Disulfide Cross-Linked Amphiphilic Copolymers Loading Doxorubicin for Controlled Drug Delivery

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ABSTRACT

An amphiphilic copolymer, PEG-b-PLA-b-(PAA-co-PNIPAM), was synthesized via atom transfer radical polymerization of tert-butyl acrylate and N-isopropyl acrylamide using PEG-b-PLA-Br as a macronitiator and CuBr/Me6TREN as a catalytic system, followed by selectively hydrolyzing tert-butyl groups to carboxyl groups. Then, doxorubicin (DOX) was loaded to the hydrophobic core, and the PAA segments were cross-linked by cystamine to get disulfide cross-linked and DOX loaded micelles. The transmission electron microscopy images showed that the cross-linked DOX loaded micelles were spherical nanoparticles, with a mean diameter of 100 nm. The drug release behavior of the cross-linked DOX loaded micelles was redox responsive. The cumulative release amount of DOX was 33.1% with the presence of 10 mM of glutathione (GSH) at 37 °C, much higher than that without the presence of GSH, which was only 4.3%. Furthermore, the cumulative release amount of DOX was 50.9% at 25 °C, higher than that at 37 °C, indicating that the cross-linked DOX loaded micelles were also thermo-sensitive.

KEYWORDS: Amphiphilic Copolymer, Disulfide Cross-Linked, DOX, Controlled Drug Delivery.

1. INTRODUCTION

Amphiphilic copolymers are ideal drug carriers to construct drug delivery systems. They could self-assemble to form micelles with core/shell structure in aqueous solution.1–3 Thus, hydrophobic drugs could be entrapped into the hydrophobic core via hydrophobic interaction. However, many factors, such as dilution, pH,4,5 temperature,6,7 and the presence of numerous charged blood components,8,9 may affect the stability of the micelles, leading to burst release of drug before reaching the targeted lesion site, which would not only decrease the therapeutic efficacy, but also induce side effects.10,11 Recently, cross-linked copolymers have been developed to improve the stability of the micelles. For example, Kissel et al. have reported that core cross-linked biodegradable micelles by photopolymerization.12 McCormick et al. have prepared cross-linked stimuli-responsive micelles.13 Liu et al. have utilized ABC tri-block copolymer to prepare core cross-linked and pH responsive micelles.14 All these micelles showed more stability than the corresponding non-cross-linked micelles. However, the cross-linked network hindered the release of loaded drugs, which would decrease the therapeutic efficacy.15–17 It is highly demanded to prepare environment responsive micelles, which are not only stable in blood circulation, but also active in lesion sites to release the loaded drug.

A disulfide bond is stable in the extracellular milieu, and will be broken by glutathione (GSH), which concentration in tumor cells (2–8 mM) is much higher than in blood plasma (1–2 μM).18–20 This unique characteristic makes disulfide bond to be an ideal drug release switcher, which would be stable in normal cells and blood plasma, and be broken in tumor tissues.21–24 Li et al. have synthesized thiolated linear-dendritic polymers, and then oxidized thiol groups to disulfide bonds, to obtain cross-linked micelles. The release rate of paclitaxel (PTX) from the cross-linked micelles was significantly slower than that from non-cross-linked micelles, but could be gradually facilitated by increasing the concentration of reducing agent.25

Herein, we synthesized a well-defined amphiphilic copolymer, PEG-b-PLA-b-(PBA-co-PNIPAM), by atom transfer radical polymerization26–28 of tert-butyl acrylate and N-isopropyl acrylamide using PEG-b-PLA-Br29,30 as a...
macrominiator and CuBr/Me₆TREN as a catalytic system. Then, the tert-butyl groups were hydrolyzed to carboxyl groups. The hydrolysis product, PEG-b-PLA-b-(PAA-co-PNIPAM), was loaded with doxorubicin (DOX), and PAA segments were cross-linked by cystamine to get disulfide cross-linked and DOX loaded micelles (Fig. 1). The in vitro drug release experiments were conducted to investigate the drug release behaviors of the disulfide cross-linked micelles.

