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Biodegradable multi-blocked polyurethane micelles for intracellular drug delivery: the effect of disulfide location on the drug release profile

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Biodegradable multi-blocked polyurethane (PU) based micelles with redox responsive properties have been widely used as anticancer drug delivery systems due to their customizable molecular structures. Disulfide bonds can be easily introduced into the hard segment of polyurethanes by conventional two-step polymerization processes, which result in a random distribution of the reduction responsive disulfide linkages on polyurethanes. We speculated that the disassembly and drug release profiles of polyurethane micelles were related to the location of the disulfide linkages on the polymer main chain. To this end, two kinds of redox responsive polyurethane micelles with the same quantity of disulfide bonds but at different locations were prepared: i) most of the disulfide bonds located at the hydrophobic core of the polyurethane micelles (PU-SS-C) and ii) disulfide bonds located primarily at the interface between the hydrophobic core and the hydrophilic shell (PU-SS-I). Paclitaxel (PTX) was chosen as the model hydrophobic drug to evaluate the loading and redox-triggered release of the PU micelles. It was demonstrated that the PU-SS-I micelles disassembled simultaneously in response to a 10 mM glutathione (GSH) stimulus and the payloads released more rapidly than that of PU-SS-C nanocarriers. The results show that the release profiles of PU based nanocarriers can be optimized by the location of the disulfide on the polyurethane main chain. The rapid redox-stimulated release properties of the PU-SS-I nanocarriers will be a promising anticancer drug delivery system to ensure sufficient drug concentration to kill the cancer cell and to prevent the emergence of multidrug resistance (MDR). In addition, confocal laser scanning microscopy (CLSM) demonstrated the cellular uptake of Doxorubicin-loaded (DOX-loaded) micelles and the GSH-responsive intracellular release of DOX. The in vitro cytotoxicity and cell uptake of the PTX-loaded micelles was also assessed in H460 cells.

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Introduction

As one of the many promising anticancer drug delivery systems, various self-assembled micelles based on amphiphilic block copolymers have been developed in recent years.[1-3] The hydrophobic core of polymer micelles improves the solubility of hydrophobic drugs by physical encapsulation, while the hydrophilic corona of polymer micelles prolongs the circulation time of the micelles and stabilizes the colloid in aqueous medium. Meanwhile, the nanocarriers can accumulate passively on the solid tumour by the enhanced permeability effect (EPR).[1, 4-7] Once internalized into cancer cells through endocytosis, the nanocarriers are expected to degrade or destabilize instantly, which facilitates rapid intracellular drug release to kill the tumour and to prevent emergence of multidrug resistance (MDR).[8-13] The degradation of nanovehicles in the intracellular environment also aids in the removal of empty carriers after drug release. Thus, it is reasonable to construct smart nanocarriers with cleavable bonds in the main chain or side chain that are cleaved simultaneously by the stimuli of the intracellular micro-environment of the tumour, i.e. pH,[14] redox potential[15, 16] or enzymatic activity[17]. Glutathione (GSH), a reducing agent that can cleave the disulfide bond at millimolar concentrations, is found at an elevated level of about 10 mM in cancer cells. The concentration of GSH in the matrix outside cancer cells is about 10 mM, 1000 times higher than that in normal cells.[18] Therefore, block polymer based micelles containing disulfide bonds have been considered a promising platform for tumour-targeting drug delivery applications because of the great difference in the redox potential between the intracellular and extracellular compartments.[19, 20]

Multi-blocked polyurethanes (MPUs) are a flexible material platform that can be designed to fit the requirements of different applications.[3, 12, 21] Recently, biodegradable multi-blocked polyurethane-based micelles with redox responsive properties have been prepared by using bis(2-hydroxyethyl) disulfide as a chain extender for anticancer drug delivery systems.[3] In the published

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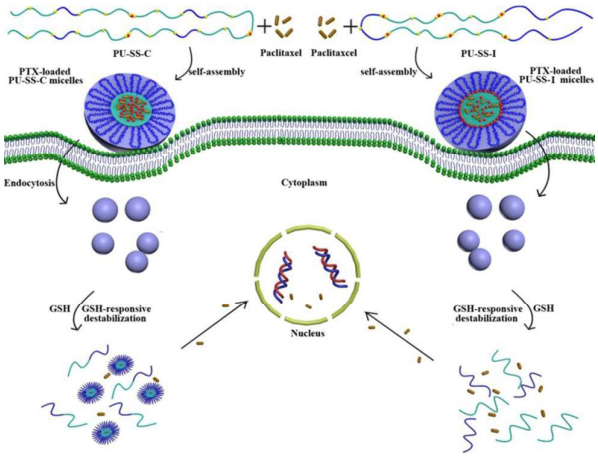


Scheme 1 Schematic illustration of the reduction sensitive polyurethane with disulfide bonds located randomly on the polyurethane main chain (PU-SS-C) or positioned mainly at the interface between the hydrophobic PCL units and the hydrophilic PEG units (PU-SS-I), and their self-assembled PTX-loaded micelles as effective intracellular drug delivery nanocarriers.

