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pH and reduction dual responsive cross-linked polyurethane micelles as an intracellular drug delivery system

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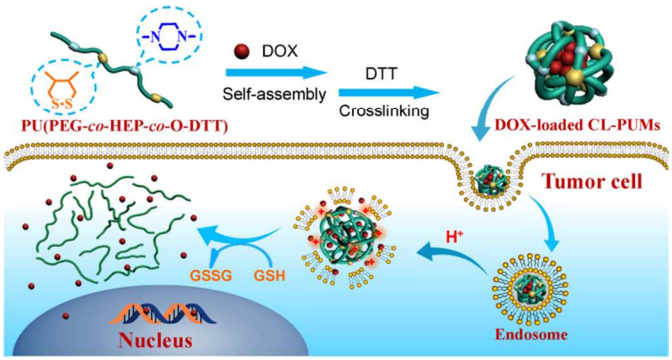
Nano-vehicles that exhibit the enhanced stability in blood circulation while spontaneously release therapeutic cargos at pathological sites in response to specific biological triggers are of interest for on-demand drug delivery. In this work, disulfide cross-linked polyurethane micelles (CL-PUMs) that respond to pH change and an intracellular reducing agent were developed. The micelles were prepared by cross-linking of poly(ethylene glycol)-polyurethane multiblock copolymers containing tertiary amino and cyclic disulfide moieties *via* a thiol-disulfide exchange reaction. The CL-PUMs tended to swell or decompose under a weakly acidic environment or in the presence of an intracellular reducing agent, glutathione (GSH), likely owing to the protonation of the tertiary amino groups and cleavage of the disulfide cross-linking bonds. The doxorubicin (DOX)-loaded CL-PUMs suppressed the initial burst release at pH 7.4 without GSH, while displayed a triggered drug release manner in response to an acidic environment and GSH. It was found that the intracellular DOX release of the DOX-loaded CL-PUMs in HepG2 cells was accelerated by an acidic environment or enhanced intracellular GSH concentration. Moreover, the time-dependent cytotoxicity against HepG2 and HeLa cells of the DOX-loaded CL-PUMs was confirmed by the MTT assay. Overall, due to the enhanced stability, selective swelling and decomposition properties in response to intracellular micro-environments, the pH- and reduction-sensitive polyurethane cross-linked nano-carriers can serve as a potential system for intracellular drug delivery.

Introduction

Nanoscale polymeric drug delivery systems have shown great potential for cancer chemotherapy owing to the enhanced permeability and retention (EPR) effect.[1-4] Nevertheless, to-date, positive clinical outcomes of cancer treatments by using polymeric nano-carriers are still limited. Among the key challenges remaining in anti-cancer chemotherapy, premature drug release from nano-vehicles at undesired sites usually leads to insufficient drug accumulation in tumor sites and enhances toxic side effects to normal tissues. [5] Therefore, the nano-carriers that remain stable in blood circulation while are able to spontaneously release cargos in response to some specific biological stimuli at tumor sites or intracellular space have attracted considerable interest for efficient anti-tumor drug delivery. Accordingly, stimuli-responsive polymeric nano-carriers that respond to various external stimuli, such as pH[6-10], glutathione (GSH)[11-14], temperature[15], light[16-18] and enzyme[19-21], have been extensively investigated. For instance, the drug release from pH-responsive nano-carriers may be triggered in the acidic endosomal/lysosomal compartments, leading to enhanced intracellular drug dose and cytotoxicity.

To reduce the premature drug release of drug-loaded nano-particles, polymeric micelles cross-linked by stimuli-cleavable linkages have been developed.[22] Bio-reversible disulfide linkage has been widely used as the cross-linker in various nano-carriers for intracellular drug delivery, attributed to its selective cleavage in the intracellular environment with relatively higher GSH concentration (1-10 mM) compared to the extracellular environment (2-20 μM).[5] Moreover, multi-stimuli-responsive nano-carriers have shown unique advantages for overcoming the multiple biological barriers during drug delivery.[23-26] Recently, polyurethane-based nano-carriers have received increasing attention due to their excellent biocompatibility and highly tunable functional moieties.[27-34] Nevertheless, to-date, reports on multi-stimuli-responsive cross-linked nano-carriers based on polyurethanes are still limited.

In this work, a series of novel type of pH- and reduction-responsive disulfide cross-linked polyurethane micelles (CL-PUMs) were prepared (Scheme 1). The change in the aggregation behavior in response to an acidic pH and GSH was studied by dynamic laser scattering (DLS). The triggered drug release behavior of the CL-PUMs loaded with an anti-cancer drug, doxorubicin (DOX), was revealed at a weakly acidic pH or in the presence of GSH. To show the environment-dependent intracellular drug release behavior, the DOX-loaded CL-PUMs were incubated with a HepG2 cell line and the intracellular drug release was observed by CLSM and flow cytometry. To further demonstrate the intracellular drug release and cytotoxicity of the DOX-loaded CL-PUMs, the cytotoxicities of the drug-loaded nano-particles against HepG2 and HeLa cells were studied at various DOX concentrations and incubation time.



Scheme 1. Schematic illustration for the formation of DOX-loaded CL-PUMs as well as the structural transitions of the nano-carriers in response to acidic micro-environment and GSH.

