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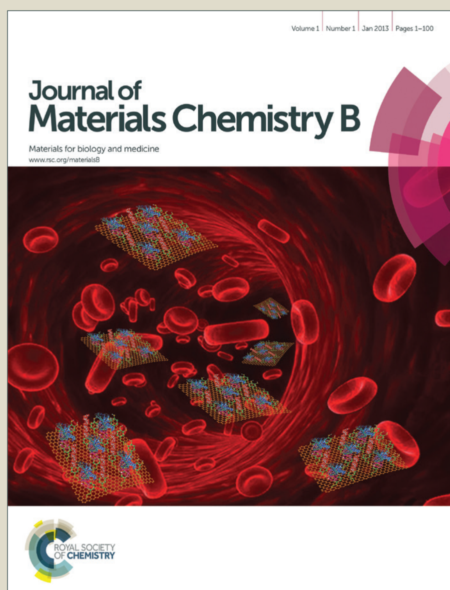

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ARTICLE

Reduction-Triggered Release of Paclitaxel From *In Situ* Formed Biodegradable Core-Crosslinked Micelles†

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Paclitaxel-loaded reduction-responsive core-crosslinked micelles were in situ prepared in aqueous media via “click” chemistry. An amphiphilic block copolymer with multiple pendant azide groups was first synthesized through the controlled ring-opening copolymerization of ε-caprolactone (CL) and 5,5-dibromomethyl trimethylene carbonate (DBTC) in the presence of methoxy poly(ethylene glycol) (mPEG) as macroinitiator, followed by azidation. Then this amphiphilic block copolymer could self-assemble into micelles and encapsulated paclitaxel (PTX) into the micellar core to form PTX-loaded micelles, which were in situ core-crosslinked by propargyl dithiopropionate via “click” chemistry, to develop a reduction-responsive polymeric drug delivery system. The in vitro release studies showed a minimized release of PTX under physiological conditions, while a burst release of PTX in response to reductive conditions. The core-crosslinked micelles display an efficient cell-uptake and reduction-responsive drug release due to the nanoscale diameter and the splitting of disulfide bonds under reductive environment, which was confirmed by confocal laser scanning microscopy using Nile red as a fluorescent probe. Hence, this kind of polymeric nano-carriers with excellent biocompatibility and quick reduction-response has opened a new avenue to intracellular anticancer drug delivery.

Introduction

Nowadays, a steadily increasing attention has been paid on the treatment of cancer, which is one of the major causes of mortality in the world.[1] Although the conventional chemotherapy has proved partially successful in cancer therapy, it is still limited by the lack of tumor-selectivity of anticancer drugs, resulting in severe side effects to normal tissues. To circumvent these limitations, tremendous attempt has been concentrated on the development of smart anticancer drug delivery systems for targeted delivery of anticancer drugs.[2-7] Particularly, self-assembled micelles with nanoscale size and core/shell architecture, enabling encapsulation of hydrophobic biomolecules and minimizing the dose-limiting systemic toxicity by preferential accumulation in tumors via the enhanced permeability and retention (EPR) effect has received widespread attention.[8-11]

However, polymeric micelles, which were formed by self-assembly of amphiphilic copolymers above the critical micelle concentration (CMC), might be disintegrated into unimers *in vivo* and resulted in premature release of the encapsulated drug in unexpected location.[12, 13] Aiming to improve the stability of micelles and achieve controlled drug release, various stimuli-sensitive crosslinkable micelles have been developed in recent years.[14-17] These smart nanocarriers are except to stably encapsulate therapeutics in circulation and release them at a desired site in response to specific microenvironmental condition within the target tumor tissues.[18, 19] Particularly, smart reversible disulfide crosslinkable micelles have attracted extensive attention due to the fact that intracellular concentration of GSH (1-10 mM) is substantially higher than that extracellularly with only 10-100 μM.[20] These considerable differences in the redox potential may selectively trigger the reduction (cleavage) of the disulfide crosslinker and resulting in a controlled release of the loaded drugs intracellularly.[21-27]