literature, the disulfide bonds were randomly located at the polyurethane main chain[22] or only at the hydrophobic core[3]. Jung Kwon Oh et al.[1, 2] developed a multi-location stimuli-responsive degradation concept, which synergistically enhanced the release of encapsulated anticancer drugs. We speculated that the disassembly and drug release profiles of polyurethane micelles were related to the location of the disulfide linkage on the polymer main chain. To the best of our knowledge, however, there is no report to study the drug release profiles of polyurethane micelles with different location of disulfide linkage on the main chain. Therefore, two kinds of redox responsive polyurethane micelles with the same amount of disulfide bonds but in different locations were prepared in this study: i) most of the disulfide bonds located at the hydrophobic core of polyurethane micelles (PU-SS-C); and ii) disulfide bonds located primarily at the interface between the hydrophobic core and the hydrophilic shell (PU-SS-I). As shown in Scheme 1, the amphiphilic multi-blocked polyurethanes with redox-responsive properties self-assembled in aqueous solution into micelles having a hydrophobic PCL core and a hydrophilic PEG shell. PTX was chosen as a model hydrophobic drug to evaluate the loading- and redox-triggered release profiles of the PU micelles. The drug release profiles of the PU-SS-I and PU-SS-C micelles in response to a 10 mM GSH stimuli was studied in this report. The *in vitro* cytotoxicity and cell uptake of the PTX-loaded micelles was also assessed in H460 cells.

Experimental

Materials

Polyethylene glycol (PEG) [M_n=1000, Sunshine Biotechnology (Nanjing) Co., Ltd. China] and poly (ε-caprolactone) diol (PCL) (M_n=2000, Dow Chemical, USA) were dehydrated under reduced pressure at 100 C for 2-3 h before use. N, N-Dimethylacetamide (DMAc) was dried over CaH_2 and vacuum distilled before use. L-Lysine ethyl ester diisocyanate (LDI) was synthesized from L-lysine ethyl ester and triphosgene and purified by vacuum distillation. Triethylamine (TEA, Aladdin Industrial Corporation, China) was distilled under vacuum, and cystaminedihydrochloride (Cys, Aladdin Industrial Corporation, China) was used as received. PTX (99.5%) was obtained from AstaTech (Chengdu) Pharmaceutical Co. Ltd. China. Doxorubicin hydrochloride (DOX, -$NH_3^+Cl^-$ salt form, >98%) was obtained from AstaTech (Chengdu) Pharmaceutical Co. Ltd. China.

Synthesis of polyurethanes

The two kinds of polyurethane were synthesized from the same amount of PEG, PCL, LDI, and Cys but with different feed processes, which resulted in different locations of disulfide bonds on the polyurethane, i.e. PU-SS-C and PU-SS-I. The feed ratios are listed in Table S1. PU-SS-C was synthesized by the traditional two step solution polymerization process, i.e. pre-polymerization and chain extension steps. In brief, anhydrous PEG and PCL-diol were dissolved in DMAc under a dry nitrogen atmosphere. LDI was added into the DMAc solution of PCL and PEG and pre-polymerized in the presence of 0.1% stannous octoate [$Sn(Oct)_2$] at 90 C for 4 h with stirring. Then, the prepolymer solution was cooled to 0 C in ice water and the chain extender Cys with an equal amount of triethylene amine (TEA, used to neutralize the hydrogen chloride of Cys) was added, and the mixture was kept at 0 C for 1 h and then at room temperature for 2 h. Finally, the mixture was incubated at 80 C for 4 h to complete the chain extending reaction. After that, the reaction mixture was cooled to room temperature. To remove the organic solvents and impurities, the crude product subsequently was poured into a 3:1 water and methanol mixture (v:v). The precipitant was redissolved in DMAc and precipitated again by the water and methanol mixture. The polymer was harvested and dried at 40 C in atmosphere for 5 h and then at 60 C at reduced pressure for 2 days to get the final product.

To synthesize the polyurethane with most of the disulfide groups located at the interface between the hydrophobic PCL segment and the hydrophilic PEG segment, i.e. PU-SS-I, a different reactant addition process was conducted as listed in Table S1. Briefly, anhydrous PCL-diol was dissolved in DMAc under a dry nitrogen atmosphere. LDI was added into the DMAc solution of PCL and pre-polymerized in the presence of 0.1% $Sn(Oct)_2$ at 90 C for 4 h with stirring, resulting in the **prepolymer 1** solution. Then, the **prepolymer 1** solution was cooled to 0 C by ice water and the chain extender Cys was added with an equal amount of TEA, and the mixture was kept at 0 C for 1 h, at room temperature for 2 h, and finally at 80 C for 4h to form the **prepolymer 2**. After that, the **prepolymer 2** solution was cooled to room temperature, the LDI was added, and the mixture was reacted at 60 C for 2 h. Next, the anhydrous PEG was added and remained at 90 C for 5h to complete the reaction. After that, the reaction mixture was cooled to room temperature and the polyurethane was purified using the same procedure as that to purify the PU-SS-C. The final product was denoted as PU-SS-I.

Characterization of polyurethanes

^{1}H NMR was recorded on an Agilent-NMR-VNMRS 400 (400 MHz) spectrometer using deuterated dimethyl sulfoxide (DMSO-d_6) and deuteroxide (D_2O) as the solvent. The molecular weights and molecular weight distributions of PU-SS-C and PU-SS-I were determined by a Waters-1515 gel permeation chromatograph. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1 mL min^{-1} at 40 C and the molecular weights were reported relative to polystyrene (PS) standards. Elemental analysis was conducted on an elemental analyser (Vario MICRO cube, Elementar,

Germany) to investigate the chemical composition of the synthesized PUs.