Experimental

Materials

Bis-1,4-(hydroxyethyl) piperazine (HEP), 1,6-hexamethylene diisocyanate (HDI), poly(ethylene glycol) (PEG, Mn = 2000), *trans*-4,5-dihydroxy-1,2-dithiane (O-DTT), glutathione (GSH) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich. 1,2-dichloroethane ($(CH_2)_2Cl_2$) and dibutyltin dilaurate (DBTDL) were purchased from Alfa. Prior to use, PEG was dried by an azeotropic distillation in toluene. Toluene was dried and distilled from sodium/benzophenone under a nitrogen atmosphere before use. Other reagents were commercially available and used as received. Doxorubicin hydrochloride (DOX·HCl) were obtained from Zhejiang Hisun Pharmaceutical Co., Ltd. All the other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd., China and used as obtained.

General Characterization

^{1}H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer. FT-IR spectra were recorded on a Bio-Red Win-IR instrument. Gel permeation chromatography (GPC) was performed by using a Waters 515 HPLC pump with a series of Shodex columns (KD802.5 and KD804) and OPTILAB DSP Interferometric Refractometer (Wyatt Technology) was used as the detector. The eluent was DMF containing 0.05 M LiBr at a flow rate of 1.0 mL/min at 50 °C. Linear polystyrene standards were used for calibration. Dynamic light scattering (DLS) measurements were performed on a WyattQELS instrument with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology). The scattering angle was fixed at 90°. Transmission electron microscopy (TEM) measurement was performed on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 KV. A drop of the micelle solution (0.2 g L^{-1}) was deposited onto a 230 mesh copper grid coated with carbon and allowed to dry in air at 25 °C before measurements. Zeta potential of drug-loaded NPs at pH 7.4 was measured with a zeta potential/BI-90plus particle size analyzer at room temperature, and the concentration of the NPs was 1.0 mg mL^{-1}.

Synthesis of polyurethane multiblock copolymers

The multiblock polyurethanes containing 1,2-dithiane and tertiary amino groups (PU(PEG-*co*-HEP-*co*-O-DTT)) was synthesized according to the method of our previous work with slight modification.[32, 33] The typical reaction procedure is shown in Scheme 2 and the feed ratios of all original materials are listed in Table S1. Briefly, *trans*-4,5-dihydroxy-1,2-dithiane (O-DTT), bis-1,4-(hydroxyethyl) piperazine (HEP) and PEG (Mn 2000) were completely dissolved in $(CH_2)_2Cl_2$ at 60 °C in a flask under a dry nitrogen atmosphere. DBTDL (0.5 mol% relative to hydroxyl groups) was added into the flask as a catalyst. Then, the requisite amount of hexamethylene diisocyanate (HDI) was added into the flask under stirring and the polymerization reaction was kept at 60 °C for 5 h. After the flask was cooled to room temperature, the resulting copolymer was isolated by precipitating in a 10-fold excess of diethyl ether. The product was further purified by repeatedly precipitation into diethyl ether from *N*,*N*-dimethylformamide (DMF) solution, and then dried under vacuum at room temperature for 48 h. The yield of the resulting copolymer was over 80%.

Acid-base titration

The pH-responsive property of the multiblock polyurethanes was determined by acid-base titration according to the literature method.[32] Briefly, PU(PEG-*co*-HEP-*co*-O-DTT)s was dissolved in 30 mL of deionized water at a concentration of 1 mg/mL and the pH was adjusted to ~ 3 using 1 M HCl. Then, the solution was titrated with 0.1 M NaOH solution, and the pH change was monitored by a pH-211 Microprocessor pH Meter (HANNA Instruments).

Preparation of disulfide cross-linked polyurethane micelles (CL-PUMs)

Polyurethane multiblock copolymer (50 mg) was fully dissolved in 10 mL of DMF. Then, 40 mL of deionized water

was added dropwise into the DMF solution with mild ultrasonication for 2 h. The organic solvent was removed by dialysis against deionized water for 24 h and the polyurethane micelles (PUMs) were obtained by filtering the aqueous solution through a 0.45 μm filter. The cross-linked polyurethane micelles (CL-PUMs) were prepared according to the procedure reported by Zhong and co-workers with slight modification.[12] The pH of above PUM solution was adjusted to 8.5 using phosphate buffer (PB, final PB concentration: 5 mM) and the dispersion was purged with nitrogen for 30 min. The cross-linking reaction was initiated by adding dithiothreitol (DTT, 10 mol % relative to the amount of dithiane units) under nitrogen at room temperature. After stirring for 24 hours, the above solution was dialyzed against distilled water for 1 day. Then, the CL-PUMs were obtained by filtering the solution through a 0.45 μm filter.

