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Mercaptan acids modified amphiphilic copolymers for efficient loading and release of doxorubicin

You-Mei Li, Xiu-Peng Chang, Yin-Jia Cheng, Shu Chen, Feng He*, Ren-Xi Zhuo

Key Laboratory of Biomedical Polymers of Ministry of Education, Department of Chemistry, Wuhan University, Wuhan 430072, China

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ABSTRACT

In this paper, four different kinds of mercaptan acids modified amphiphilic copolymers mPEG-*b*-PATMC-*g*-SRCOOH (R = —CH_2—, —CH_2CH_2—, —$(CH_2)_{10}$— and —$CH(COOH)CH_2$—) were successfully synthesized by thiol-ene "click" reaction between pendent carbon-carbon double bonds of PEG-*b*-PATMC and thiol groups of thioglycolic acid, 3-mercaptopropionic acid, 11-mercaptoundecanoic acid or 2-mercaptosuccinic acid. DLS and TEM measurements showed that all the mPEG-*b*-PATMC-*g*-SRCOOH copolymers could self-assemble to form micelles which dispersed in spherical shape with nano-size before and after DOX loading. The positively-charged DOX could effectively load into copolymer micelles *via* synergistic hydrophobic and electrostatic interactions. All DOX-loaded mPEG-*b*-PATMC-*g*-SRCOOH micelles displayed sustained drug release behavior without an initial burst which could be further adjusted by the conditions of ionic strength and pH. Especially in the case of mPEG-*b*-PATMC-*g*-$S(CH_2)_{10}COOH$ (P3) micelles, the suitable hydrophobility and charge density were not only beneficial to improve the DOX-loading efficiency, they were also good for obtaining smaller particle size, higher micelle stability and more timely drug delivery. Confocal laser scanning microscopy (CLSM) and MTT assays further demonstrated efficient cellular uptake of DOX delivered by mPEG-*b*-PATMC-*g*-SRCOOH micelles and potent cytotoxic activity against cancer cells.

1. Introduction

In recent decades, polymer micelles have gained significant attentions as anticancer drug delivery carriers and several types have already been used in clinical trials [1–3]. As drug delivery carriers, polymer micelles have many superiorities, such as unique core-shell structure, tunable shape and size, as well as improved drug solubilization and stabilization. In general, amphiphilic copolymers could self-assemble to form micelles in aqueous solution. The shell formed by hydrophilic moieties could protect the loaded drugs from the aqueous environment and uptake by mononuclear phagocytes, while polyethylene glycol (PEG) was considered to be one of the best choices due to its several interesting features, including enhanced permeability and retention (EPR) effect [4,5]. The hydrophobic micellar core could incorporate the hydrophobic drug molecules, while drug loading capacity was one of the key factors for micelles as drug delivery carriers [6,7]. For most micellar drug delivery systems in water, hydrophobic interactions are the major driving forces for molecular self-assembly and drug incorporation [7], but the drug contents generally cannot exceed 10% by using hydrophobic incorporation approach alone [8,9]. Many strategies have been carried to improve the drug loading capacity, such as adjustment of hydrophilic-hydrophobic balance, crosslinking and conjugation [10–12]. Our previous work and other studies have shown that improving the hydrophobility of micellar core could lead to higher drug loading capacity, such as hydrophobic side-chain chemical modification of hydrophobic micellar core [13,14].

Moreover, the introduction of non-covalent interactions in drug delivery systems is another powerful strategy. The non-covalent interactions between drugs and carriers, such as hydrogen bond interactions, π–π interactions and electrostatic interactions, could easily improve the drug loading capacity, thermodynamic and kinetic stability without complicated syntheses [7,15]. Nondirectional electrostatic interactions result from the attractions between oppositely charged groups, which also provide an effective approach for controlled drug-release due to their environmental sensitivity (such as pH and ionic strength) [7,16]. The increase in ionic strength or deviation from neutral pH could weaken the strength of electrostatic interactions, thus leading to micelle destabilization and drug release. As shown in our previous work

* Corresponding author at: Wuhan University, Luo-jia-shan, Wuchang, Wuhan, China.
E-mail address: hefeng@whu.edu.cn (F. He).

