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A comparative study of linear, Y-shaped and linear-dendritic methoxy poly(ethylene glycol)-*block*-polyamidoamine-*block*-poly(L-glutamic acid) block copolymers for doxorubicin delivery *in vitro* and *in vivo* ☆

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ABSTRACT

The linear, Y-shaped, and linear-dendritic block copolymers of methoxy poly(ethylene glycol)-*block*-polyamidoamine-*block*-poly(L-glutamic acid) (MPEG-*b*-PAMAM-*b*-PGA) with one, two, four, and eight PGA arms but similar MPEG/PGA weight ratios (*W*/*W*) (named as P1PA, P2PA, P4PA and P8PA, respectively) were synthesized and comparatively investigated for doxorubicin hydrochloride (DOX) delivery. All the obtained block copolymers were highly biocompatible and could efficiently load DOX into nanoparticles (NPs) through electrostatic interaction. The NPs formed by linear (P1PA) or Y-shaped (P2PA) block copolymers and DOX were spherically shaped with smaller sizes, while the NPs formed from linear-dendritic block copolymers (P4PA and P8PA) were irregular in shape and larger in size. The P1PA/DOX and P2PA/DOX NPs exhibited better DOX protection and slower DOX release profile. However, cell cytotoxicity assays indicated that all the DOX-loaded NPs exhibited similar cytotoxicities with free DOX, indicating effective DOX release after cellular uptake. The NPs from linear and Y-shaped block copolymers greatly extended the blood circulation time, and displayed more accumulation in tumor site and less accumulation in the liver and kidney compared with the linear-dendritic counterparts. In addition, the P1PA/DOX and P2PA/DOX NPs also exhibited higher anti-tumor efficacy and less toxicity than the other DOX formulations. All these results indicated that the linear and Y-shaped MPEG-*b*-PAMAM-*b*-PGA block copolymers displayed better DOX delivery ability in anti-tumor treatment than the linear-dendritic copolymers.

Statement of Significance

Polymeric NPs derived from block copolymers have emerged as effective vehicles for drug delivery. However, the majority of the researches in this field have involved simple linear block copolymers and there are very few comparative studies on the self-assembly, *in vitro*, and *in vivo* drug delivery by the block copolymers with similar composition but different architectures. In this study, a series of linear, Y-shaped, and linear-dendritic polypeptide-based block copolymers were prepared and thoroughly investigated for DOX delivery. These block polymers loaded DOX into NPs with different sizes and morphologies, and exhibited different anti-tumor capabilities both *in vitro* and *in vivo*. The results indicated that the architecture of the block copolymers played an important role in their drug delivery behaviors.

1. Introduction

To reduce premature drug release and degradation, and increase drug accumulation in tumor site, various drug delivery systems have been developed. Since firstly proposed by Ringsdorf's group in the early 1980s, polymeric nanoparticles (NPs) derived from block copolymers have attracted wide interest as delivery

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vehicles for anticancer drugs over the past several decades [1–3]. Polypeptide-based block copolymers showed great potential due to their excellent biocompatibility and biodegradability. Various polypeptide-based block copolymers have been developed and their self-assemble behaviors have been investigated [4–8]. In most occasions, the polypeptide-based block polymers for drug delivery are amphiphilic polymers consisting hydrophilic poly (ethylene glycol) (PEG) and hydrophobic polypeptide blocks. They could self-assemble into micelles with hydrophilic PEG shell to prolong the circulation time in the blood and hydrophobic core to serve as a reservoir to encapsulate hydrophobic drugs [9]. Ionic polypeptide-based block polymers with good water solubility were another important type of polypeptide-based block copolymers for drug delivery. They could self-assemble into core-shell nanostructures with oppositely charged components through electrostatic or chelate interactions between the ionic polypeptide block of the polymeric carrier and oppositely charged drugs [10]. These interactions could result in high drug loading efficiency and pH responsive drug release behavior [11].

The majority of the researches on block copolymers have involved simple linear block copolymers. Recent advances in synthetic methodologies have provided efficient routes to prepare block copolymers with more complex architectures, such as Y-shaped copolymers, star copolymers, brush copolymers, linear-dendritic block copolymers, *etc.* [12–16]. The introduction of extra branching points might affect the conformational entropy of the block copolymers, which further impart the assembled vectors with other unique properties in drug delivery [17–19]. Some previous studies have noticed that linear, Y-shaped, and linear-dendritic block copolymers self-assembled into NPs with different sizes and morphologies [20–22]. Up to now, there are still quite limited comparative studies on the self-assembly, *in vitro*, and *in vivo* delivery behaviors between the block copolymers with similar composition but different molecular architectures.

Herein, a series of MPEG-*b*-PAMAM-*b*-PGA block copolymers were prepared for DOX encapsulation due to their good water solubility and promising DOX delivery capabilities. The block copolymers were of similar composition but different macromolecular architectures. They loaded DOX into NPs through ionic interactions. The size, morphology, drug-loading property, drug release behavior, cellular uptake and intracellular drug release, cytotoxicity, pharmacokinetics, *in vivo* distribution and anti-tumor efficacy of these DOX-loaded NPs were thoroughly evaluated and compared.