In vitro drug loading and release

DOX was loaded into polyurethane nanoparticles as a model drug by a co-precipitation method according to our previous method.[32, 33] DOX·HCl (50 mg), equimolar amounts of triethylamine and polyurethane (500 mg) were dissolved in 20 mL of DMF. The mixture was allowed to stand at room temperature for 2 h. Then, 60 mL of phosphate buffer (PB, pH = 8.5, 5 mM) was added dropwise into the mixture solution, followed by mild ultrasonication for 2 h. Most of the organic solvent was removed by dialysis against deionized water for 5 h at room temperature (MWCO 3500 Da). Then, the precursor solution was separated into two parts to prepare DOX-loaded PUMs and DOX-loaded CL-PUMs, respectively. DOX-loaded PUMs were obtained by further dialysis of the precursor solution against deionized water for 24 h at room temperature, followed by filtering the aqueous solution through a 0.45 μm filter. On the other hand, to prepare DOX-loaded CL-PUMs, the pH of the precursor solution was adjusted to 8.5 by addition of phosphate buffer (final PB concentration: 5 mM), and the dispersion was purged with nitrogen for 30 min. The crosslinking of the DOX-loaded micelles was then carried out according to the procedure to prepare CL-PUMs. Finally, the DOX-loaded CL-PUMs were obtained by filtering the solution through a 0.45 μm filter.

The amount of drug loaded in the nano-particles was determined by a Perkin-Elmer LS50B luminescence spectrometer. The drug loading content (DLC%) and drug loading efficiency (DLE%) were calculated based on the equations below:[35]

DLC% = (weight of drug in nano-particles / total weight of drug-loaded nano-particles) × 100%

DLE% = (weight of drug in nano-particles / total weight of drug in feed) × 100%

In vitro DOX release behavior from the DOX-loaded nano-particles was investigated in PBS at pH 5.5 or 7.4 with or without the presence of GSH. The pre-weighed drug-loaded nano-particles were suspended in 6 mL of PBS with or without GSH, and the particle solution was immediately transferred into a dialysis bag (MWCO 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag into 50 mL of PBS containing 0 or 10 mM GSH at 37 °C with continuous shaking at 72 rpm. At predetermined time intervals, 2 mL of external release medium was taken out and the volume withdrawn was replenished with an equivalent volume of fresh release medium. The amount of released DOX was assayed by using fluorescence measurement (λ_{ex} = 480 nm). The release experiments were performed in triplicate.

Confocal laser scanning microscopic observation

The intracellular release of DOX-loaded micelles was observed by confocal laser scanning microscopy (CLSM) using a HepG2 cell line. The cells were seeded in 6-well plates at a density of 1×10^5 cells per well in 2 mL of complete Dulbecco's modified Eagle's medium (DMEM) and cultured at 37 °C in 5% CO_2 atmosphere for 24 h. After removing the culture medium, the cells were incubated with DOX-loaded nanoparticles at a final DOX concentration of 10 mg L^{-1} in DMEM at 37 °C for additional 6 h. Then, the culture medium was removed and the cells were washed three times with PBS. After that, the cells were fixed with 4% formaldehyde for 20 min at room temperature, and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 5 min and the cell membranes were stained with Alexa Fluor 488 for 30 min. CLSM images of cells were obtained through a Carl Zeiss LSM 780 confocal microscope.

Flow cytometric analysis

The cellular uptake of DOX-loaded nano-particles in HepG2 cells was analysed by flow cytometric analysis. The cells were first seeded in 6-well plates at a density of 2×10^5 cells per well in 2 mL of complete DMEM and cultured at 37 °C in 5% CO_2 atmosphere for 24 h. The cells were then incubated with the DOX-loaded nano-particles under different conditions. The cells for the GSH-pretreated group were treated with 10 mM GSH for 2h and then washed with PBS three times before adding fresh DMEM containing drug-loaded nano-particles. On the other hand, the cells for the acid-treated group were incubated in acidic culture media (pH 6.8) containing the DOX-loaded nano-particles for 3 h. The final DOX concentration was 10 mg L^{-1}. The cells without additional treatment were used as control. After the culture medium was removed, the cells were washed with PBS three times and treated with trypsin. Then, 1 mL of PBS was added to each culture well, and the solutions were centrifuged for 5 min at 3000 rpm. After removal of the supernatants, the cells were resuspended in 0.5 mL of PBS. Data for 1×10^4 gated events were collected, and analysis was performed by flow cytometer (Beckman, California, USA).

Cell viability assays

The relative cytotoxicities of the polyurethane multiblock copolymer and the DOX-loaded polyurethane nano-particles against HepG2 and HeLa cells were evaluated *in vitro* by MTT assay. Generally, the cells were seeded in 96-well plates at a density of 7,000 cells per well in 200 μL of complete DMEM containing 10% fetal bovine serum, supplemented with 50 U

mL^{-1} penicillin and 50 U mL^{-1} streptomycin, and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. After removing culture media, the plates were supplied with fresh culture media containing polyurethane at different concentrations (0~1000 mg L^{-1}) or DOX-loaded nanoparticles with different DOX concentrations (0~5 mg L^{-1} DOX). The cells were subjected to MTT assay after incubation for additional 24, 48 and 72 h, respectively. The absorbency of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated based on the following equation: ($A_{sample}/A_{control}$) × 100%, where A_{sample} and $A_{control}$ represent the absorbencies of the sample and control wells, respectively.