2. EXPERIMENTAL DETAILS

2.1. Materials

Doxorubicin hydrochloride (DOX·HCl, >98.0%) was purchased from Beijing Huafeng United Technology Co., Ltd. DL-Lactide was purchased from TCI Co., Ltd. Methoxypolyethylene glycol (MePEG, Mₑ = 5000 g/mol, 99%), 2-bromoisobutyl bromide (99%), and copper (I) bromide (CuBr, >99%) were purchased from Sigma–Aldrich. N-Isopropyl acrylamide (NIPAM, 99%), tert-butyl acrylate (tBA, 98.5%), trifluoroacetic acid (TFA, 99%), cysteamine hydrochloride (98%), and tris(2-aminoethyl) amine (TREN, 95%) were purchased from J&K CHEMICAL. 4-Dimethylamino pyridine (DMAP, 98%), stannous octanoate (SnOct₂) and 1-ethyl-3-(3dimethylaminopropyl)carbodiimide (EDC, 98%) were purchased from Shanghai Chemical Reagent Co., Ltd. 4-Dimethylamino pyridine (DMAP, 98%), stannous octanoate (SnOct₂) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 98%) were purchased from Aladdin Reagent Inc. Triethylamine (TEA, 99%), formaldehyde (37–40%), formic acid (>88%), N,N-dimethyl formamide (DMF, 99%), dimethyl sulfoxide (DMSO, 99%), and dichloromethane were purchased from Shanghai Chemical Reagent Co., Ltd. Triamine was synthesized according to Ref. [31]. All the chemicals were used without further treatment.

2.2. Synthesis of PEG-b-PLA-Br

0.5 g of MePEG, 25 mg of DL-lactide, and a toluene solution containing 1.3 mg of SnOct₂ were added into a Schlenk flask. Then, the flask was sealed and heated at 160 °C for 2 h. After reaction, the mixture was cooled to room temperature and dissolved into dichloromethane. Then, the solution was precipitated into diethyl ether. The resultant white powder, PEG-b-PLA-Br, was dried in vacuo overnight.

4.0 g of PEG-b-PLA (0.435 mmol) was dissolved into 0.053 g of DMAP (0.435 mmol), the solution was degassed and charged with nitrogen. Then, 15 mL of dichloromethane and 0.12 mL of TEA (0.87 mmol) were added. The mixture was stirred under ice-water bath, followed by adding 0.11 mL of 2-bromoisobutyl bromide (0.87 mmol). The reaction was conducted under ice-water bath for 1 h, and then at room temperature for 24 h. After reaction, the mixture was diluted with 80 mL of dichloromethane, and consecutively washed with saturated sodium hydrogen carbonate and saturated sodium chloride aqueous solutions for three times. Then, the mixture was treated with anhydrous magnesium sulfate overnight, and precipitated into diethyl ether. The resultant white powder, PEG-b-PLA-Br, was dried in vacuo overnight.

2.3. Synthesis of PEG-b-PLA-b-(PAA-co-PNIPAM)

PEG-b-PLA-b-(PAA-co-PNIPAM) was obtained by ATRP of tBA and NIPAM, using PEG-b-PLA-Br as a macroiniator and CuBr/Me₆TREN as a catalytic system. Typically, a dry 10 mL of Schlenk flask (flame-dried three times under vacuum prior to use) was charged with PEG-b-PLA-Br (0.5 g, 0.044 mmol) and CuBr (7.8 mg, 0.054 mmol). Then, NIPAM and tBA at a certain ratio, DMF/H₂O (4 mL, v/v = 7:1), Me₆TREN (12.5 mg, 0.054 mmol) were added into the flask. The mixture was degassed with three freeze-evacuate-thaw cycles and kept at 20 °C for 13.5 h. After reaction, the mixture was diluted with CH₂Cl₂ and precipitated into diethyl ether. The resultant white solid, PEG-b-PLA-b-(PAA-co-PNIPAM) was obtained by drying in vacuo overnight.

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2.4. Drug Loading and Cross-Linking

Doxorubicin hydrochloride (DOX·HCl, >98.0%) was purchased from Beijing Huafeng United Technology Co., Ltd. DL-Lactide was purchased from TCI Co., Ltd. Methoxypolyethylene glycol (MePEG, Mₑ = 5000 g/mol, 99%), 2-bromoisobutyl bromide (99%), and copper (I) bromide (CuBr, >99%) were purchased from Sigma–Aldrich. N-Isopropyl acrylamide (NIPAM, 99%), tert-butyl acrylate (tBA, 98.5%), trifluoroacetic acid (TFA, 99%), cysteamine hydrochloride (98%), and tris(2-aminoethyl) amine (TREN, 95%) were purchased from J&K CHEMICAL. 4-Dimethylamino pyridine (DMAP, 98%), stannous octanoate (SnOct₂) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 98%) were purchased from Shanghai Chemical Reagent Co., Ltd. Triamine was synthesized according to Ref. [31]. All the chemicals were used without further treatment.
The micelles obtained above were transferred into a 50 mL of flask, and then cystamine (1.69 mg), EDC (2.8 mg, 0.018 mmol), NHS (2.55 mg, 0.022 mmol) were added. After stirred at room temperature for 24 h, the solution was transferred into a dialysis bag with MWCO of 3500. The bag was immersed in deionized water for 48 h with constantly shaking. After freeze-drying, DOX loaded and disulfide cross-linked micelles were obtained.

