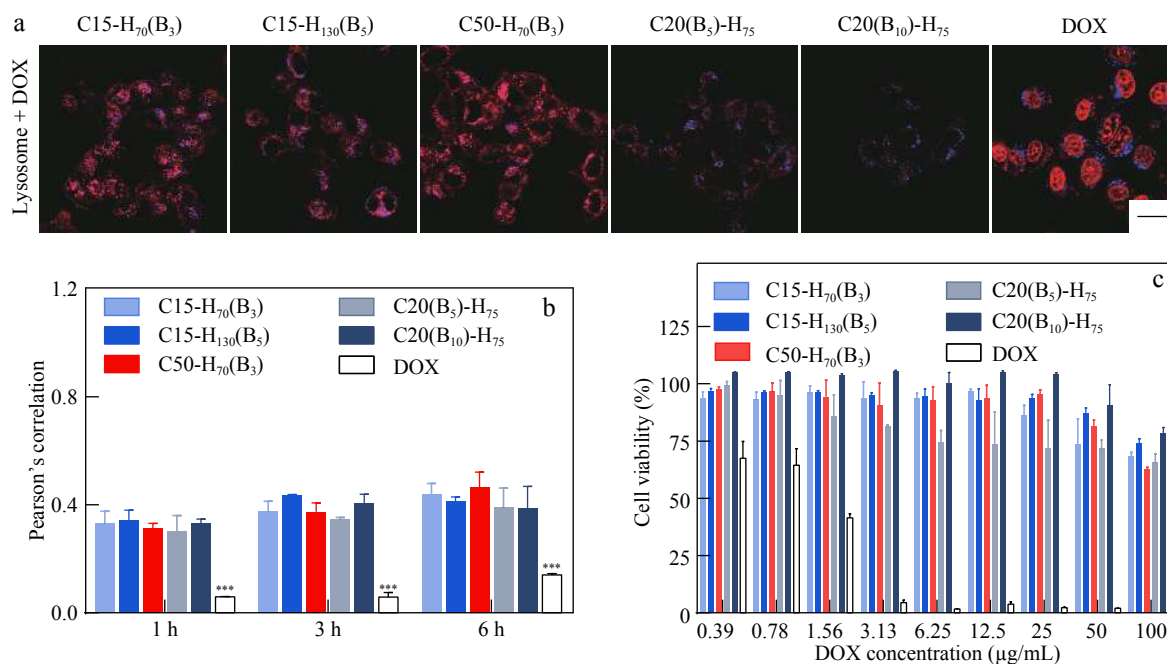


Fig. 5 (a) Subcellular trafficking study of comb-like PHPMA-DOX conjugates after 6 h co-incubation with 4T1 cells. Lysosomes were stained by LysoTracker blue DND-22. The scale bar represents 25 μm; (b) The degree of co-localization was presented by Pearson's correlation calculated from Image Pro Software. *** $p < 0.001$ for free DOX compared with the rest conjugates; (c) Cell viability of comb-like PHPMA-DOX conjugates and free DOX at different concentrations after 48 h incubation

during 48 h for C15-H₁₃₀(B₅)-DOX, the FID of the rest conjugates increased in the initial 3–6 h and then decreased. The final FID of C15-H₁₃₀(B₅)-DOX at 48 h was about 4 times higher than the other conjugates, indicating its strong tumor retention ability. The *ex vivo* distribution and the related quantitative analysis at 48 h after injection (Figs. 6b and 6d) further corroborated the highest tumor accumulation of C15-H₁₃₀(B₅)-DOX conjugate. Further determination of DOX content inside tumor tissue revealed that the DOX accumulation of C15-H₁₃₀(B₅)-DOX conjugate reached 5.64% of the injected dose (ID %) and was 7–8 times higher than that of the rest conjugates (Fig. 6f).

From the topological aspect, as-prepared conjugates belonged to comb-like structures. However, due to the relatively short backbone length, the conjugates tended to form globular shape micelles other than cylindrical ones^[64]. Therefore, the transportation and clearance of these conjugates should be influenced by the characteristics of both kinds of structures. In addition, larger aggregates, whose formation and stabilities were greatly influenced by polymer architectures and compositions, were also observed for these comb-like conjugates. For example, C15-H₇₀(B₃)-DOX conjugates exhibited rapid blood clearance and low tumor accumulation for the small micelle size of 3 nm. This enlightened us that at least in one direction, the size of the conjugates could be as small as 3 nm after compression, which was small enough to pass the glomerular fenestrations^[35]. Accordingly, in the case of C20(B₅)-H₇₅-DOX conjugates, even though larger micelle size (19 nm) was yielded, rapid clearance and kidney accumulation were still observed when moving DOX moieties from side chains

to backbones. Moreover, for the rest conjugates with 70–75 repeating units in the side chains, obvious kidney accumulation and rapid blood clearance were also observed.