Importantly, for any drug delivery systems to be practically useful in biomedical, the nevertheless fundamental consideration is to tailor a polymer with great biocompatibility and appropriate biodegradability.[28] PEGylated polyesters, as a major material for micelle preparing and applied for drug delivery, have received much attention contributing to their biodegradability, biocompatibility, nontoxicity and facile preparation via ring-opening polymerization (ROP) of cyclic monomers with PEG as macroinitiator.[29-32] Among these, polymeric micelles self-assembled based on PCL and PEG have been extensively studied in biomedical area for both of them are approved by U.S. Food and Drug Administration (FDA).[33-36] Moreover, various stimuli-responsive agents have been incorporated into PEGylated polyesters to develop stimuli-sensitive drug delivery systems by using functionalized cyclic esters or carbonates as comonomer for ring-opening polymerization (ROP).[37-39] However, most of these monomers are difficult to synthesize and needed protection and deprotection procedure, resulting in multiple synthetic and purification steps.

Recently, our research group reported a facile synthesis of poly(ester-carbonate) bearing multiple bromo groups by ROP based on 5,5-dibromomethyl trimethylene carbonate (DBTC), which could be synthesized in a much easier and faster strategy within one step.[40] Particularly, the pendent bromide groups in resulting copolymer can be easily modified by quaternization[41] and azidation[42, 43] for further applications. Based on our previous work, herein we reported a facile strategy to synthesize amphiphilic methoxy poly(ethylene glycol)-*b*-poly((ε-caprolactone)-*co*-(5,5-diazidomethyl trimethylene carbonate)) (mPEG-*b*-PDATCL) by the ring-opening

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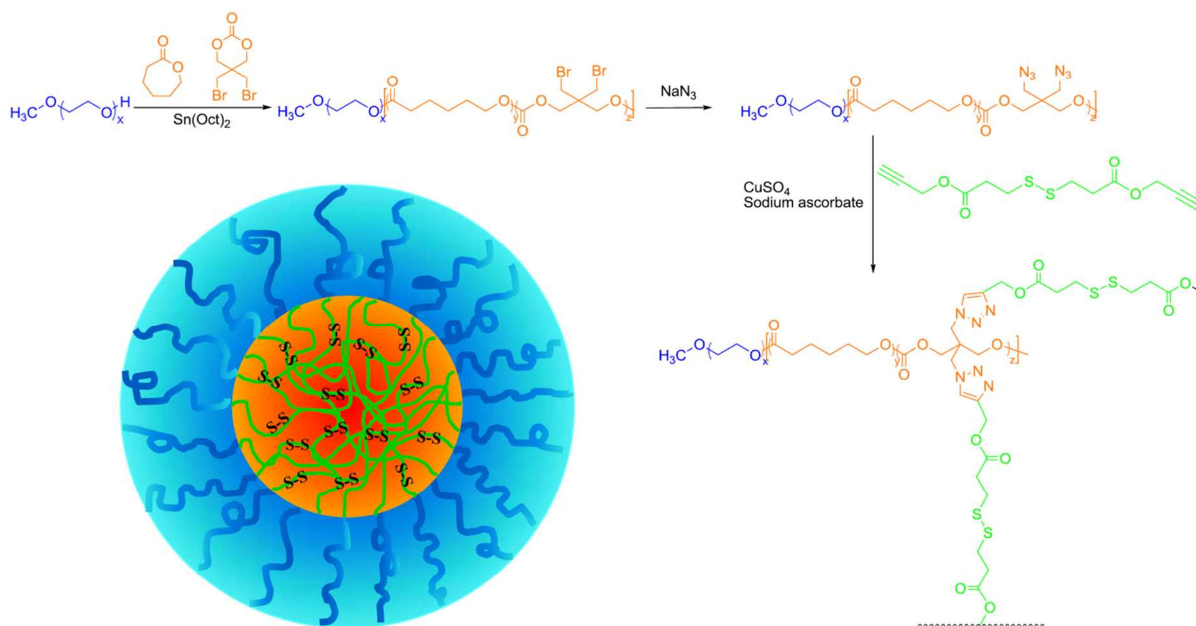
copolymerization of CL and DBTC in the presence of mPEG as macroinitiator, as well as azidation. Then paclitaxel-loaded reduction-sensitive CCL micelles were *in situ* prepared in aqueous media by "click" chemistry using propargyl 3,3'-dithiopropionate as crosslinker. Notably, the disulfide CCL micelles showed excellent stability under the extracellular environment, but were prone to de-crosslinking under an intracellular reductive environment. *In vitro* PTX release from PTX-loaded CCL micelles showed an accelerated release in the presence of 10 mM DL-dithiothreitol (DTT) mimicking the intracellular conditions. Moreover, the cytotoxicity, cellular uptake and apoptosis ability of CCL micelles against Hela cells were also determined using CCK-8 assay and confocal laser scanning microscopy (CLSM), respectively.