2. Experimental section

2.1. Materials

Methoxy poly(ethylene glycol) (MPEG5000, M_n = 5000 Da) was purchased from Sigma-Aldrich Co., Ltd. (Shanghai, P. R. China). The amino-terminated methoxy poly(ethylene glycol) (MPEG5000-NH_2 or MPEG5000-*b*-PAMAM(G0)) and γ-benzyl-L-glutamate-N-carboxyanhydride (BLG-NCA) were synthesized with a similar method as described in our previous work [23]. Methyl acrylate and ethylenediamine were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, P. R. China) and purified by vacuum distillation before use. Doxorubicin hydrochloride (DOX) was purchased from Beijing Huafeng United Technology Co, Ltd. (Beijing, P. R. China). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and polyethylenimine (PEI, 25 kDa) were purchased from Sigma-Aldrich Co., Ltd. (Shanghai, P. R. China) and used as received. *N*,*N*′-Dimethylformamide (DMF) was firstly stored over calcium hydride (CaH_2) for a week and then further purified by vacuum distillation with CaH_2. Clear polystyrene tissue culture treated 96-well plates were Corning Costar cell culture plates from Sigma-Aldrich Co., Ltd. (Shanghai, P. R. China). Deionized water was prepared by the Milli-Q plus system (Millipore Co., Billerica, MA, USA). All other reagents and solvents were of analytical grade and used without further purification.

2.2. Synthesis of MPEG-*b*-PAMAM macroinitiators

The macroinitiator, MPEG5000-*b*-PAMAM(G0) (MPEG5000-NH_2) was firstly synthesized. Then it was used as the core to prepare MPEG5000-*b*-PAMAM(G1.0), MPEG5000-*b*-PAMAM(G2.0), and MPEG5000-*b*-PAMAM(G3.0) via repeated Michael addition and amidation at room temperature according to the similar method as described in our previous work [24,25]. MPEG5000-*b*-PAMAM(G0), MPEG5000-*b*-PAMAM(G1.0), MPEG5000-*b*-PAMAM (G2.0) and MPEG5000-*b*-PAMAM(G3.0) were named as P1, P2, P4 and P8 according to the number of terminal amines for short.

2.3. Synthesis of MPEG-*b*-PAMAM-*b*-PGA block copolymers

MPEG-*b*-PAMAM-*b*-PGA block copolymers were synthesized through the ring-opening polymerization (ROP) of BLG-NCA monomer with P1, P2, P4, and P8 as macroinitiators in dry DMF at 25 °C for 3 days, and then the benzyl groups were deprotected according to the literature procedure [26]. A typical example is shown here: for the synthesis of MPEG-*b*-PAMAM(G1.0)-*b*-PGA block copolymer, MPEG-*b*-PAMAM(G1.0) (3.14 g, 0.6 mmol) and BLG-NCA (10.1 g, 38.4 mmol) were dissolved in 70 mL of dry DMF in a flame-dry flask. The molar ratio of MPEG-*b*-PAMAM(G1.0) and BLG-NCA was 1/64. The polymerization was performed at 25 °C for 3 days. Then, the solution was precipitated into excess amount of cold diethyl ether for 3 times to give the block copolymer, methoxy poly(ethylene glycol)-*b*-poly(amidoamine(G1.0))-*b*-poly(γ-benzyl-L-glutamate), which was marked as MPEG-*b*-PAMAM (G1.0)-*b*-PBLG. With similar method, MPEG-*b*-PAMAM(G0)-*b*-PBLG, MPEG-*b*-PAMAM(G2.0)-*b*-PBLG and MPEG-*b*-PAMAM (G3.0)-*b*-PBLG block copolymers were also obtained. They were denoted as P1PB, P2PB, P4PB and P8PB according to the number of PBLG arms for short. Subsequently, MPEG-*b*-PAMAM(G1.0)-*b*-PBLG block copolymer was dissolved in dichloroacetic acid at 25 °C in a flask. After the addition of HBr/acetic acid (33 wt.%), the solution was slowly stirred at 30 °C for 1 h and then the final product was precipitated into excessive diethyl ether. After dried under vacuum, the precipitate was dialyzed against distilled water and freeze-dried, yielding a white solid. It was MPEG-*b*-PAMAM (G1.0)-*b*-PGA. MPEG-*b*-PAMAM(G0)-*b*-PGA, MPEG-*b*-PAMAM (G2.0)-*b*-PGA and MPEG-*b*-PAMAM(G3.0)-*b*-PGA were all obtained with similar method. These block copolymers were denoted as P1PA, P2PA, P4PA and P8PA according to the number of PGA arms for short.

2.4. Preparation of DOX-loaded NPs

The weighted lyophilized powders of P1PA, P2PA, P4PA, and P8PA were dissolved in deionized water. The pH value was adjusted to 7.4 with 0.1 M NaOH. After the addition of DOX (10 wt.% of the block copolymer), the solution was further stirred for 2 h. Free DOX and salt were removed by dialysis (molecular weight cutoff (MWCO) = 3500 Da) against deionized water for 24 h (The dialysis medium was changed five times), and the drug-loaded NPs were obtained by lyophilization in the dark. To determine the drug loading content (DLC) and drug loading efficiency (DLE), the lyophilized DOX-loaded NPs were dissolved in DMF and measured by ultraviolet–visible (UV–vis) spectrometer

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