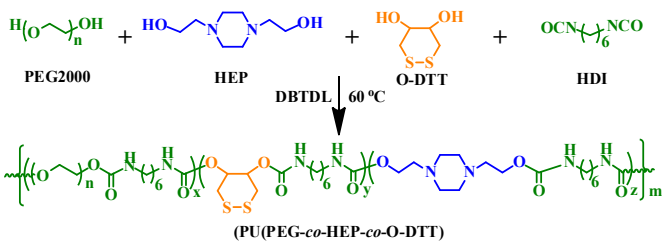
Statistical analysis

The paried student's t-test was used to analyze the statistical differences of the cytotoxicities between treated groups. The date were presented as mean ± standard deviation which were obtained from three separate experiments.

Results and discussion

Synthesis and characterization of PEG/polyurethane multiblock copolymers

Polyurethanes have been widely used in biomedical field for their good biocompatibility. Recently, stimuli-responsive polyurethane-based micelles were developed and have shown huge potential as triggered drug delivery systems.[23, 29, 30, 36] Herein, PEG-polyurethane multiblock copolymers containing tertiary amino groups and cyclic disulfide moieties were synthesized *via* the condensation polymerization of HEP, O-DTT, PEG and HDI (Scheme 2).



Scheme 2. Synthesis route of PU(PEG-*co*-HEP-*co*-O-DTT) multiblock copolymers.

The chemical structures of the final products were identified by ^{1}H NMR, FT-IR and GPC, respectively. Figure 1 shows the ^{1}H NMR spectrum of the block copolymers. The peak centered at 3.65 ppm (peak a) is attributed to the methylene protons of PEG. The resonances at 3.12 ppm (peak b), 1.45 ppm (peak c) and 1.29 ppm (peak d) are assigned to the methylene protons of HDI units. The peaks at 4.14 ppm (peak g), 2.59 ppm (peak h) and 2.52 ppm (peak i) are ascribed to the methylene protons of HEP units. Moreover, peaks e and f confirmed the existence of O-DTT units in the copolymer. Additionally, the absence of any absorbance at 2275 - 2250 cm^{-1} in FT-IR spectrum suggested the complete consuming of the isocyanate groups during the reaction (Figure S1 in supporting information). The chemical compositions of the multiblock copolymers were calculated based on the NMR result. As listed in Table S1 in supporting information, the contents of PEG segments in the copolymers were 49 wt% - 54 wt%, and the dithiane/piperazine molar ratio changed from 1:2.4 to 2.6:1. Moreover, the molecular weights (MWs) and polydispersities (PDIs) of the multiblock copolymers were determined by GPC measurement as listed in table S1. All above data suggested that the mutiblock polyurethanes have been successfully synthesised.

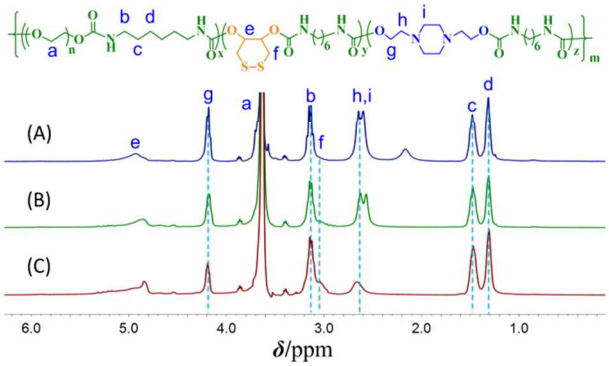


Figure 1. ^{1}H NMR spectra of PU(PEG-*co*-HEP-*co*-O-DTT)-1 (A), -2 (B) and -3 (C) in $CDCl_3$.

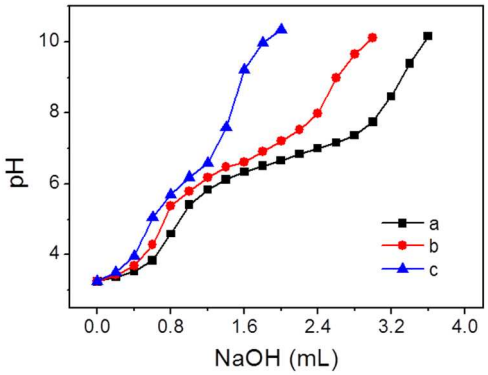


Figure 2. Acid-base titration curve of the acidified solution of PU(PEG-*co*-HEP-*co*-O-DTT)-1 (a), -2 (b) and -3 (c) titrated with 0.1 N NaOH.

The pH buffering capacity of the multiblock polyurethanes was checked by a titration method. It was found that the copolymers displayed different extents of buffering capacity in the pH region of 5.5 – 7.5, suggesting a protonation-to-deprotonation transition of the tertiary amino groups. As shown in Figure 2, the pH buffering effect of the polymers was dependent on the content of HEP units. With the increase in the HEP content, the pH buffering capacity increased obviously. Moreover, the pK_a values of the polyurethanes were estimated to be around 6.6 from the derivative values of the titration curves, which based on the inflection point.[37, 38]

Characterization of pH- and reduction-sensitive cross-linked polyurethane micelles (CL-PUMs)

It has been established that the size and stability of drug-loaded nanoparticles play a key role in drug delivery behavior *in vivo*.[1, 39] Herein, a dialysis method was used to prepare the polyurethane micelles (PUMs) according to our previous experiment. [32] The disulfide-cross-linked polyurethane micelles (CL-PUMs) were then prepared by the intermolecular cross-linking of the polyurethane copolymers through a thiol-disulfide exchange reaction.[33] The cross-linking of the polyurethane micelles was carried out by addition of a catalytic amount of DTT (10 mol % relative to the amount of dithiane units) under a nitrogen atmosphere in PB (pH 8.5, 5 mM) according to the literature method.[12]

Table 1. Characterization of PUMs and CCL-PUMs.