Scheme 1 Synthetic routes of disulfide CCL micelles

Experimental

Materials

Methoxy poly(ethylene glycol) (Fluka; M_n = 5 K) denoted as $mPEG_{113}$ were dried by azeotropic distillation in the presence of anhydrous toluene. DBTC was synthesized according to our previous report.[44] Triethylamine (TEA) and *N,N*-dimethylformamide (DMF) were dried over CaH_2 and distilled just prior to use. ε-Caprolactone (99 %; Acros) was distilled under reduced pressure before use. $CuSO_4 \cdot 5H_2O$ (Sinopharm Chemical Reagent; China), Suberic acid (99 %; Energy Chemical; China), 3,3'-dithiodipropionic acid (99 %; Aladdin; China), propargyl bromide (99 %; Aladdin; China), sodium ascorbate (99 %; Aladdin; China), stannous octoate ($Sn(Oct)_2$; 97 %; Sinopharm Chemical Reagent; China), Nile red (NR; 99 %; Sigma), PTX (99.5 %; Haoxuan Bio-technique Co. Ltd; China) and other reagents were used as received.

Synthesis of mPEG-*b*-PDATCL

1.0 g of $mPEG_{113}$ (0.2 mmol), 0.81 g of DBTC (2.8 mmol) and 0.68 g of CL (6 mmol) were added into a round-bottom reactor, which was sealed, evacuated, and back-filled with argon for three times. Then 18 mg of $Sn(Oct)_2$ (0.045 mmol) was added under argon atmosphere. The reactor was immersed into a thermostated oil bath and stirred for 12 h at 130 ºC. Then crude product was dissolved in methylene chloride and precipitated into cold ethyl ether, isolated by filtration, dried under high vacuum to constant weight at room temperature. Yield: 2.34 g (94 %). 2.0 g of poly(ethylene glycol)-*block*-poly((ε-caprolactone)-*co*-(5,5-dibromomethyl trimethylene carbonate)) (mPEG-*b*-PDBTCL; 4.5 mmol of bromo groups) and 0.58 g of NaN_3 (9.0 mmol) were dissolved in 30 mL DMF. The reaction mixture was stirred for 100 h at 80 ºC, then filtered and evaporated to remove DMF. The crude product was dissolved in 50 mL methylene chloride and washed three times with distilled water, dried with anhydrous $MgSO_4$, and filtered. The filtrate was concentrated and poured into ethyl ether to precipitate mPEG-*b*-PDATCL, which was dried in vacuum overnight. Yield: 1.8 g (98 %).

