Construction of polymer–paclitaxel conjugate linked via a disulfide bond

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**A R T I C L E   I N F O**

Article history:
Received 5 July 2015
Received in revised form 26 August 2015
Accepted 7 September 2015
Available online 10 September 2015

Keywords:
Reduction responsive
Drug delivery system
PTX
Polymer–drug conjugate

**A B S T R A C T**

Covalently linked amphiphilic polymer–paclitaxel (PTX) could self-assemble into micelles to overcome many drawbacks of existing delivery systems of PTX by virtue of tunable compositions, variable sizes, high drug loading content and zero burst release. Moreover, a reduction-responsive system based on glutathione (GSH) can be established by introducing disulfide bonds into the polymeric carriers to improve the selectivity for cancer cells. Herein, we reported a disulfide linked polymer-PTX, P(PEGMEA)-co-P(PDPHEMA)-g-PTX with a high PTX loading content of 43.7 wt% in vitro. In vitro cell assay showed that the polymer carrier has almost no cytotoxicity. The half maximal inhibitory concentration (IC50) values of the polymer-PTX conjugate against HEK-293 cells was about 10 times higher than that of HeLa cells after incubation for 72 h. Such a dramatic selectivity for cancer and normal cells provides a promising strategy to improve the therapeutic efficacy and decrease the side effects of PTX in chemotherapy.

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1. Introduction

Over the past decades, immense efforts have been devoted to improve the therapeutic efficacy of anti-cancer drugs [1]. As a potent anti-cancer drug, paclitaxel (PTX) has attracted much attention owing to its characteristic diterpenoid structure, resulting in the ability to stabilize microtubules from tubulin dimers and prevent depolymerization [2,3]. However, due to the low water-solubility of PTX, adjuvant Cremophor EL® was inevitably needed in the intravenous administration formation, which would induce some serious side effects, such as hypersensitivity reaction and neurological toxicity [4–6]. Thus, a great deal of alternative carriers, such as liposomes [7], microspheres [8], nanoparticles [9], and polymer conjugates [10], have been used to improve the water-solubility and avoid the side effects of PTX [11]. Till now, many kinds of functional carriers have been developed to meet the requirements and afford new functions to the PTX delivery system, such as biocompatibility [12], biodegradability [13], targeting effect [14], and admirable drug loading capacity [15]. Because of the flexible synthetic technology and feasible multi-functionality, functional polymers exhibit huge potential for application as drug carriers [16,17]. Polymers mainly loaded drugs via non-covalent or covalent strategies. Although non-covalently loading strategy possesses the advantage of facile handling, it suffers from burst release of the loaded drug. Thus, proper covalently loading strategy was highly required to resolve this problem [18,19].

To covalently load PTX, it was required to carefully design the linking form of the polymer–drug conjugate, to ensure that the loaded drug could be released [20,21]. Given that the concentration of glutathione (GSH) in tumor cells is 7–10 folds higher than that in normal cells in spite of the extra- and intra-cellular redox difference and the concentration of glutathione (GSH) in the tumor tissue is at least 4-fold higher than that in normal tissue [22,23], the noteworthy gradient concentration of GSH provides an attractive opportunity to design a reduction-responsive drug delivery system [24]. In recent years, many drug delivery systems were based on reduction-responsive polymers by introducing GSH-responsive disulfide bonds into the backbones or side groups of the polymeric carriers [25–27]. Nevertheless, most reduction-responsive drug carriers contain only one single disulfide bond at the polymer backbone or constructed by disulfide cross-linking the shell of the drug with non-covalently encapsulated polymer micelles. Obviously, these methods had a limited drug loading amount or insufficient stability [28,29]. For instance, Li et al. and Shao et al. [46] synthesized reversible disulfide cross-linked PTX loaded polymer micelles, which have a drug loading lower than 40% and possible burst release due to the unstable non-covalent encapsulation. In addition, most researches previously have not shown the obvious selectivity of PTX loaded polymer micelles between normal and cancer cells [27].

Thus, a rational design of polymer–PTX conjugate based on disulfide linker with high PTX loading amount, low cytotoxicity and high selective reduction-responsibility remains a challenging work.

Herein, we constructed a novel polymer–PTX conjugate based on disulfide linker with a high PTX loading amount of 43.7 wt.% and excellent anti-cancer effect. Firstly, we synthesized a monomer containing disulfide bond. Then the monomer was copolymerized with PEGMEA to give P(PEGMEA)-co-P(PDPHEMA). After thiol–disulfide reaction, the copolymer was functionalized with carboxyl groups, which could react with PTX via esterification reaction (Scheme 1). This conjugate was
characterized by $^1$H NMR spectra, GPC, and other methods. The amphiphilic copolymer could self-assemble into micelles, whose size and morphology were studied by DLS and TEM. In vitro cell assay was further performed to evaluate the cytotoxicity of the drug carrier and the highly discriminating intracellular anticancer drug release of polymer–drug conjugate in HEK-293 cells and HeLa cells.