Preparation of polyurethane micelles

A dialysis method was used to prepare the polyurethane micelles. Typically, polyurethane (PU-SS-C or PU-SS-I, 20mg) was completely dissolved in 4 mL DMAc. Afterwards, the solution was added drop wise into 10mL distilled water under vigorous stirring. Subsequently, the micelle solution was transferred to the dialysis bag (MWCO, 3.5 kDa) and dialysed against distilled water for 72 h to eliminate the organic solvent at room temperature. The micelle solution was passed through a 0.45 μm pore-sized syringe filter (Millipore, Carrigtwohill, Co. Cork, Ireland)and stored at 4 C.

Characterization of polyurethane micelles

The critical micelle concentration (CMC) of the obtained polyurethanes in distilled water was determined by fluorescence spectroscopy using pyrene as a probe. The polyurethane concentration was varied from 1.0×10^{-5} to 0.2 mg mL^{-1} and the final pyrene concentration was fixed to 5.2×10^{-6} M. The combined solution of pyrene and micelles was sonicated for 4 h in the dark before the fluorescence measurement. Fluorescence excitation spectra were recorded by a fluorometer (Varian Cary Eclipse Fluorescence Spectrophotometer,USA) at a wavelength range of 285 to 355 nm, with the emission wavelength at 372 nm and slits at 5 nm for both excitation and emission. Size and zeta potentials of the nanoparticles in aqueous solution were measured with a Zetasizeranalyzer (Malvern Zetasizer Nano, Zen 3690+MPT2, Malvern, UK). The morphologies of PU-SS-C and PU-SS-I micelles were observed by transmission electron microscopy (TEM) (Tecnai G^2 F20 transmission electron microscope, Royal Dutch Philips Electronics Ltd., Holland) at an accelerating voltage of 200 kV.

Preparation of PTX-loaded micelles

A micelle extraction technique was used to load PTX into reduction-sensitive polyurethane micelles. Prior to loading, the given amount of PTX was dissolved in 5 mL of acetone to a concentration of 5 mg ml^{-1} and placed in a vial. The acetone was allowed to evaporate from the vial under nitrogen atmosphere to form a drug film. Then 10 mL of micelle solution (1 mg ml^{-1}) was transferred into the vial containing the drug film with different amounts of PTX and ultrasonicated for 2 h to achieve maximum loading. The excess PTX was removed by a 0.45 μm filter. The drug concentration of the loaded micelles was evaluated using a high-performance liquid chromatography (HPLC) system (Waters Isocratic HPLC Pump, US) equipped with a reverse-phase C18 column (4.6 × 250 mm, 5 μm). An acetonitrile-water (60/40 v/v) mixture was used as the mobile phase and the flow rate was 1.0 mL min^{-1}. The UV adsorption of PTX outflow from the chromatographic column was recorded by a Waters 2489 UV/Visible Detector at a wavelength of 227 nm. The loading content (%) and encapsulation efficiency (%) were calculated based on the equations below:

Loading Content (LC) (%) = Mass of drugs in micelles / Total mass of loaded micelles × 100%

Encapsulation Efficiency (EE) (%) = Mass of drugs in micelles / Initial amount of feeding drugs × 100%.

Preparation of DOX-loaded micelles using dialysis method

Water (5 mL) was added drop-wise to a clear solution consisting of the purified, dried PU (10 mg), DOX (2 mg), and Et_3N (3 molar equivalents to DOX) in DMF (2 mL). The resulting dispersion was dialyzed over water (500 mL) for 2 days, yielding DOX-loaded micelles of PU at 1.0 mg/mL. The excess DOX was removed by a 0.45 μm filter. The drug concentration of the loaded micelles was evaluated using a high-performance liquid chromatography (HPLC) system (Waters Isocratic HPLC Pump, US) equipped with a reverse-phase C18 column (4.6 × 250 mm, 5 μm). An acetonitrile-water (32/68 v/v) mixture was used as the mobile phase and the flow rate was 1.0 mL min^{-1}. The UV adsorption of DOX outflow from the chromatographic column was recorded by a Waters 2489 UV/Visible Detector at a wavelength of 254 nm. The loading content (%) and encapsulation efficiency (%) were calculated based on the equations below:

Loading Content (LC) (%) = Mass of drugs in micelles / Total mass of loaded micelles × 100%

Encapsulation Efficiency (EE) (%) = Mass of drugs in micelles / Initial amount of feeding drugs × 100%.

***In vitro* release of PTX**

In vitro drug release from the drug-loaded micelles was performed using the dialysis method. In brief, 10 mL (PU-SS-C 3.1%, PU-SS-I 2.7%) of drug loaded micelle solution was added to a dialysis bag (MWCO: 3.5 kDa) and immersed in 100 mL of PBS (0.01 M, pH 7.4) containing 0.1 M sodium salicylate with or without 10 mM GSH. The dialysis system was kept at 37 C in a thermostatic incubatorwith a shaking speed of 110 rpm. Samples were removed and replaced with the same volume of fresh medium at the desired time interval. The concentration of PTX released from drug loaded micelles was analyzed by HPLC.

Cell culture

The human liver cell line H460 was cultured in RPMI 1640 media supplemented with 2 mM L-glutamine, 100 U mL^{-1} penicillin and 10% foetal bovine serum (FBS) (FBS, HyClone, Logan, UT) at 37 C in a humidified atmosphere containing 5% CO_2 (Sanyo Incubator, MCO-18AIC, Japan).