Synthesis of Crosslinker Propargyl 3,3'-Dithiopropionate and Dipropargyl Suberate

2.1 g of 3,3'-dithiodipropionic acid (10 mmol) and 1.2 g of KOH (21.5 mmol) were dissolved in 25 mL DMF, stirred at 100 °C for 1.5 h. Then 2.38 g of propargyl bromide (20 mmol) was added dropwise to the solution during 0.5 h. After that, the mixture was stirred and reacted at 70 °C for 72 h, then terminated the reaction by cooling to room temperature. After filtration and concentration on a rotary evaporator, the residue was dissolved in CH_2Cl_2 and then washed three times with distilled water. Dried the organic layer with anhydrous $MgSO_4$, and then removed CH_2Cl_2 in vacuum to obtain the product. 2.49g of a brown viscous liquid in 87% yield was obtained after drying the product under vacuum overnight. Dipropargyl suberate was synthesized based on 2.1 g of suberic acid (10 mmol), 1.2 g of KOH (21.5 mmol) 2.38 g of propargyl bromide (20 mmol) in the same manner as described above. Yield: 2.65 g (88 %).

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Preparation of PTX-loaded CCL Micelles

25 mg of mPEG-*b*-PDATCL (0.062 mmol of azide group), 8.8 mg of propargyl 3,3'-dithiopropionate (0.031 mmol) and 2.5 mg of PTX were dissolved in 15 mL of THF and stirred for 0.5 h. Then 15 mL of phosphate buffer (PBS, 0.01 M, pH 7.4) was added dropwise to the solution under stirring. The resulting solution was stirred for 2 h and evaporated to remove THF under vacuum. Then sodium ascorbate (12.3 mg; 0.062 mmol) and copper sulfate (15.4 mg; 0.062 mmol) was added to the reactor under argon atmosphere. The mixture was stirred for 24 h, then dialyzed (MWCO 3500, Fisher Scientific) against PBS (0.01 M, pH 7.4) over 24 h. The final concentration of the micellar solution was adjusted to 1.0 mg/mL. The solid state of drug-loaded micelles was retrieved by lyophilization, the residue was dissolved in ethanol and the amount of PTX trapped in the micelles was determined by UV (Fig. S4). Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following equations:

$$DLC\ (\%) = W_D/W_P \times 100\ \% \qquad (1)$$

$$DLE\ (\%) = W_D/W_{D0} \times 100\ \% \qquad (2)$$

W_D is the weight of PTX calculated from UV, W_P is the weight of copolymer, W_{D0} is the total weight of PTX used in drug-encapsulated experiment.

***In vitro* PTX Release from CCL Micelles**

5 mL of PTX-loaded micellar solutions (1.0 mg/mL) in PBS (0.01 M, pH 7.4, used except additional statement) were transferred into a dialysis membrane bag (MWCO 3500, Fisher Scientific), which were then immersed in 50 mL of PBS with or without DTT (10 mM), and suspended in a water bath at a constant temperature of 37 °C with horizontal shaking. At each predetermined time interval, 10 mL of incubated solution was taken out and replenished with an equal volume of corresponding PBS. PTX release profiles were determined by measuring the absorbance in ethanol at a wavelength of 227 nm using a Shimadzu UV2550 UV-vis spectrophotometer. Calibration curves were established from known concentrations of PTX in ethanol solution.

Cytotoxicity Assay

The cytotoxicity of disulfide CCL micelles was evaluated by CCK-8 (Dojindo, Japan) assay. CCL micelles were prepared in PBS and then sterilized by filtration (0.22 μm). Hela cells (Cell bank of the Chinese Academy of Science, China) were pre-incubated in a 96-well plate (5×10^3 cells/well) with culture medium 10% FBS/1640 (Invitrogen Co., Carlsbad, CA) in a humidified 5% CO_2-containing atmosphere at 37 °C for 24 h. Then cells were further incubated with CCL micelles with four concentrations, 0.1 mg/L, 1 mg/L, 10 mg/L, and 100 mg/L for 48 h. Subsequently, media was aspirated and replenished with 100 μL of fresh culture medium. 10 μL CCK-8 reagents were added into each well, and the cells were incubated at dark for another 1 h to 3 h. The absorbance at a wavelength of 450 nm of each well was measured using a microplate reader (Sunrise™ Basic; TECAN, Zurich, Switzerland). Cells treated with 10μL of PBS were used as a negative control and relative cell viability was calculated as follows:

$$\text{Cell viability}\ (\%) = [(OD_{450_{sample}} - OD_{450_{blank}})/(OD_{450_{control}} - OD_{450_{blank}})] \times 100\%$$

Data are presented as average ± SD (n = 3).

The overall cytotoxicity of PTX-loaded disulfide CCL micelles against Hela cells with different drug concentrations was also evaluated by CCK-8 assays, and free PTX was used as a positive control. Meanwhile, to further confirm the reduction-responsive of the disulfide CCL micelles, the *in vitro* cytotoxicity of PTX-loaded non-disulfide CCL micelles was also determined by CCK-8 assays, which was prepared in the same manner as describe above using dipropargyl suberate as crosslinker (Supporting Information). Similarly, cells were cultured for 24 h to permit cell attachment, and then PTX-loaded CCL micelles with different PTX concentrations from 0.005 to 10 mg/L were added. After incubation for 48 h, the cells were treated with CCK-8 reagents. The absorbance was measured on the microplate reader at a wavelength of 450 nm. All tests were repeated three times.

Cellular Uptake and Intracellular Release of CCL Micelles

The cellular uptake and intracellular release experiments of disulfide CCL micelles were carried out using Nile red (NR) as a hydrophobic fluorescence probe instead of PTX, free NR was used as a control. The non-disulfide CCL micelle was also studied as a compare, to further certify the reduction-responsive of disulfide CCL micelles. NR (0.1 mg), crosslinker (dipropargyl 3,3'-dithiopropionate or dipropargyl suberate) and mPEG-*b*-PDATCL (25 mg, 0.062 mmol of azide group) were dissolved in 15 mL of tetrahydrofuran (THF) and stirred for 30 min at room temperature. 15 mL of PBS was added dropwise to the solution under stirring and then evaporated to remove THF. Then copper sulfate and sodium ascorbate was added into the mixture under nitrogen atmosphere. The mixture was stirred for 24 h at room temperature, and dialyzed against PBS. The solution was filtered to remove excess NR and the concentration of micelles was adjusted to 1.0 mg/mL.

The cellular uptake and intracellular release of NR-loaded micelles in response to GSH were investigated against Hela cells using CLSM (BX61W1-FV1000, OLYMPUS, Japan). Autoclave sterilized coverslips were placed in 6-well plate. Hela cells were seeded with a concentration of 2×10^4 cells/well in 1.8 mL of culture media and precultured for 24 h. Then NR-loaded micelles or free NR were added, and cultured for 1 h, 4 h. Then the culture media was removed, followed by rinsed the cells three times with PBS and fixed with 4 % paraformaldehyde at room temperature for 15 min. The cell nuclei were stained by Hoechst 33342 (blue fluorescence; Sigma-Aldrich). After being mounted with neutral balsam, samples were observed with 60× magnification microscope.

The amount of NR in cells was analyzed by Spectramax M5 plate reader (Molecular Devices, LLC, USA). 180 μL suspension of the Hela cells were seeded into each well of 96-well plate (1×10^4 cells) and incubated for 24 h. Then 20 μL PBS containing free NR or NR-loaded micelles were added. After 1 or 4 h's incubation, cells were washed with PBS twice, and finally 100 μL of PBS was added. The content of NR inside cells was measured by fluorescence spectroscopy (Spectramax M5, ex: 550 nm, em: 605 nm). Each group was tested in quintuplicate.