2. Experimental section

2.1. Chemicals

PTX was purchased from Beijing Huafeng United Technology Co., Ltd. Poly(ethylene glycol) methyl ether methacrylate (PEGMEA, $M_n$ = 480, 99%, Aldrich) was passed through a neutral alumina column to remove inhibitors. 2-Hydroxyethyl methacrylate (HEMA, 98%), 2,2′-dipyridyl disulfide (PDS, 98%), N,N′-dicyclohexylcarbodiimide (DCC, 98%), 3-mercaptopropionic acid (98%), dimethylaminopyridine (DMAP, 99%) and N,N′-diisopropylcarbodiimide (DIC, 98%) were purchased from Aldrich. 2,2′-Azobisisobutyronitrile (AIBN) was recrystallized twice from ethanol. Dichloromethane (DCM) was dried over calcium hydride and then purified by distillation. Toluene was dried by sodium before use. A dialysis bag with a molecular weight cutoff of 3.5 kDa was purchased from Shanghai Green Bird Science & Technology Development Co., Ltd. Cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. 3-(Pyridin-2-yldisulfanyl)propanoic acid (PDP) was prepared according to previous literature [30,31]. All the other chemicals were used without further treatment.

2.2. Preparation of monomer 1 PDPHEMA

2.02 g of PDP (9.4 mmol), 1.13 mL of HEMA (9.4 mmol), and 0.30 g of DMAP (2.4 mmol) were added into 50 mL of dichloromethane. Subsequently, the solution was mixed with 2.12 mL of DIC (14.2 mmol) and stirred at room temperature for 4 h. After reaction, the solvent was removed under reduced pressure and purified by column chromatography on silica gel with hexane/ethyl acetate (4:1, v/v) as eluent, to obtain the desired product, PDPHEMA (2.2 g, yield 72%).

2.3. Preparation of 2 P(PEGMEA)-co-P(PDPHEMA)

P(PEGMEA)-co-P(PDPHEMA) was prepared by free radical polymerization. Typically, 0.2 g of PDPHEMA (0.612 mmol), 0.273 mL of PEGMEA (0.614 mmol), and 9.9 mg of AIBN (0.0604 mmol) were added into 3 mL of toluene. The mixture was bubbled with nitrogen for 30 min. Subsequently, the mixture was kept at 60 °C for 4 h under nitrogen atmosphere. P(PEGMEA)-co-P(PDPHEMA) (0.27 g, yield 54%) was obtained by precipitation into cold diethyl ether three times and dried in vacuum at room temperature overnight.

2.4. Preparation of 4 P(PEGMEA)-co-P(PDPHEMA)-g-PTX

0.24 g of P(PEGMEA)-co-P(PDPHEMA) (containing 0.297 mmol of dithio group) was dissolved in 10 mL of methanol, and then the solution was deoxygenized with nitrogen for 30 min. After dropwise adding 78.8 mg of 3-mercaptopropionic acid (0.743 mmol) dissolved in 4 mL of methanol, the mixture was stirred at room temperature for 24 h. P(PEGMEA)-co-P(PDPHEMA)-g-COOH (0.21 g, yield 88%) was obtained by precipitation into diethyl ether four times and dried in vacuum at room temperature overnight.

50 mg of P(PEGMEA)-co-P(PDPHEMA)-g-COOH (containing 62 μmol of carboxyl group), 53 mg of PTX (62 μmol), 3 mg of DMAP (15.6 μmol) and 5 mL of DCM was added to a pre-dried round-bottom flask with a magnetic stirring bar, followed by adding 14 mg of DCC (67.9 μmol). After stirring at room temperature for 24 h, the mixture was filtrated to remove the undissolved substance. The desired product, P(PEGMEA)-co-P(PDPHEMA)-g-PTX (80 mg, yield 78%), was obtained by precipitation into diethyl ether twice and dried in vacuum at room temperature overnight.

Scheme 1. The synthesis procedure of polymer–drug conjugates of P(PEGMEA)-co-P(PDPHEMA)-g-PTX.
2.5. Preparation of polymer–drug conjugate micelles

Briefly, 15 mg of P(PEGMEA)-co-P(PDPHEMA)-g-PTX was dissolved in 3 mL of THF, and then the solution was added dropwise into 3 mL of deionized water under stirring. Subsequently, the solution was exposed to air at room temperature for 40 h to volatilize the THF.