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[17], biodegradable carboxyl-modified amphiphilic copolymer mPEG-*b*-PATMC-*g*-SCH_2COOH was successfully synthesized and employed as a novel powerful controlled drug delivery system. The positively-charged DOX could be effectively loaded into the negatively-charged micelles with high drug loading capacity, while DOX release could be reached faster in an acidic environment owing to the weaker electrostatic interactions at a lower pH.

Not only that, it is well known that blood vessel wall and blood cells are negatively-charged [18]. Owing to the electrostatic repulsion interactions, the negatively-charged micelles could effectively block protein adsorption and thus resulting in good biocompatibility and high stability for long circulation time in blood [19,20]. However, the introduction of excess negatively-charged carboxyl groups would reduce hydrophobic interactions in the micellar hydrophobic core thus be disadvantageous to micelle self-assembly and drug incorporation, while the electrostatic repulsion interactions between negatively-charged micelles and cell membrane would further hinder the uptake of drug delivery micelles *in vivo* [19,21,22].

In this paper, biodegradable amphiphilic block copolymer based on methoxy poly(ethylene glycol)-*b*-poly(5-allyloxy-1,3-dioxan-2-one) (mPEG-*b*-PATMC) was synthesized according to our previous report using immobilized *porcine pancreas* lipase (IPPL) as the catalyst [23,24]. After thiol-ene "click" reactions, mercaptan acids were efficiently grafted onto the hydrophobic PATMC segments and four different kinds of pendent mercaptan acids modified copolymers mPEG-*b*-PATMC-*g*-SRCOOH (R = —CH_2—, —CH_2CH_2—, —$(CH_2)_{10}$— and —$CH(COOH)CH_2$—) were successfully synthesized. DOX, a widely-used amine-containing hydrophobic anticancer drug, was employed as the model drug and loaded into carboxyl-modified micelles *via* synergistic hydrophobic and electrostatic interactions. Base on the selective and suitable hydrophilic-hydrophobic balance and charge density, mPEG-*b*-PATMC-*g*-SRCOOH were proposed as powerful controlled drug delivery systems with good stability, high-efficiency drug loading, efficient cell uptake and controlled DOX release properties.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) (mPEG, M_n = 5000) was obtained from Acros. Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd (Zhejiang, P. R. China). Thioglycolic acid (Sinopharm Chemical Reagent Co., Ltd), 3-Mercaptopropionic acid (Aladdin), 11-Mercaptoundecanoic acid (J&K. Scientific Ltd) and 2-Mercaptosuccinic acid (TCI) were used as received. 2,2-dimethoxy-2-phenylacetophenone (DMPA) from Aladdin was used without further purification. Immobilized *porcine pancreas* lipase (IPPL) and ATMC were prepared according to He [25,26]. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's phosphate buffered saline (PBS), 3-Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Corp and used for cytotoxicity without further purification. HeLa cells were incubated in DMEM containing 10% FBS and 1% antibiotics (penicillin-streptomycin, 10,000 U/mL) at 37 °C and a humidified atmosphere containing 5% CO_2. Other reagents were of analytical grade and purified by general methods.