2.5. In Vitro Drug Release

The drug release behavior of DOX loaded micelles was studied. Typically, DOX loaded micelles were dissolved in deionized water, and then the solution was transferred into a dialysis bag with MWCO of 3500. The bag was immersed in 30 mL of pH 7.4 PBS at 37 °C with constantly shaking. GSH (10 mM) was added to the test group, while the control group didn’t add GSH. Periodically, 3 mL of external buffer solution was removed and replaced with equal volume of fresh medium. The released amount of DOX was quantified by measuring the UV absorbance at 480 nm. The release ratio of DOX was determined by the flowing equation:

Release ratio of DOX (%) = \( \frac{I_{\text{sample}}}{I_{\text{control}}} \times 100 \)

Where \( I_{\text{sample}} \) and \( I_{\text{control}} \) represent the absorbance intensity at 480 nm with or without the presence of GSH, respectively.

2.6. Characterization

\(^1\)H NMR spectra were recorded on a Bruker Avance 500 spectrometer using DMSO and CDCl\(_3\) as solvents, trimethylsilane (TMS) was used as internal standard. Fourier transformed infrared (FT-IR) measurements were performed on a Nicolet 6700 spectrometer. The molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). The GPC was performed in THF at 35 °C with an elution rate of 1.0 mL/min on Agilent 1100 equipped with a G1310A pump, a G1362A refractive index detector, and a G1314A variable wavelength detector. Polystyrene standard samples were employed for the GPC calibration. Transmission electron microscopy (TEM) images were obtained on a JEOL JEM 2100 F transmission electron microscope, and samples for TEM measurements were prepared by casting one drop of sample water solution on carbon copper grids. The size distribution of the micelles and zeta potential were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS90 (Malvern Instruments, U.K.). UV-vis absorbance spectra were measured with a Perkin–Elmer Lambda 35 spectrophotometer, and the wavelength was 480 nm.

3. RESULTS AND DISCUSSION

The synthetic route of PEG-b-PLA-b-(PAA-co-PNIPAM) was showed in Scheme 1. During the polymerization reaction, a small quantity of solution was extracted by a gastight syringe under nitrogen protecting when the reaction reached the setting time of 2 h, 4 h, 6 h and 13.5 h, and then the solution was precipitated in ether. After dried, the solid product was characterized by \(^1\)H-NMR and GPC. The conversion fractions of NIPAM and tBA were determined by the signal integration ratio of peaks at 4.01 ppm (tertiary protons of isopropyl at NIPAM) and 1.38 ppm (tert-butyl protons at tBA), respectively, relative to the peak at 3.50 ppm (methyl protons at the terminal of PEG). The results were listed in Table I. Calculating by \(^1\)H-NMR, after reaction for 13.5 h, the copolymer could be denoted as PEG\(_{114}\)-b-PLA\(_{57}\)-b-(P\(_{89}\)tBA-cop NIPAM\(_{58}\)). The GPC data of different reaction time was also listed Table I. Both the monomer conversion percent and \(M_n\) of resultant copolymers increased with the increase of reaction time. The activity of tBA is much higher than that of NIPAM, more than 80% of the tBA was reacted in less than 2 h, and even after 13.5 h, the conversion percent of NIPAM was lower than 60%. Furthermore, the distribution of molecular weight kept narrow throughout the process of polymerization.

The FT-IR spectra of PEG-b-PLA-Br, PEG-b-PLA-b-(PrBA-co-PNIPAM), and PEG-b-PLA-b-(PAA-co-PNIPAM) were shown in Figure 2. It could be seen that the FT-IR spectra of PEG-b-PLA-b-(PrBA-co-PNIPAM) (Fig. 2(b)) contained all the absorption peaks of
**Table I.** ATRP reaction of tBA and NIPAM using PEG-**b**-PLA-Br as the macroinitiator in solution of DMF:H₂O = 7:1.α

<table>
<thead>
<tr>
<th>Time/h</th>
<th>Conv. % (tBA)</th>
<th>Conv. % (NIPAM)</th>
<th>Mₙ, NMR</th>
<th>Mₙ, GPC</th>
<th>PDI</th>
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<td>89</td>
<td>58</td>
<td>27200</td>
<td>29900</td>
<td>1.40</td>
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</table>

Notes: αPolymerization reaction was conducted with the molar ratio of [tBA]₀/[NIPAM]₀/[PEG-**b**-PLA-Br]₀/[CuBr]₀/[ME6TREN]₀ = 100:100:1:1:1 under nitrogen atmosphere at 20 °C.