2.6. In vitro cell assay

The cytotoxicity of P(PEGMEA)-co-P(PDPHEMA)-g-PTX against HeLa cells (human cervical cancer cells) and HEK-293 cells (human embryo kidney cells) were evaluated in vitro by CCK-8 assay [30,31]. The HeLa cells (human cervical cancer cells) and HEK-293 cells (human embryonic kidney cells) were seeded in 96-well plates at a density of 5 × 10³ cells/well in 100 μL of DMEM (high glucose) culture medium and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h. Thereafter, in order to investigate the toxicity of the polymer carrier to HEK-293 cells and of the polymer–drug conjugate to HeLa cells and HEK-293 cells, the medium was replaced with fresh one containing the indicated concentration of P(PEGMEA)-co-P(PDPHEMA)-g-COOH or P(PEGMEA)-co-P(PDPHEMA)-g-PTX. The cells were further incubated for 48 h and 72 h. After incubation, the medium was replaced by 100 μL of fresh medium containing 10 μL of CCK-8. The cells were incubated for another 1 h (for HeLa cells) or 2 h (for HEK-293 cells) at 37 °C in dark. Then, the absorbance at a wavelength of 450 nm of each well was measured by a microplate reader (Synergy NEO HTS). The statistical significance was analyzed by Student’s t test with p < 0.05 considered statistically significant. Data are presented as mean ± SD.

2.7. Characterizations

1H NMR spectra were recorded on a Bruker Avance III HD 400 MHz spectrometer using DMSO-d₆ as solvents and tetramethylsilane as an internal standard. The Mₙ and the polydispersity (Mₚ/Mₙ) were determined by gel permeation chromatography (GPC), which was measured in DMF at 50 °C with an elution rate of 1.0 mL · min⁻¹ on Agilent 1100 equipped with a G1310A pump, a G1362A refractive index detector and a G1314A variable wavelength detector. The system was calibrated with linear polymethylmethacrylate standards. An FEI Tecnai G2 20 TWIN transmission electron microscope was used to obtain transmission electron microscopy (TEM) images. Samples were prepared by drop-casting micelle solutions onto ultrathin carbon-coated grids and then dried in vacuum at room temperature before measurement. Fourier transform infrared (FT-IR) spectra were measured by the KBr sample holder method on a Nicolet 6700 spectrometer. Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer Nano ZS90 instrument to measure the size distribution of the micelles.

3. Results and discussions

To obtain a polymerizable monomer containing disulfide bond, PDP, containing a pyridyldithio group and a carboxyl group, was synthesized according to the literature [32]. Then, PDPHEMA was synthesized through an esterification reaction between PDP and HEMA, a widely used monomer containing a hydroxyl group. The chemical structure of synthesized PDPHEMA was characterized by 1H NMR. As shown in Fig. 1, peaks d (6.0 ppm) and e (5.7 ppm) were assigned to the two protons of –C=CH₂; the four protons of the pyridyl group could be characterized by peaks a (8.5 ppm), b (7.8 ppm) and c (7.3 ppm). All the characteristic peaks in Fig. 1 could be attributed to the protons of PDPHEMA or the solvent. In addition, the integral area ratio of these peaks were also in accord with proton number ratio of PDPHEMA (a:b:c:d:e:f:g:h:l = 2:2:1:1:4:2:2:3). Thus, the 1H NMR result indicated that the PDPHEMA was successfully synthesized.

Due to the similar reactivity, PDPHEMA and PEGMEA can be facilely copolymerized by free radical polymerization to obtain P(PEGMEA)-co-P(PDPHEMA). The Mₙ and the PDI of P(PEGMEA)-co-P(PDPHEMA) determined by GPC were 16.1 kDa and 2.0, respectively (Fig. 2A). Fig. 3A displayed the 1H NMR spectrum of P(PEGMEA)-co-P(PDPHEMA). The integral area ratio of peaks a and f was about 1:1, which demonstrated that the copolymer consisted of about 50% PDPHEMA and 50% PEGMEA. The FT-IR spectrum of P(PEGMEA)-co-P(PDPHEMA) in Fig. 4 also confirmed the structure. The peak at 1107 cm⁻¹ was attributed to the vibration of C–O–C in a side chain, while the v C= N peak at 1570 cm⁻¹ is indicative of pyridyldithio group from the segment of PDPHEMA [33,34]. All these results indicated that PDPHEMA and PEGMEA were successfully copolymerized.