Characterization

^{1}H NMR spectra were recorded on a Bruker Avance DMX500 spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. The molecular weight and molecular weight distribution were measured by gel permeation chromatography (GPC), on a Waters degasser, a Waters 1515 Isocratic HPLC pump, and columns: PL gel 5 μm MIXED-C, 300×7.5 mm. THF was used as the mobile phase with a flow rate of 1.0 mL/min at 60 °C and standard narrow PDI polystyrene (PS) was used for calibration. The hydrodynamic diameter and size distribution of micelles were determined by dynamic light scattering (DLS) at 90° angle to the incident beam on a Brookhaven 90 Plus particle size analyzer. All micellar solutions had a final polymer concentration of 1.0 mg/mL and were filtered

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through a 0.45 µm filter. TEM images were obtained using JEM-1230 operating at an acceleration voltage of 60 kV. A drop of 1.0 mg/mL micellar solution was put onto the surface of Formvar-carbon film-coated copper grids. Excess solvent was quickly removed away with a filter paper and then stained by 2 wt-% phosphotungstic acid aqueous solution (for particles in water) or the vapor of osmium tetroxide (for particles in DMF). The amount of PTX was measuring by the absorbance in ethanol at a wavelength of 227 nm using a Shimadzu UV2550 UV-vis spectrophotometer.

Results and discussion

Synthesis and characterization of mPEG-*b*-PDATCL

mPEG-*b*-PDBTCL was synthesized via the ring-opening copolymerization of DBTC and CL with mPEG as macroinitiator and $Sn(Oct)_2$ as catalyst. The ^1H NMR spectrum of the product is shown in Fig. 1A with all the relevant signals well labeled. The composition of the polymer can be calculated from the integral ratio of H^b, H^e and H^h. According to the result of ^1H NMR, mPEG-*b*-PDBTCL containing 113 EO units, 30 CL units and 14 DBTC units (28 bromo groups) has been successfully prepared, which is in great agreement with the feeding molar ratio. Then the azidation of mPEG-*b*-PDBTCL was carried out at 80 ºC, giving completely azidated product (mPEG-*b*-PDATCL), which was also characterized by ^1H NMR as shown in Fig. 1B. Comparing with the bromo-pendant precursor, the shift of the signal from δ 3.44 ppm (H^h) to δ 3.53 ppm ($H^{h'}$) clearly demonstrate that the pendant bromo groups have been completely transformed into azides.

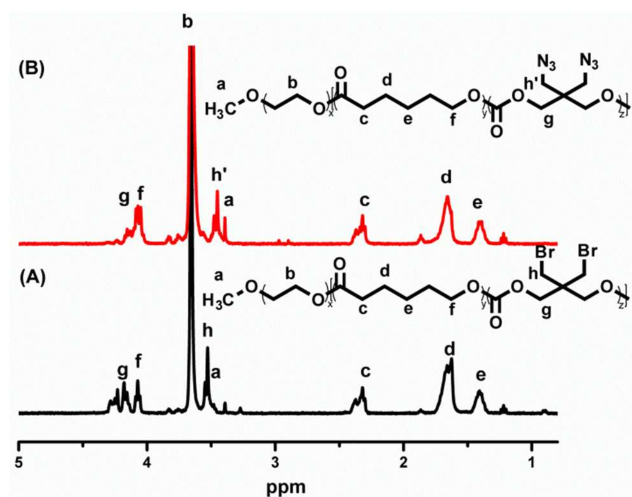

Fig. 1 ^1H NMR spectra of: (A) mPEG-*b*-PDBTCL, (B) mPEG-*b*-PDATCL.

The molecular weight and molecular weight distribution of the copolymers were determined by GPC, as shown in Fig. 2. The results intelligently indicated that the curve of mPEG-*b*-PDBTCL was unimodal with reasonably narrow molecular distributions. After azidation, the GPC trace of mPEG-*b*-PDATCL present a unimodal curve with similar broadness and a slightly shifted retention time compared with that of mPEG-*b*-PDBTCL, probably attributing to the transformation of pendant moieties. Obviously, the unimodal peaks and narrow molecular weight distributions clearly demonstrate that the final products are block copolymers without any byproduct.