HUVEC were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life, Grand Island, NY, USA) supplemented with 10% (v/v) foetal bovine serum (FBS, HyClone, Logan, UT), 2 mM L-glutamine and 1% (v/v) antibiotic mixture (10000 U of penicillin and 10 mg of streptomycin) (Gibco). The cells were incubated in a humidifiedatmosphere of 5% CO_2 at 37 C (Sanyo Incubator, MCO-18AIC, Japan).

Cellular uptake and intracellular release of payloads

Confocal laser scanning microscopy (CLSM) was employed to examine the cellular uptake and intracellular release behaviour of polyurethane micelles. The DOX-loaded micelles were incubated with H460 cells for 1h at 37 C. After removal of the medium, the cells were washed three times with cold PBS, fixed with 1 mL of 4% paraformaldehyde for 30min at 4 C, and stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Roche) for 10 min. Finally, the slides were mounted with a 10% glycerol solution and observed by a Leica TCS SP8 (Leica Microscopy Systems Ltd., Germany).

Cell viability assay

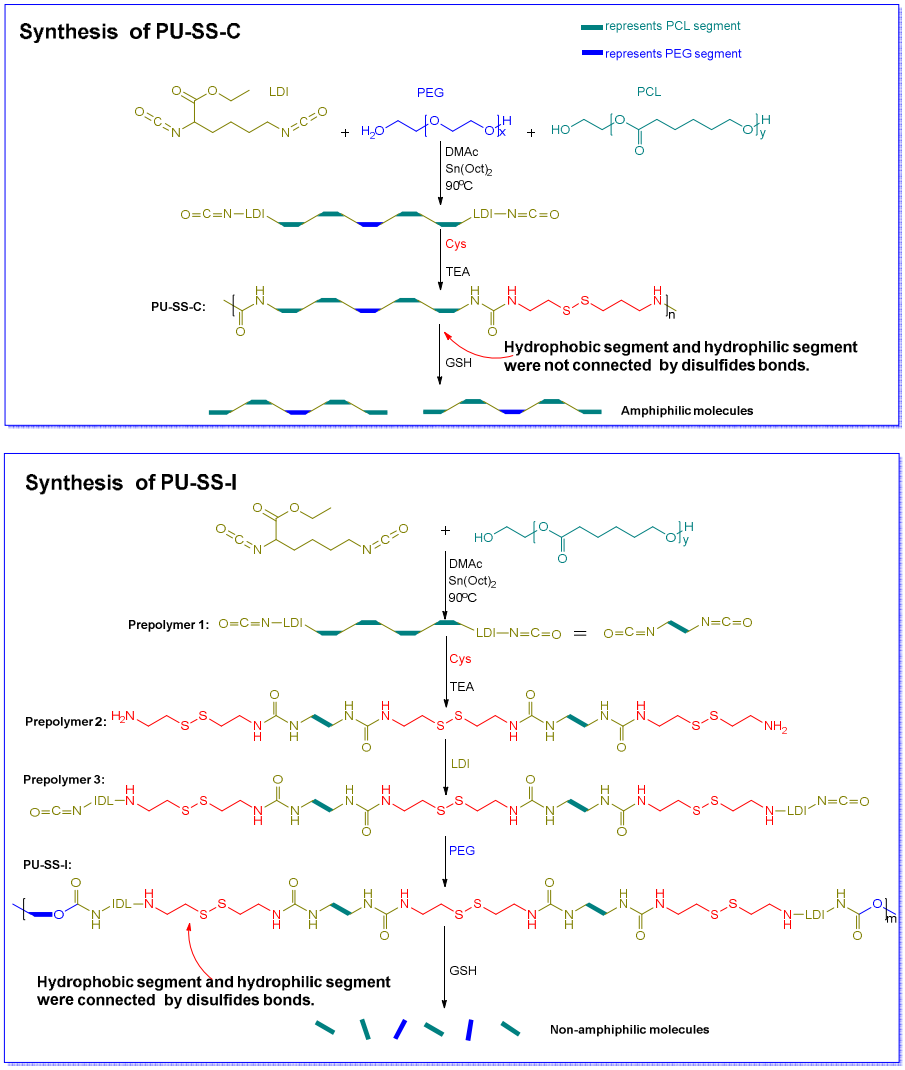
To evaluate the antitumor activity of PTX-loaded polyurethane micelles and the cytocompatibility of drug-free micelles, H460 cells and HUVEC were seeded in 96-well plates at 4×10^3 cells per well and incubated for 24h. The culture medium was removed and replaced with 100 μL of medium containing various concentrations of micelle solutions and incubated for another 24 h. Then, 10 μL of

Cell Counting Kit-8 (CCK-8) solution (Shanghai Qcbio Science & Technologies Co., Ltd.) was added to each well. After incubating the cells for 4 h, the absorbance was measured at a wavelength of 450 nm. The cell viability was normalized to that of cells cultured in the full culture media. The dose-effect curves were plotted and the median inhibitory concentration (IC_{50}) was determined using IBM SPSS Statistics software (SPPS, Inc., USA).