Sample code	Molar ratios in Polymer [a] PEG/O-DTT/HEP/HDI	R_h [b] (nm)	DLC (%)	DLE (%)
PUMs-1	0.74/1.0/2.4/4.1	99.4 ± 30.6	3.49	38.4
PUMs-2	0.33/1.0/0.98/2.3	72.5 ± 22.3	3.31	36.4
PUMs-3	0.74/2.6/1.0/4.4	81.5 ± 25.1	3.99	44.0
CL-PUMs-1	/	56.1 ± 16.7	4.19	46.1
CL-PUMs-2	/	58.8 ± 13.1	4.53	49.8
CL-PUMs-3	/	66.2 ± 13.1	4.16	45.8

[a] Determined by NMR. [b] Determined by DLS at pH = 7.4.

The polyurethane nanoparticles were studied by DLS and TEM (Figure 3 and supporting information: Figure S2). It was found that all the CL-PUMs displayed reduced sizes compared to their precursor PUMs at pH 7.4 (Figure 3 and Table 1), likely due to the formation of more compact structures after cross-linking.[40] The TEM observation indicated that the CL-PUMs took spherical morphology (Figure 3 (F)). Moreover, to reveal the stability of the cross-linked nano-particles, freeze-dried CL-PUMs and PUMs were dispersed in DMF, which is a good solvent for the polyurethane block copolymers, and the solutions were characterized by DLS and TEM. As shown in Figure S2, CL-PUMs-2 maintained spherical nano-aggregate morphology in DMF, in contrast to no aggregation was observed for the un-cross-linked polyurethane solution in DMF. The small peak around 5 nm in the CL-PUMs DMF solution shown in Figure S2 may be attributed to the small aggregates that were cross-linked incompletely.[41] These results indicated that the CL-PUMs obtained enhanced stability due to the disulfide cross-linking structure.[42]

Furthermore, to evaluate the pH and reduction-responsive properties of the polyurethane nano-particles, DLS measurements were carried out under different conditions. As shown in Figure 3, the aggregation behaviors of both PUMs and CL-PUMs were influenced markedly by the pH. As pH declined from 7.4 to 5.5, the R_h of the PUMs-2 increased obviously from 72.5 nm to 165 nm, and that of CL-PUMs-2 showed an increase from 59 nm to 88 nm (Figure 3(C, D)). This may be attributed to the swelling of the nano-particles caused by the protonation of the tertiary groups at acidic pH. Additionally, CL-PUMs-2 exhibited lower swelling ratio compared to PUMs-2 at pH 5.5, attributed to the cross-linked structure of CL-PUMs (Figure 3 (D)). Moreover, the reduction-triggered conformation change of CL-PUMs was evaluated under a reducing condition mimicking the intracellular circumstance. The change in the size of CL-PUMs in response to GSH was monitored over time in PBS (pH 7.4) containing 10 mM GSH. As shown in Figure 3 (E), the addition of GSH (10 mM) resulted in a marked increase in the R_h of the nano-particles, likely due to the gradual dissociation of nano-particles caused by the cleavage of disulfide cross-linking linkages.[12, 43]

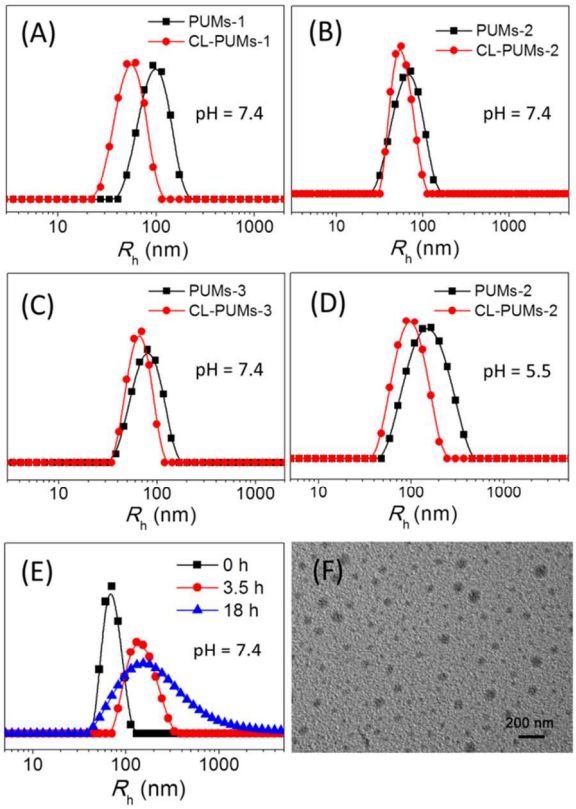


Figure 3. The hydrodynamic radii (R_h) of PUMs-1 and CL-PUMs-1 (A), PUMs-2 and CL-PUMs-2 (B), PUMs-3 and CL-PUMs-3 (C) at pH 7.4; PUMs-2 and CL-PUMs-2 (D) at pH 5.5; change in R_h of CL-PUMs-2 after addition of 10 mM GSH at pH 7.4 (E); TEM image taken after drying of the CL-PUMs-2 aqueous solution (F).