The increase of side chain length from 70 to 130, *i.e.* C15-H₁₃₀(B₅), caused longer contour length and formation of larger multi-micelle aggregates with a diameter of 85 nm. As a result, the $t_{1/2}$ of C15-H₁₃₀(B₅)-DOX conjugates was greatly prolonged to 8.35 h. However, as reported in our previous work, such aggregates may undergo disassembly after dilution by blood during circulation^[39]. The apparent molecular weight in the radial direction of the uni-molecular micelle should be about 36 kDa after disassembly. Therefore, the apparent contour length of the conjugates after compression should be about 5 nm as referred to 3 nm size of C15-H₇₀(B₃)-DOX conjugates, making it pass through the pore structures in kidney by deformation^[35]. Moreover, the highest tumor accumulation and retention of C15-H₁₃₀(B₅)-DOX conjugates might be attributed to the formation of stable aggregates due to its long side chains^[39]. In contrast to C15-H₁₃₀(B₅)-DOX conjugates, the rest conjugates, which possessed shorter side chain length, underwent easy destabilization of the aggregates after blood dilution. Compared with the kidney accumulation, lower liver accumulation could be observed for all conjugates due to the ‘stealth’ properties of PHPMA.

Above all, these results revealed that, without the sophisticated synthetic procedures like star-like PHPMA polymer conjugates, prolonged blood circulation, efficient tumor accumulation and possible renal excretion could also be realized with comb-like PHPMA conjugates by proper control of polymer architectures and compositions. However,