Results and Discussion

Synthesis and characterization of polyurethanes

In order to prepare polyurethanes with the same amount of PCL, PEG, LDI, and especially Cys, but different locations of the redox active disulfide bonds, i.e. PU-SS-C and PU-SS-I, two different synthesis processes were conducted as listed in **Scheme 2**. For PU-SS-C, The disulfide bonds were mainly located at the connection between the hydrophobic PCL segments. The feed ratio of PCL to PEG was 4:1 as listed in Table S1. It is expected that most of the disulfide groups were located between the PCL segments, which resulted in the core location of disulfide groups in the self-assembled micelles of PU-SS-C. In another synthetic process, PCL and LDI were first polymerized and formed an isocynateendcapped PCL (**Prepolymer 1**). After the reaction, the theoretical residual isocynates were 0.67 mmol. Then**prepolymer 1** was further reacted with 1 mmol (0.33 mmol morethan the residual isocynates) Cys to form an amine group endcappedprepolymer (**Prepolymer 2**).For the reaction process of **prepolymer 2**, the stoichiometric ratio r (-NCO/-NH_2) was equal to 0.67, then the number of monomer units incorporated into **prepolymer 2** was 5.06 calculated by the step growth polymerization equation. So, the sequence of prepolymer 2 was -cys-**prepolymer 1**-cys-**prepolymer 1**-cys-. Based on this, we predicted that a third of the disulfide groups were connected by a hydrophobic PCL segment that would position at the core of the self-assembled micelles, while another two thirds of the disulfide bonds were located at the end of prepolymer 2, which resulted in the disulfide bonds being positioned at the interface between the PEG and PCL segments in PU-SS-I. The theoretical residual of amine in prepolymer 2 was 0.33 mmol. **Prepolymer 2** was further reacted with 1.13 mmol LDI to endcap prepolymer 2 with isocynates (Prepolymer 3, residual -NCO: 0.80 mmol). Finally, **prepolymer 3** was reacted with 0.8 mmol PEG to form the final product.



Scheme 2 Synthesis of reduction-sensitive biodegradable multi-block polyurethanes

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The unimodal GPC curves of purified PU-SS-C and PU-SS-I (SI: Figure S1) confirmed successful polymerization. The average molecular weight and polydispersity of PU-SS-C and PU-SS-I are listed in Table S1. As shown in Table S1, PU-SS-C had M_n = 19150 g/mol with M_w/M_n = 1.65, While the M_n of PU-SS-I was 24121 g/mol with M_w/M_n = 1.69.

The representative ^{1}H NMR spectra of PU-SS-C and PU-SS-I and the assignment of the peaks are presented in Fig. 1. The ^{1}H NMR spectra of PU-SS-C and PU-SS-I are nearly identical arising from their identical composition. The resonance peak centred at 3.51 ppm (peak h)was assigned to the protons on the PEG units while the peaks at 3.97 ppm (peak g), 2.26 ppm (peak c), 1.52 ppm (peak d and f) and 1.29 ppm (peak e) are attributed to the protons of the PCL units. The resonances at 4.06 ppm (peak c) and 1.16 ppm (peak d) are ascribed to the methylene and methyl protons in the ethoxyl group of the LDI units in the products. The peaks at 3.25 ppm (peak a) and 2.79 ppm (peak b) are attributed to the methylene protons of the Cys units, which indicated the presence of the Cys units in the resulting polyurethanes.

The chemical composition of the purified PU-SS-I and PU-SS-C are listed in Table S2. As shown in Table S2, thetwo PU samples have a similar content of N, C, H and especially S elements.The same chemical composition of PU-SS-I and PU-SS-C indicates that the PU-SS-C and PU-SS-I has the same amount of redox-active disulfide bonds.

Characterization of PU micelles

The amphiphilic multi-blocked polyurethanes with redox-responsive properties self-assembled in aqueous solution into micelles having a hydrophobic PCL core and a hydrophilic PEG shell.[23] The core-shell structure of the polyurethane micelles was confirmed by the ^{1}H NMR resonance difference of the hydrophilic PEG and hydrophobic PCL signals when DMSO-d6 and D_2O were used as solvents (SI: Fig. S2), and from fluorescence measurements using pyrene as a probe (SI: Fig. S3A).[3]Most of the disulfide groups located between the PCL segments, which resulted in the core location of the disulfide groups in the PU-SS-C micelles as illustrated in Scheme 2. However,

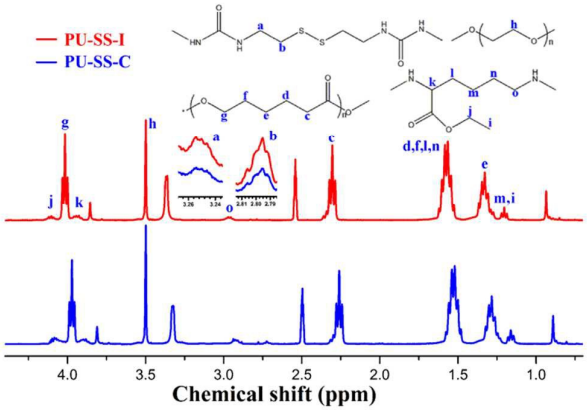

Fig. 1 ^{1}H NMR spectra of PU-SS-C (a) and PU-SS-I (b) in DMSO-d_6

as presented in Scheme 2, two thirds of the disulfide linkages in PU-SS-I micelles were positioned at the interface between the hydrophobic PCL core and the hydrophilic PEG shell, while the remaining one third were located at the PCL core. As presented in Table S1, the hydrodynamic diameter of polyurethane micelles determined by DLS are in the range of 132-137 nm with unimodal size distribution (Fig. 2C). Notably, as shown in Table S1, the zeta potential of PU-SS-I micelles is -22mV, much lower than that of PU-SS-C micelles (-7mV). Since there is no ionic group in the polymer structure, the negative zeta potential may be attributed to the delocalization of negative charges on ester bonds or the polarization of water molecules under the effect of PEG.[24, 25] This indicates that more PEG chains stretch out from PU-SS-I micelles than from PU-SS-C micelles, which may result in the longer circulation time of PU-SS-I micelles than that of PU-SS-C micelles.[26-28] TEM images indicate spherical micelles with an average diameter of 100 nm with relatively broad size distribution (Fig. 2A,B), which is smaller than the size determined by DLS. The difference in micelle sizes between DLS and TEM can be attributed to the dehydrated state of the micelles.