PTX is highly hydrophobic and cytotoxic to both cancer cells and normal cells, which greatly limits its bioavailability and clinical applications [35-37]. In order to covalently link PTX to the amphiphilic copolymer, carboxyl group was introduced to P(PEGMEA)-co-P(PDPHEMA) copolymer through thiol–disulfide reaction between the thiol of 3-mercaptopropionic acid and the pyridyldithio group of P(PEGMEA)-co-P(PDPHEMA). According to the 1H NMR result in Fig. 3B, all the signals of protons of pyridyl group disappeared, and two new peaks at 2.6 and 2.9 ppm, ascribed to –CH₂CH₂ near the carboxyl group on the
copolymer, appeared. The $M_n$ of P(PEGMEA)-co-P(PDPHEMA)-g-COOH also increased to 27.5 kDa (Fig. 2B). The successful reaction was also confirmed by FT-IR characterization. The $\nu$ C = N peak at 1570 cm$^{-1}$ disappeared in the FT-IR spectrum of P(PEGMEA)-co-P(PDPHEMA)-g-COOH (Fig. 4B), which suggested that the pyridyl group was replaced by the carboxyl group.

Utilizing the carboxyl group as an active reaction site, PTX could be linked to P(PEGMEA)-co-P(PDPHEMA)-g-COOH via an esterification reaction. Due to the successful reaction, the $M_n$ of P(PEGMEA)-co-P(PDPHEMA)-g-PTX increased to 42.6 kDa (Fig. 2C). Furthermore, in the $^1$H NMR spectrum of Fig. 3C, the signal of PTX from 4.5 to 10 ppm appeared. The loading content of PTX was about 43.7%, calculated from the integral area ratio of peak b (4.6 ppm, characteristic peak of PTX) with peak c (3.5 ppm) or a (3.2 ppm), characteristic peaks of PEG. The high drug loading content was ascribed to the high content of carboxyl groups via disulfide bond linked with polymer backbone, which is much higher than those in the most previous reports (Fig. S5). As seen in FT-IR spectra of Fig. 4, the $\nu$ C = O (in amides) peak at 1732 cm$^{-1}$, the $\delta$ N-H peak at 711 cm$^{-1}$ and the peaks at 1400–1600 cm$^{-1}$ of benzene group in P(PEGMEA)-co-P(PDPHEMA)-g-PTX also suggested the successful loading of PTX [38,39].

The synthetic process was also confirmed by the UV–vis measurement. As shown in Fig. 5, the copolymer, P(PEGMEA)-co-P(PDPHEMA), has two characteristic peaks derived from the pyridyl group at 236 nm and 261 nm [40]. After the exchange reaction, the peaks relating to pyridyl disappeared in P(PEGMEA)-co-P(PDPHEMA)-g-COOH without new peaks, suggested that the pyridyl group was replaced by the propanoic acid group, and P(PEGMEA)-co-P(PDPHEMA)-g-PTX showed the characteristic peak related to PTX at 227 nm [41,42].

Due to the amphiphilic property, P(PEGMEA)-co-P(PDPHEMA)-g-PTX could self-assemble into micelles in an aqueous solution with hydrophilic PEG chain as the shell and hydrophobic PTX as the core. The size of the micelles was characterized by DLS and TEM. As seen from the TEM images in Fig. 6A, the micelles were spherical with an average diameter of approximately 40 nm. The DLS measurement results (Fig. 6B) exhibited that the micelles were approximately 300 nm with a PDI of 0.34. The different results of the size measured by DLS and TEM may be ascribed to the measurement state of polymeric micelles and the molecular chemical structure [43]. The size determined by
DLS was hydrated diameter and the micelles may be micellar aggregates instead of a single micelle [44]. However, the TEM image was obtained in a high vacuum environment, which may render the size of micelles smaller than the result of DLS [43]. Thus, P(PEGMEA)-co-P(PDPHEMA)-g-PTX micelles were suitable for passive delivery of PTX targeted to the tumor tissue due to the enhanced permeability retention (EPR) effect [45].

The in vitro cytotoxicity of drug carrier, P(PEGMEA)-co-P(PDPHEMA)-g-COOH, against HEK-293 cells was evaluated by CCK-8 assay. Fig. 7 shows the HEK-293 cell viability after incubation with P(PEGMEA)-co-P(PDPHEMA)-g-COOH at a concentration ranging from 0.005 μg·mL⁻¹ to 1000 μg·mL⁻¹ for 48 and 72 h. Compared with the normal incubated cells, the cell viability was near to 100% even at the concentration of P(PEGMEA)-co-P(PDPHEMA)-g-COOH up to 1000 μg·mL⁻¹ after incubation for 72 h. This result demonstrated that P(PEGMEA)-co-P(PDPHEMA)-g-COOH was highly biocompatible and suitable as a drug carrier.