In vitro pH- and reduction-triggered drug release

DOX was loaded into the polyurethane nano-particles as a model drug. The *in vitro* drug release behavior of the DOX-loaded polyurethane nano-particles was evaluated at different pH with or without the presence of GSH. The drug loading content (DLC) and drug loading efficiency (DLE) of the CL-PUMs were determined to be 4.16% - 4.53% and 45.8% -

49.8%, respectively (Table 1). Generally, the CL-PUM examples showed enhanced DLCs and DLEs compared to their un-cross-linked precursor micelles, likely attributed to the increased stability of the DOX-loaded micelles after crosslinking.[44]

The drug release behaviors of DOX-loaded PUMs and CL-PUMs in response to pH change and GSH were investigated (Figure 4). As shown in Figure 4 (A), obvious burst release was observed from the DOX-loaded un-cross-linked PUMs in PBS at pH 7.4 without the presence of GSH. About 30 % of DOX was released from the DOX-loaded PUMs with different compositions in 5 h and about 37% were released in 24 h at pH 7.4 without GSH. In contrast, the initial burst release of DOX was markedly supressed for the DOX-loaded CL-PUMs at pH 7.4 without GSH, and only 10 - 17 % of DOX was release in 24 h under the same condition. This suggested that DOX-loaded disulfide cross-linked micelles displayed enhanced stability in PBS (pH 7.4) without GSH compared to the un-cross-linked counterpart.

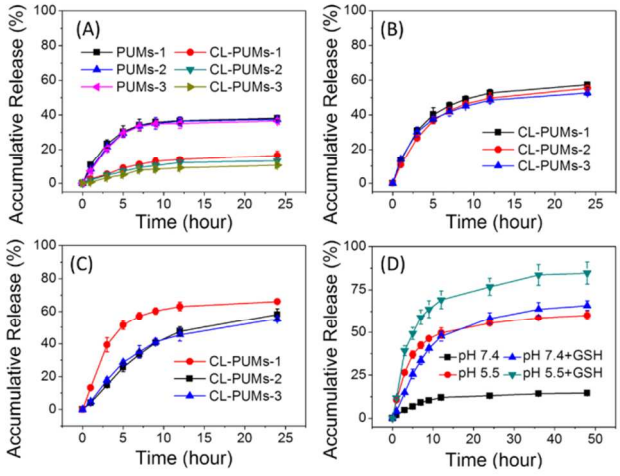


Figure 4. The drug release behaviors of DOX-loaded PUMs and CL-PUMs in PBS at different conditions. (A) DOX-loaded PUMs and CL-PUMs at pH 7.4 without GSH; (B) DOX-loaded CL-PUMs at pH 5.5 without GSH; (C) DOX-loaded CL-PUMs at pH 7.4 with 10 mM GSH; (D) DOX-loaded CL-PUMs-2 under different stimuli. Data are presented as the average ± standard deviation (n = 3).

On the other hand, it was found that the DOX release from the DOX-loaded CL-PUMs was obviously accelerated by either lowering the pH or adding GSH, indicating a triggered release manner in response to acidic environment or GSH. As show in Figure 4 (B), with reducing the pH from 7.4 to 5.5, the amount of DOX released from the DOX-loaded CL-PUMs in 24 h was markedly increased to 52% - 57%, due to the swelling of the micelles caused by the ionization of the piperazine units. Additionally, addition of GSH (10 mM) also led to the obvious increase in the drug release rates of DOX-loaded CL-PUMs (Fig 4 (C)). The accelerated drug release of DOX-loaded CL-PUMs with addition of GSH should be attributed to the dissociation of the cross-linked nano-particles resulted from the cleavage of the disulfide cross-linking bonds. It is noteworthy that the sample containing less dithiane units (denoted as CL-PUMs-1 in Table 1), which implied less cross-linking density, displayed a higher drug release rate compared to those containing relatively higher dithiane units (CL-PUMs-2 and CL-PUMs-3) with the presence of 10 mM GSH (Figure 4 (C)). This should be attributed to the faster de-cross-linking for the DOX-loaded CL-PUMs-1 in response to GSH. Notably, the highest drug release rate was detected when dual stimuli were applied. As shown in Figure 4 (D), at pH 5.5 and with the presence of 10 mM GSH, ~ 85% of DOX was released at 48 h. This phenomenon may be reasonably attributed to the faster decomposition of the micelles under the dual stimuli.

Overall, the *in vitro* drug release study clearly revealed an enhanced stability of the DOX-loaded CL-PUMs in the PBS (pH 7.4) without GSH, but triggered drug release profiles in response to an acidic environment and GSH. It is noteworthy that the DOX-loaded CL-PUMs-2 and CL-PUMs-3 displayed slower drug release profiles at pH 7.4 with or without GSH compared to DOX-loaded CL-PUMs-1, which contains less dithiane units for cross-linking (Figure 4 (A) and (C)). Moreover, a higher drug loading efficiency was obtained for the DOX-loaded CL-PUMs-2 (Table 1). Therefore, DOX-loaded PUMs-2 and CL-PUMs-2 were selected for the further intracellular drug delivery and cytotoxicity tests.