The critical micellar concentration (CMC) was determined by fluorescence spectroscopy with a pyrene probe. The ratio of the intensity of the peak at 337 nm to that at 333.5 nm is plotted against the log of polymer concentration and the concentration corresponding to the intersection of the two tangential lines is the CMC value (SI: Fig. S3B). The CMC of PU-SS-C and PU-SS-I was determined to be 6.8738×10^{-4} mg mL^{-1}and 2.7868×10^{-4} mg mL^{-1},respectively.

3.3 Stimuli-responsiveness of polyurethane micelles

We incorporated redox active disulfide linkages into the polyurethane hard segment that will cleave in the presence of 10mM GSHand result in the destabilization of the polyurethanemicelles.As discussed previously, most of the disulfide linkages located at the interface between the hydrophilic corona and the hydrophobic core in the PU-SS-I micelles result in easier access for the GSH than in the PU-SS-C micelles where the disulfide linkages are mostly positioned at the hydrophobic core. Furthermore, after the reduction cleavage of the disulfide linkages, the amphiphilicity of the PU-SS-I is lost while that of the PU-SS-C remained. So, it is reasonable to expect that the PU-SS-I micelles disassemble and aggregate more rapidly than the PU-SS-C micelles in the presence of intracellular levels of GSH (10 mM).

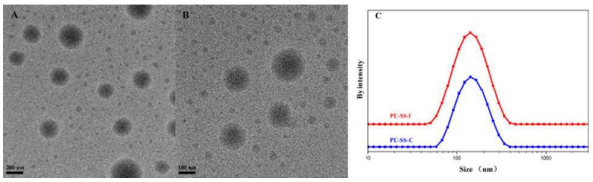

Fig. 2 TEM micrograph of PU-SS-I (A) and PU-SS-C (B) micelles. Size distribution of reduction-sensitive polyurethanes determined by DLS (C).

To confirm the above hypothesis, the change of size and size distributions of micelles in response to an intracellular level of GSH (10 mM) was carried out. As is shown in Fig. 3B, the size of the PU-SS-C micelles increase gradually in the presence of 10 mM GSH, suggesting the reduction cleavage of the disulfide bonds. However, the size of PU-SS-I micelles increases dramatically with an intracellular level of GSH (Fig. 3A). As can be seen from Fig. 3A, a large amount of visible aggregates were present in the PU-SS-I colloid after 7.5 min, suggesting the rapid cleavage of the disulfide bonds and disassembly and aggregation of the micelles, which may facilitate rapid drug release from PU-SS-I micelles. In addition, as seen in Figure S1, the disulfide linkages in micelles cleaved to the corresponding thiols in response to reducing agents such as DTT and cellular GSH. Such cleavage of the disulfide linkages in PU-SS-C and PU-SS-I were demonstrated further by a decrease in molecular weight from M_n= 19,150 to M_n = 2,590 and M_n= 24,121 to M_n = 8,588, respectively, when the polyurethanes were treated with DTT in THF.

Drug loading and release of the polyurethane micelles

PTX was used as a model hydrophobic drug to investigate the loading capacity of the redox active polyurethane micelles. A micelle extraction technique was used to load the PTX into polyurethane micelles. The excess PTX was removed by filtration through a 0.45 μm filter. The loading level of PTX for PTX-loaded micelles was determined using HPLC. The drug loading content (LC) and encapsulation efficiency for PTX loaded micelles are in the range of 1.04–5.30% and 2.1-11.2%, respectively. The loading content (LC) and encapsulation efficiency for DOX loaded micelles are in the range of 1.02–1.65% and 5.15-8.39%, respectively. It should be noted that the size and size distribution of the PTX and DOX loaded micelles are in the same range (SI: Fig. S4).

The drug release behaviour of the PTX-loaded polyurethane micelles was investigated in PBS (pH 7.4) at 37 C in the presence and absence of GSH (10 mM). Fig. 4 shows the cumulative drug release profiles as a function of time. As shown in Fig. 3, the release of PTX from the polyurethane micelles was correlated markedly with the presence or absence of GSH. Without GSH, PTX release from PU-SS-C and PU-SS-I micelles was suppressed significantly due to the good stability of the polyurethane micelles, with only 5.45% and 6.73% of the PTX released in the first 4 h, respectively and less than 18.5% released within 48 h. However, the PTX release from both PU-SS-C and PU-SS-I micelles was enhanced dramatically when 10 mM GSH was present in the PBS solution, causing 18.54% and 43.75% to be released from PU-SS-C and PU-SS-I micelles in 4 h, respectively. Within 48 h, the PTX release was nearly 57.45% and 70.95% for PU-SS-C and PU-SS-I micelles, respectively. The enhanced drug release in the presence of GSH arose from the

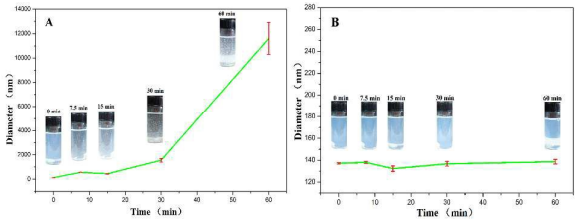

Fig. 3 Change in size and size distribution of PU-SS-I (A) and PU-SS-C (B) micelles in the presence of 10 mM GSH over time.