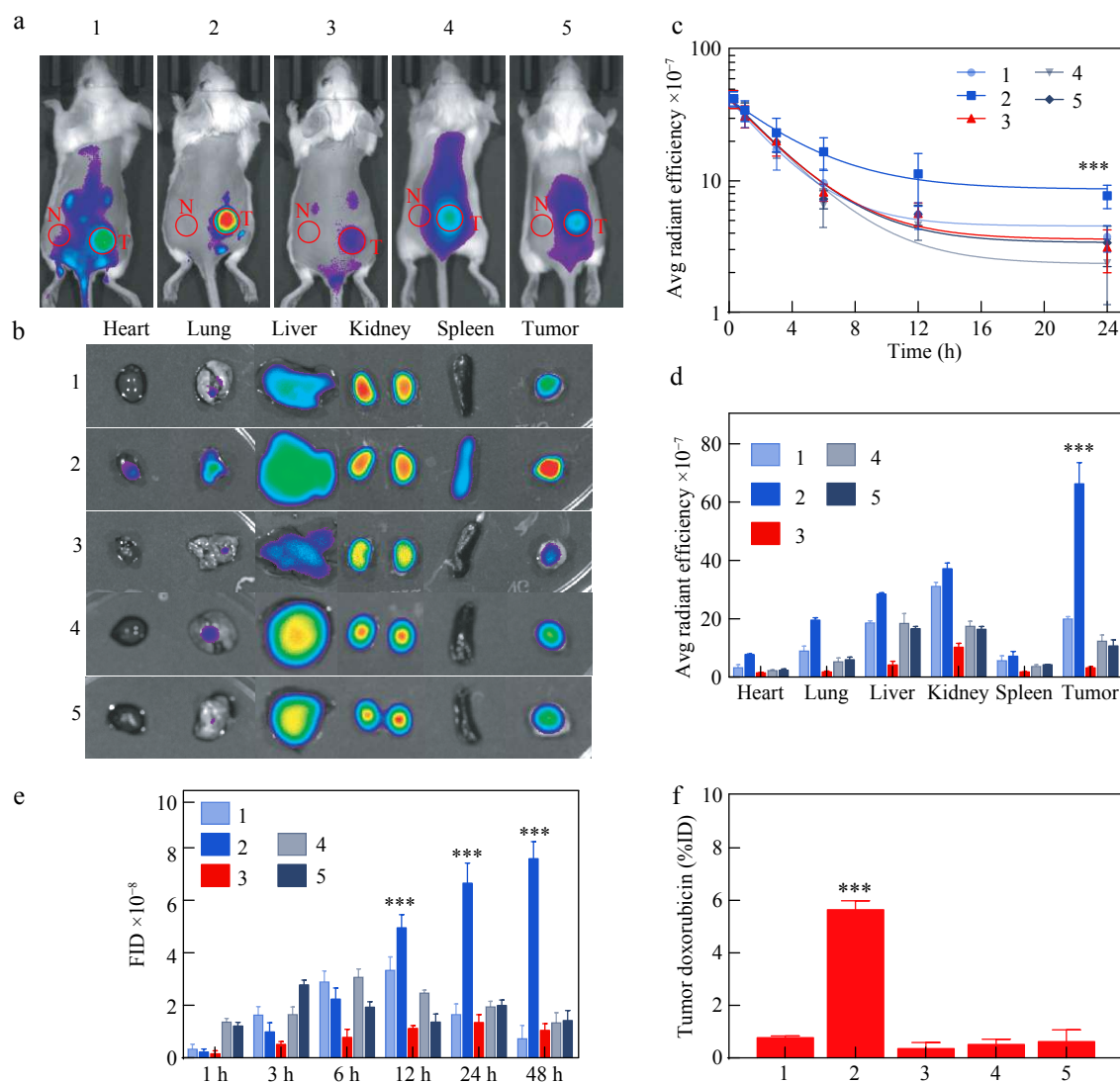


Fig. 6 (a) The whole-body NIRF images, (b) the *ex vivo* organ distribution analysis at 48 h, (c) the plasma fluorescent intensity versus time, (d) the quantitative analysis of the *ex vivo* fluorescent intensity in major organs and tumor at 48 h, (e) the fluorescent intensity difference (FID) between tumor region (T) and normal region (N) at different time points post injection and (f) the DOX content in tumor at 48 h for 4T1 tumor-bearing BALB/c mice treated with DOX conjugates of (1) C15-H70(B₃), (2) C15-H130(B₅), (3) C50-H70(B₃), (4) C20(B₅)-H₇₅ and (5) C20(B₁₀)-H₇₅

the low endocytic ratio and low cytotoxicity might be the major constraints for further application of as-prepared conjugates. Therefore, we did not carry out more in-depth exploration like *in vivo* anticancer capacity. Further studies will focus on the design of comb-like PHPMA conjugates with diverse functions and responsiveness, such as receptor-mediated endocytosis to enhance internalization, biodegradable spacers (*i.e.* GFLG peptide) introduction to improve polymer elimination efficiency, GSH/ROS responsive drug release or combination therapies to improve the drug efficacy, *etc.*

CONCLUSIONS

In this work, we demonstrated the synthesis of comb-like PHPMA *via* the combination of RAFT polymerization and Me₄Cyclam-Cu(I)Br mediated ATRP. The kinetic study

showed that the ATRP process remained controllable in the initial polymerization stage at low temperatures. Therefore, by controlling the reaction conditions and introducing fractional precipitation method, well-defined comb-like PHPMA copolymers with distinct polymer architectures, chemical compositions and low PDI were successfully prepared. The model drug DOX was bounded to the BHMAGG residues *via* a hydrazone bond so that pH-responsive drug release could be achieved. Small micelles and large multi-micelle aggregates were observed for all comb-like conjugates. The micelle size of as-prepared conjugates largely depended on the comb number, side chain length and location of DOX. Longer backbone or side chain length led to larger micelle size. No direct correlation was observed between the aggregate size and the polymer architectures or compositions. The *in vivo* studies revealed that the side chain other than backbone played more

important roles in prolonging blood circulation and increasing tumor accumulation. C15-H₁₃₀(B₅)-DOX conjugates with the longest side chain but shorter backbone exhibited the longest blood circulation and the highest tumor accumulation. However, low cellular uptake and the bypass of lysosomal endocytic pathway limited the acid-induced intracellular drug release, thus all comb-like conjugates exhibited lower cytotoxicity than that of free DOX against 4T1 cells. Therefore, further studies will focus on DDS with similar structures but higher cellular uptake level and more efficient intracellular drug release properties or on theranostics with brachytherapy or surgical navigation capabilities.

Electronic Supplementary Information

Electronic supplementary information (ESI), which includes polymer synthesis, cell culture, tumor model, additional data of ¹H-NMR spectra, ¹³C-NMR spectra, GPC elution curves, size distribution by intensity, drug release profiles, co-localization between conjugates and lysosomes, whole-body biodistribution images, and ATRP polymerization of HPMA under different conditions, is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-018-2159-y>.

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