Intracellular drug release

The cellular uptake and the intracellular drug release of DOX-loaded polyurethane nano-particles in HepG2 cells were measured by using flow cytometric analysis and confocal laser scanning microscopy (CLSM) under different conditions.

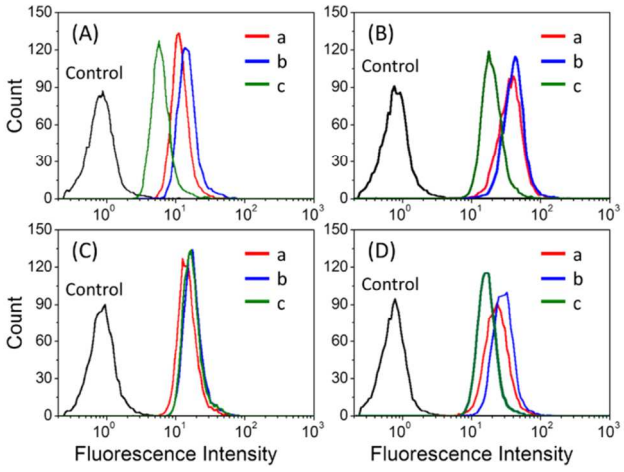


Figure 5. Flow cytometric profiles for HepG2 cells incubated with Free DOX (a), DOX-loaded PUMs-2 (b) and DOX-loaded CL-PUMs-2 (c) under different conditions: incubated at pH 7.4 for 1.5 h (A) and 6 h (B); incubated at pH 6.8 for 3 h (C); incubated at pH 7.4 for 3 h and cells were pretreated with 10 mM GSH before addition of free DOX or the DOX-loaded nano-particles (D). The DOX concentration was fixed at 10 mg L^{-1}.

As show in Figure 5 (A), when the HepG2 cells were incubated with free DOX or DOX-loaded nano-particles at pH 7.4 without GSH for 1.5 h, the lowest fluorescence intensity of DOX was observed in the cells incubated with DOX-loaded CL-PUMs. This may be attributed to the enhanced extracellular stability and the time-dependent intracellular triggered de-cross-linking and swelling process for the DOX-loaded CL-PUMs.[10, 12] Notably, as shown in Figure 5 (A), it was found that the group treated with the DOX-loaded PUMs-2 displayed slightly higher DOX fluorescence intensity compared to the free DOX treated group in the initial stage (1.5 h). This phenomenon may be due to the enhanced endocytosis of the DOX-loaded PUMs caused by the weakly positively charged surface of the piperazine-containing nano-particles with a zeta potential of 1.53 ± 0.93 mV at pH 7.4, as well as the fast intracellular drug release.[31, 45, 46, 47] Whereas, for the DOX-loaded CL-PUMs (zeta potential: 1.95 ± 1.09 mV at pH 7.4), the time-dependent de-cross-linking of the nano-particles in the intracellular environment led to a delayed DOX release and probably the self-quenching of DOX fluorescence at the initial stage (1.5 h). [48, 49] The DOX release from CL-PUMs was confirmed by further incubation of the cells for 6 h (Figure 5 (B)). CLSM observation showed a similar trend of the intracellular DOX release from the polyurethane nano-particles (Figure 6).

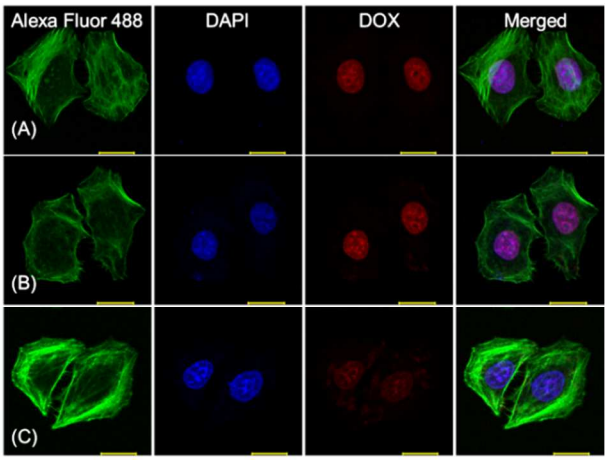


Figure 6. CLSM images of HepG2 cells incubated with Free DOX (A), DOX-loaded PUMs-2 (B) and DOX-loaded CL-PUMs-2 (C) at pH 7.4 for 6 h. For each panel, the images from left to right show cell membrane stained by Alexa Fluor 488, cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the three images.