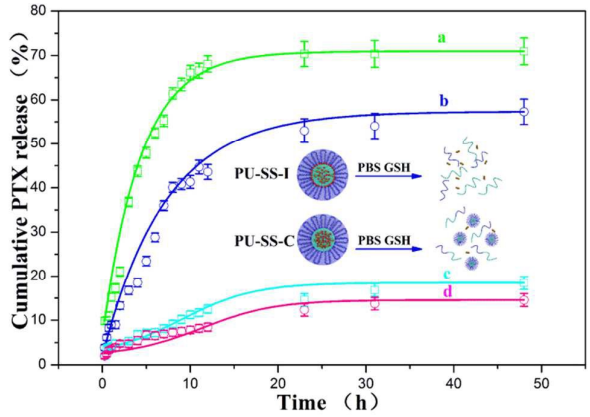

Fig. 4 Time dependent cumulative release of PTX from reduction-sensitive polyurethane micelles in PBS buffer solutions (pH 7.4, 10 mM) with and without 10 mM of GSH. (a) PU-SS-I micelles in PBS with 10mM GSH; (b) PU-SS-C micelles in PBS with 10mM GSH; (c) PU-SS-C micelles in PBS and (d) PU-SS-I micelles in PBS

disassembly of polyurethane micelles due to the GSH-induced disulfide cleavage.[29]

Notably, as compared in Fig. 4, the PTX release from PU-SS-I micelles was faster than PU-SS-C micelles in the presence of 10 mM GSH, especially within the first 10 h. For example, within 10 h, 66.15% PTX was released from PU-SS-I micelles in the presence of 10 mM GSH, whereas about 41.44% was released from PU-SS-C micelles. The enhanced reduction-induced release and greater early burst of PTX release from PU-SS-I micelles is presumably attributed to the efficient reductive cleavage of disulfide linkages at interfaces that resulted from the instant disassembly of the micelles as proven in Fig. 3. These results demonstrate that the location of the redox-responsive disulfide bonds in multi-block polyurethane micelles has great influence on their drug release profile and that drug release can be easily adjusted by the position of the disulfide linkages in the polyurethane.

Internalization and intracellular release of the polyurethane micelle payload

The cellular uptake of the micelles and the intracellular location of the encapsulated payloads was monitored by CLSM in H460 cells. Since PTX molecules are not fluorescent, doxorubicin (DOX, red

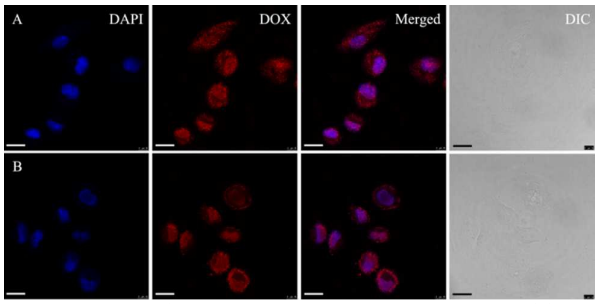

Fig. 5 CLSM images of H460 cells incubated with DOX-loaded PU-SS-I (A) and PU-SS-C (B) micelles for 2 h. Scale bar=20 μm.

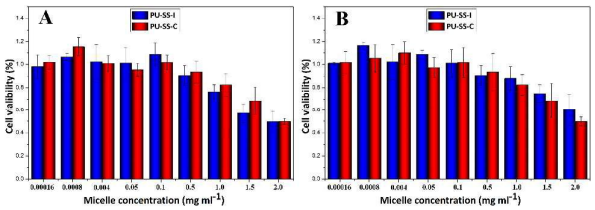

Fig. 6 Viability of H460 cells (A) and HUVECs (B) after 48 h of incubation with various concentrations of empty reduction-sensitive polyurethane micelles determined by the CCK8 assay.

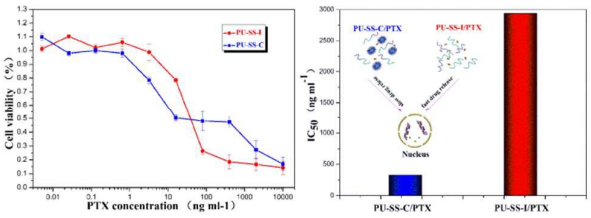

Fig. 7 Cytotoxicity (A) and IC_{50} values (B) of PTX-loaded polyurethane micelles against H460 cells after incubation for 24 h.

fluorescence) was encapsulated in the hydrophobic micellar cores by dialysis to label the nanocarriers and to track the internalization and intracellular localization of DOX in H460 cells.[30] The nuclei of H460 cells were stained by DAPI, which emits blue fluorescence to distinguish from the red fluorescence of DOX. Fig. 5 shows CLSM images of H460 cells incubated with DOX-loaded polyurethane micelles for 2 h. As shown in Fig. 5, the images of DOX fluorescence suggest that DOX-loaded polyurethane micelles were internalized and DOX was released to reach cell nuclei.[31, 32]