2.2. Synthesis of *mPEG*-b-*PATMC*-g-*SRCOOH*

2.2.1. Synthesis of *mPEG*-b-*PATMC*

mPEG-*b*-PATMC diblock copolymer was prepared in bulk *via* enzymatic ring-opening polymerization using mPEG as macroinitiator and IPPL as catalyst [13,17]. mPEG and ATMC (EG: ATMC molar feed ratio of 5:1) were mixed in a vessel containing IPPL (0.3 wt% of ATMC) and a magnetic stirring bar. After dried *in vacuo* with anhydrous phosphorus pentoxide at room temperature for 24 h, the vessel was sealed under *vacuo* and immersed into an oil bath at 140 °C for 24 h. The resulting copolymer was dissolved in CH_2Cl_2 and filtered to remove the insoluble IPPL. Then the crude product was condensed and dissolved in THF, and dialyzed against distilled water (MWCO 14,000) for 48 h at room temperature. The distilled water was refreshed every 4 h. Finally, the block copolymer mPEG-*b*-PATMC was obtained by lyophilization (Yield: 72%).

2.2.2. Synthesis of *mPEG*-b-*PATMC*-g-*SRCOOH*

mPEG-*b*-PATMC-*g*-SRCOOH (R = —CH_2—, —CH_2CH_2—, —$(CH_2)_{10}$— and —$CH(COOH)CH_2$—) were synthesized by the thiol-ene "click" reaction [27–30]. In detail, mPEG-*b*-PATMC (1 molar equivalent of allyl groups), DMPA (0.05 molar equivalent) and 5 molar equivalent of mercaptan acids (thioglycolic acid, 3-Mercaptopropionic acid, 11-Mercaptoundecanoic acid or 2-Mercaptosuccinic acid) were mixed in a vial containing anhydrous tetrahydrofuran. The vials were degassed with N_2 for 20 min and then irradiated with full spectrum UV-vis light (125WE27) for 2 h at room temperature. After filtration, the solutions were condensed and precipitated into diethyl ether twice. The obtained mPEG-*b*-PATMC-*g*-SCH_2COOH, mPEG-*b*-PATMC-*g*-SCH_2CH_2COOH, mPEG-*b*-PATMC-*g*-$S(CH_2)_{10}COOH$ and mPEG-*b*-PATMC-*g*-$SCH(COOH)CH_2COOH$ were dried and kept for future use (Yield: 75%, 74%, 78% and 72%, respectively).

2.3. Measurement

^1H NMR spectra were recorded by Mercury VX-300 spectrometer using DMSO-d_6 as the solvent and tetra-methylsilane (TMS) as an internal reference. The molecular weights and distributions of block copolymers were determined by a gel permeation chromatography (GPC) system comprising a model 2690D separation module and a 2410 refractive index detector. DMF with a flow rate of 0.3 mL/min was used as the eluent. 20 microliters of 1.0% (w/v) sample solutions were injected for each analysis. Waters Millennium module software was calibrated by narrow molecular weight distribution poly(methyl methacrylate) standards and used to calculate molecular weights. Fluorescence spectra were recorded using a RF-5301 PC (Shimadzu) spectrofluorometer (slit widths: 5 nm). The morphology and particle size of the micelles (negatively stained with phosphotungstic acid) were observed by transmission electron microscope (TEM, JEM-2100 HR). Samples were prepared by placing a drop of micellar solution (0.1 mg/mL) onto the copper grid with Formvar film and dried at room temperature. The average hydrodynamic diameters, size distributions and zeta potentials of copolymer micelles (1.0 mg/mL) were measured by dynamic light scattering (DLS, Nano-ZS 3600, Malvern Instruments, UK). The samples were passed through a 0.45 μm pore-sized syringe filter prior to measurements. The micelle stability was also analyzed by DLS using P3 micelle solution in PBS and 100% FBS at 1 mg/mL concentration. The influence of ionic strength on the electrostatic interactions was determined by Fluorescence spectra. An average value was determined by three repeated measurements at 25 °C for each sample.

2.4. Hemolysis test

Hemolysis test was performed in fresh blood anticoagulated with sodium citrate solution (2% final concentration of red blood cells, RBCs). 0.9% Saline water and distilled water were used as the negative control and the positive control. 1.0 mL of saline water, distilled water and micelle solutions with different concentrations