To further demonstrate the triggered drug release behavior in response to the environment mimicking intracellular acidic or reduction condition, the drug-loaded nano-particles were incubated with HepG2 cells at pH 6.8 or with the cells pretreated by GSH. As shown in Figure 5 (C), it was observed that the fluorescence intensity of DOX-loaded CL-PUMs group was obviously enhanced at pH 6.8 and comparable to the DOX-loaded PUMs group, indicating the swelling of the nano-particles under weakly acidic condition. Additionally, it was found that, after pretreatment of the cells with GSH, the DOX fluorescence intensity in the cells incubated with the DOX-loaded CL-PUMs was also increased (Figure 5 (D)). The results suggested that the drug release from the DOX-loaded CL-PUMs was accelerated by the acidic environment or the enhanced intracellular GSH concentration.[33]

In vitro cytotoxicity of the DOX-loaded nano-particles

The *in vitro* cytotoxicity of the polyurethane multi-block copolymers against HepG2 and HeLa cell lines was evaluated by MTT assay. As shown in Figure 7 (A), the viabilities of both cells maintained over 95% after incubation with the block copolymer at all concentrations up to 1000 mg L^{-1} for 72 h, demonstrating low cytotoxicity of the copolymer.

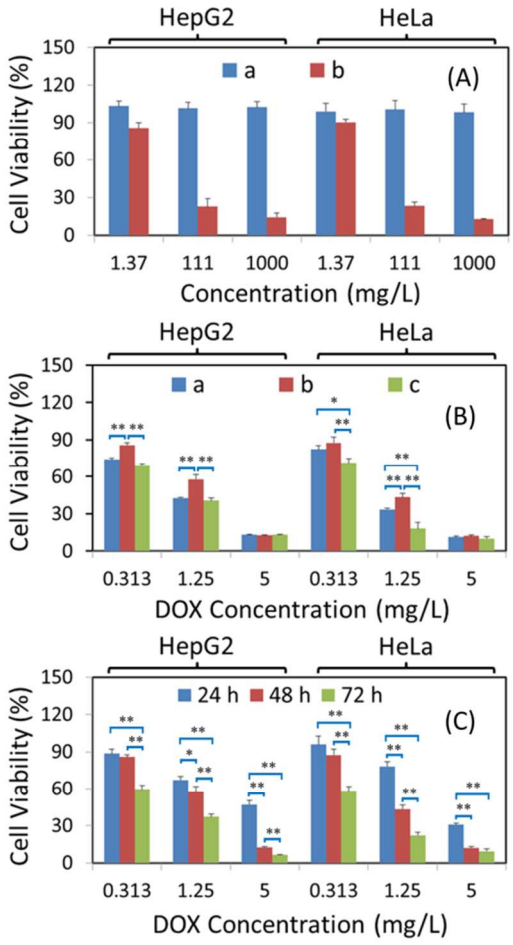


Figure 7. (A) *In vitro* cytotoxicity of PU(PEG-*co*-HEP-*co*-O-DTT)-2 (a) with PEI-25K (b) as a positive control. HepG2 and HeLa cells were incubated with the copolymer for 72 h. (B) Viabilities of HepG2 and HeLa cells after incubation with DOX-loaded PUMs-2 (a), DOX-loaded CL-PUMs-2 (b) and free DOX (c) at various DOX concentrations for 48 h. (C) Viabilities of HepG2 and HeLa cells after incubation with DOX-loaded CL-PUMs-2 at various DOX concentrations for 24 h, 48 and 72 h. Date were presented as mean ± standard deviation (n=3) (*$p < 0.05$, **$p < 0.01$).

The *in vitro* cytotoxicities of DOX-loaded polyurethane nano-particles were evaluated by MTT using HepG2 and HeLa cell lines. As shown in Figure 7 (B), the DOX-loaded CL-PUMs-2 displayed lower cytotoxicity compared to the DOX-loaded un-cross-linked counterpart after incubated 48 h. This could be reasonably attributed to the time-dependent swelling and de-cross-linking process after cellular uptake of the DOX-loaded CL-PUMs.[10, 12] The drug release from the DOX-loaded CL-PUMs-2 could be confirmed by the time-dependent reduction of the cell viability (Figure 7 (C)). It is noteworthy that, even though the DOX-loaded cross-linked micelles showed lower cytotoxicity compared to free DOX or the un-cross-linked counterpart in the cytotoxicity test *in vitro*, the environment-responsive cross-linked micelles with enhanced stability may facilitate the tumor accumulation of the DOX and showed relatively lower toxic side-effects *in vivo*. [9, 12, 33]

Conclusions

In this work, pH- and reduction-responsive disulfide cross-linked micelles based on PEG-polyurethane multiblock copolymers were developed. The DOX-loaded CL-PUMs exhibited obvious suppression on the initial burst release compared to the un-cross-linked counterpart in PBS at pH 7.4 without GSH. In contrast, the disulfide-cross-linked nano-particles tended to swell or decompose in response to acidic environment or intracellular reducing agent, leading to triggered drug release profiles. The intracellular DOX release from the DOX-loaded cross-linked nano-carriers was accelerated by acidic environment or enhanced intracellular GSH concentration. Despite the enhanced stability of the disulfide cross-linked nano-carriers at pH 7.4 without GSH, the time-dependent increase in the cytotoxicity against tumor cells was displayed. Due to the selective swelling and de-cross-linking behaviors under the conditions mimicking intracellular microenvironments, the pH- and reduction-responsive polyurethane cross-linked nano-vehicles may serve as a platform for intracellular drug delivery.

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Notes and references

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