***In vitro* cytotoxicities of polyurethane micelles and PTX-loaded polyurethane micelles**

The *in vitro* cytotoxicity of drug-free and drug-loaded polyurethane micelles was evaluated in the CCK8 assay. Fig. 6 showed the effect of PU-SS-I and PU-SS-C concentration on the viability of HUVEC and H460 cells. The results demonstrate that empty PU-SS-I and PU-SS-C micelles show very low cytotoxicity (greater than 90% cell viability) in two different cell lines even at micelle concentrations up to 0.5 mg/mL; suggesting the non-toxic nature of polyurethane micelles to HUVEC and H460 cells. However, the empty PU-SS-I and PU-SS-C micelles show high cytotoxicity (60-70% cell viability) at concentration higher than 1.0 mg/mL. Further, the *in vitro* cytotoxicity of PTX-loaded PU-SS-I micelles was compared to that of PTX-loaded PU-SS-C micelles. Fig. 7A shows the cytotoxicity results given as a function of PTX concentration from 0.01 to 10000 ng/mL. As the test concentration was increased to 100 ng/mL, PU-SS-I encapsulated PTX exhibited lower cell viability (30%) than PU-SS-C encapsulated PTX (50%). The IC_{50} (i.e., inhibitory concentration to produce 50% cell death) values of PU-SS-I and PU-SS-C encapsulated PTX were determined to be ~300 and ~2900 ng/mL respectively for H460 cells (Fig. 7B)($P< 0.05$). The results reveal that the PU-SS-I micelles provide more efficient intracellular delivery of PTX as compared to PU-SS-C micelles.

Conclusions

We have developed redox responsive multi-blockedpolyurethaneswithdisulfide bonds positioned mainly either at the hydrophobic PCL junctions (PU-SS-C) or at the connections between the hydrophilic PEG and hydrophobic PCL blocks (PU-SS-I). Atconcentrations above the CMC, the polyurethanes self-assembled to form stable micelles having disulfide linkages located mainly in the cores for PU-SS-C micelles or at the interfaces for PU-SS-I micelles.In response to reductive environments, the disulfide linkages of both PU-SS-C and PU-SS-I were entirely cleaved, causing destabilization of the micelles and controlled release of the anticancer drugs inside the cancer cells. However, the speed of micelle destabilization and the release rate of the encapsulated drugs were quite different. PU-SS-I micelles show rapid destabilization and quicker release of the anticancer drugs as compared with PU-SS-C micelles. Internalization into H460 cancer cells and intracellular release of anticancer drugs was shown by CLSM. The *in vitro* cytotoxicity of drug-free and drug-loaded polyurethane micelles was evaluated by the CCK8 assay. The enhanced cytotoxicity of PTX-loaded PU-SS-I micelles against H460 cancer cells reveals that the PU-SS-I micelles are more potent for intracellular delivery of PTX as compared to PU-SS-C micelles. Furthermore, the two empty polyurethane micelles exhibited no cytotoxicity against cancer cells or healthy cells at concentrations as high as 500 μg/mL. These results demonstrate that multi-blocked polyurethanes are a promising drug delivery platform and that the release profile of the drugs can easily be tuned by the location of the disulfide bonds in the polyurethane.

Acknowledgements

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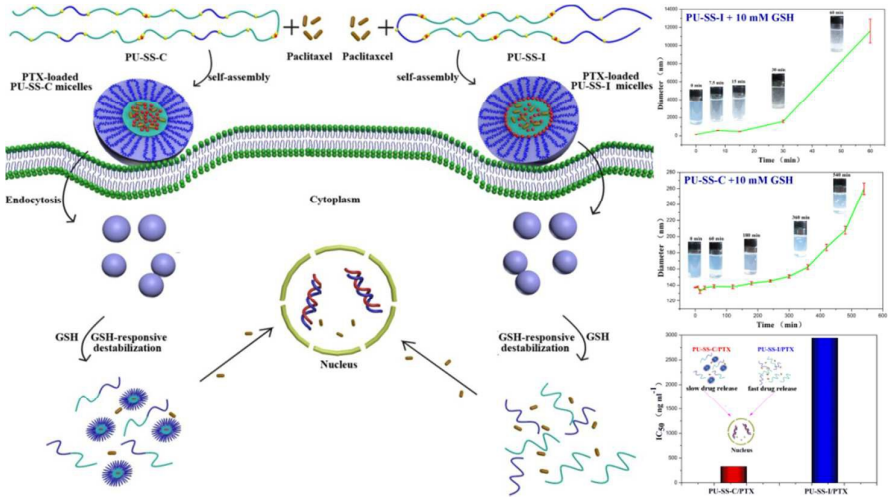
Biodegradable multi-blocked polyurethane micelles for intracellular drug delivery: the effect of disulfide location on the drug release profile

Graphical Abstract



Polyurethane micelles with disulfide bonds positioned mainly either at the hydrophobic PCL junctions (PU-SS-C) or at the connections between the hydrophilic PEG and hydrophobic PCL blocks (PU-SS-I) were developed as a antitumor drug carriers. It was demonstrated that the PU-SS-I micelles disassembled simultaneously in response to a 10 mM GSH stimulus and the payloads released more rapidly than that of PU-SS-C nanocarriers. The IC_{50} results revealed that the PU-SS-I micelles provided more efficient intracellular release of PTX as compared to PU-SS-C micelles.