PEG-**b**-PLA-Br (Fig. 2(a)). Furthermore, two new peaks appeared at 2977 cm⁻¹ and 1151 cm⁻¹, indicating the presence of PrBA, two new peaks appeared at 1651 cm⁻¹ and 1543 cm⁻¹ ascribed to the absorption of amide bond, and the characteristic C=O stretching vibration at 1726 cm⁻¹ was different from the peak at 1758 cm⁻¹ of PLA chains. After hydrolysis, the intensity of peaks at 2977 cm⁻¹ and 1151 cm⁻¹, assigned to the vibrations of C=H in tert-butyl group, remarkably decreased, suggested the successfully hydrolysis of tert-butyl groups to carboxyl groups.

The synthetic process was also confirmed by GPC and ¹H-NMR characterizations. As seen in Figure 3 of the GPC curves, the Mₙ of PEG-**b**-PLA-**b**-(PrBA-co-PNIPAM) was 19.9 KDa (PDI = 1.23), showed a remarkably increase compared with that of PEG-**b**-PLA-Br, which was 11.4 KDa (PDI = 1.23). After hydrolysis, due to the elimination of tert-butyl groups, the Mₙ of PEG-**b**-PLA-**b**-(PAA-co-PNIPAM) decreased to 13.0 KDa (PDI = 1.37). In Figure 4 of the ¹H-NMR spectra, after polymerization, besides the characteristic signals of PEG (peaks at 3.24 ppm and 3.50 ppm) and PLA (peaks at 5.2 ppm and 1.45 ppm), the characteristic peaks of NIPAM and tert-butyl group were appeared at 1.03 ppm and 1.38 ppm (Fig. 4(b)), respectively. After hydrolysis, the peak at 1.38 ppm was completely disappeared, and a new peak appeared at 12.1 ppm, assigned to the proton of the carboxyl group, which suggested that the successfully hydrolyzed tert-butyl groups to carboxyl groups.

Amphiphilic copolymers could form micelles with hydrophobic core and hydrophilic shell in aqueous solution.32,33 Thus, hydrophobic drug could be entrapped into the core by hydrophobic interaction.34–36 As it could be seen from Figure 5(a), PEG-**b**-PLA-**b**-(PAA-co-PNIPAM) could form spherical micelles with a mean diameter about 103 nm. After loading DOX, the diameter of the micelles increase to 201 nm, which was decreased to 174 nm after crosslinking. This was also confirmed by
the TEM (Figs. 5(b) and (c)). The drug loading content of PEG-b-PLA-b-(PAA-co-PNIPAM) was determined by UV-vis. Typically, 2.4 mg of DOX loaded micelles was dissolved in DMF, and then was degraded by GSH. By measuring the UV absorption at 480 nm, the DOX content was calculated as 36.7% against the standard curve.

Considering biologically relevant microenvironment, the in vitro drug release profile from cross-linked DOX loaded micelles were evaluated under simulated physiological condition. As seen from Figure 6, in the sample group (with adding 10 mM GSH), the drug released rate was high in the first 5 h, and the cumulative release amount of DOX was 33.1% at pH = 7.4 within 40 h. Whereas, in the control group (without adding GSH), the cumulative release amount of DOX was pretty low and only 4.3% until 172 h. This was because that the DOX was entrapped into the core of the micelles, and difficult to escape from the cross-linked network through diffusion, while in the sample group, GSH could effectively break the disulfide bonds, which would destroy the crosslinked network and release DOX.

On the other hand, the presence of PNIPAM in the structure of copolymer affording thermal-responsibility to the cross-linked DOX loaded micelles.37–38 Duan et al. have found that the LCST of PNIPAM with 70 repeated units was 29.3 °C, and the shorter the segment, the lower the LCST.39–41 The degree of polymerization (DP) of PNIPAM in copolymer was 58, determined by 1H-NMR. The PNIPAM segment was in hydrophobic state at 37 °C, as a result, it would be in the boundary of the core, to protect DOX from releasing. To ascertain this hypothesis, the drug release profile was studied at 25 °C. Without adding GSH, the cumulative release amount of DOX was only 5.9% at 25 °C and pH = 7.4 within 18 h. However, that was 50.9% in the situation of adding 10 mM GSH. All these results indicated that the release behavior of DOX from the cross-linked DOX loaded micelles was controllable.

4. SUMMARY
In summary, a well-defined copolymer, PEG-b-PLA-b-(PBA-co-PNIPAM), was synthesized by ATRP, and the hydrolysis product, PEG-b-PLA-b-(PAA-co-PNIPAM), was utilized to load DOX and cross-linked by disulfide bonds. The loading content of DOX could reach 36.68%. The chemical structures of the copolymer were verified by 1H-NMR, GPC, and FT-IR. The TEM and DLS results indicated that the copolymer loaded DOX could self-assemble into spherical micelles in aqueous solution. In vitro drug release experiment showed that the micelles would be stable in the situation of without reduction. Adding GSH could effectively break the disulfide bonds, destroy the cross-linked network, and release DOX. This copolymer showed great potential in the applications of controlled drug delivery.

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References and Notes