To evaluate the selective bond cleavage and intracellular drug release of P(PEGMEA)-co-P(PDPHEMA)-g-PTX, HeLa cells (human cervical cancer cells) and HEK-293 cells (human normal cells) were chosen to conduct the in vitro cell assay. This is because the concentration of GSH in the tumor cells was at least 4-fold higher than that in normal cells. The cells were treated with P(PEGMEA)-co-P(PDPHEMA)-g-PTX at different PTX concentrations from 0.005 μg·mL⁻¹ to 50 μg·mL⁻¹ for 48 and 72 h. As shown in Fig. 8, after incubation for 48 h, with the increase of PTX concentration, the cell viabilities of both HEK-293 cells and HeLa cells declined. However, at the same PTX concentration, the viability of HEK-293 cells was remarkably higher than that of HeLa cells. Particularly, at PTX concentration of 0.05 μg·mL⁻¹, the viability of HEK-293 cells was 83% while that of HeLa cells was 50%. Similar results presented after incubation for 72 h: the viability of HEK-293 cells was 78% while that of HeLa cells was 38% at PTX concentration of 0.05 μg·mL⁻¹. The cytotoxicity of P(PEGMEA)-co-P(PDPHEMA)-g-PTX to HeLa and HEK-293 cells could also be seen from the IC₅₀ value. The IC₅₀ of P(PEGMEA)-co-P(PDPHEMA)-g-PTX against HeLa cells was 0.021 μg·mL⁻¹ after incubation for 72 h. To further elevate the cytotoxicity and efficacy of P(PEGMEA)-co-P(PDPHEMA)-g-PTX, we have compared the many IC₅₀ values for HeLa cells of composites containing PTX in the works reported previously (Fig. S4). The IC₅₀ of P(PEGMEA)-co-P(PDPHEMA)-g-PTX was much lower than that of other reported works or free PTX (0.035 μg·mL⁻¹), which emphasizes the contribution of reduction-induced rapid intracellular drug release enhancing the cytotoxicity of targeted produg systems. More important, The IC₅₀ of P(PEGMEA)-co-P(PDPHEMA)-g-PTX against HeLa cells were 0.27 μg·mL⁻¹ after incubation for 72 h. The almost 10-fold distinction of IC₅₀ values between both indicated that the introduction of disulfide bond would much enhance the selectivity, which could be helpful for its potential application. All these results indicated that the drug release behavior was facile under high concentration of GSH in tumor cells, whereas the release process would be difficult to trigger under low concentration of GSH in normal cells. Therefore, the polymer–drug conjugate based on disulfide bond showed specific cytotoxicity to cancer cells, which is favorable to reduce the toxic and side effect of PTX.

4. Conclusion

A novel reduction-responsive polymer–drug conjugate based on disulfide bond was constructed. Firstly, a monomer PDPHEMA containing disulfide bond was synthesized using PDS by thiol–disulfide reaction with 3-mercaptopropionic acid followed by esterification reactions with HEMA. The structure was confirmed by ¹H NMR characterization. Then, PDPHEMA was copolymerized with PEGMEA. After another thiol–disulfide reaction of P(PEGMEA)-co-P(PDPHEMA) with 3-mercaptopropionic acid, P(PEGMEA)-co-P(PDPHEMA)-g-COOH was obtained and reacted with PTX to yield P(PEGMEA)-co-P(PDPHEMA)-g-PTX, which could self-assemble into micelles in aqueous solution. The structure of this polymer–drug conjugate was characterized by ¹H NMR, FT-IR and UV. Due to the high content of carboxyl groups via disulfide bond linked with polymer backbone, the loading content of PTX was very high, reaching 43.7%. The in vitro cell assay results indicated that the drug carrier had little cytotoxicity to HEK-293 cells and can serve as drug carrier for CDDSs. More intriguingly, the IC₅₀ of P(PEGMEA)-co-P(PDPHEMA)-g-PTX of HeLa cells and HEK-293 cells was 0.021 μg·mL⁻¹ and 0.27 μg·mL⁻¹, indicating that the active drug release can be selectively achieved owing to the introduction of reduction-responsive disulfide bond. Such a dramatic
selectivity for cancer-killing potential can serve as a promising platform that reduces the toxic and side effect of chemotherapy drugs.

Acknowledgments

The authors are thankful for the financial support from the International Science & Technology Cooperation Program of China (2014DFE40130), the National Natural Science Foundation of China (51373035, 51373040, 51573030, and 51573028).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2015.09.025.

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