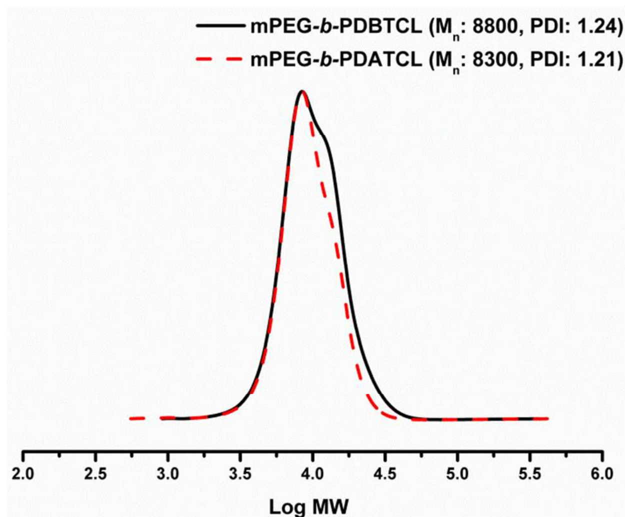

Fig. 2 GPC curves of mPEG-*b*-PDBTCL and mPEG-*b*-PDATCL.

Preparation and stability Studies of CCL Micelles

The CCL micelles were formed by self-assembly of mPEG-*b*-PDATCL via a cosolvent evaporation method, followed by core crosslinking with disulfide crosslinker through "click" chemistry to enhance their stability. The size and morphology of the micelles were studied by DLS and TEM. Non-crosslinked micelles were also prepared by self-assembly of mPEG-*b*-PDATCL without crosslinking, and studied as a control. The average diameter of the empty CCL micelles detected by DLS in water is 39 nm (Fig. 3A, PDI = 0.125), which is in great accordance with the results investigated by TEM, in which the micelles show spherical-shape with a clear boundary (Fig. 3B). Moreover, in order to verify the enhanced stability of the micelles after core crosslinking, DMF, a good solvent for both of the two block of mPEG-*b*-PDATCL, was used as solvent to prepare CCL micelles by dialysis method. Interestingly, the results from DLS and TEM (Fig. 3C, D) all show that CCL micelles in DMF can still maintained their nanostructures just as in water, indicating the covalent crosslinking of hydrophobic block can incredibly increase the stability of the micelles. Non-crosslinked micelles have similar size to corresponding CCL micelles in water. But when these non-crosslinked micelles were dialysized against DMF, no particle was detected (Fig S3), which further confirms the successful "locking" of the micellar structure for CCL micelles. Notably, the average size of the CCL micelles in DMF was slightly larger than that in water, probably attributed to the swelling of the core as a result of being penetrated by DMF molecules.

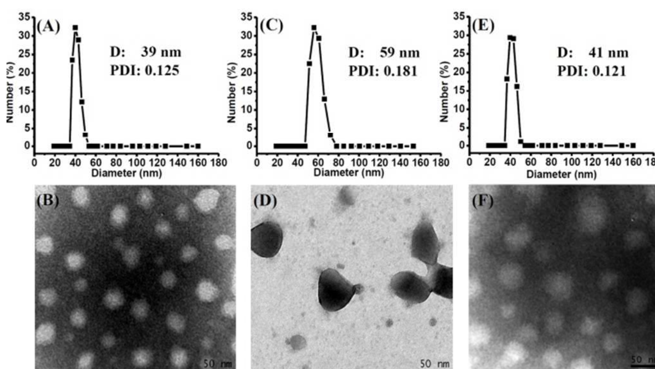

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Fig. 3 Particle size, distribution and morphology of empty CCL micelles in water by DLS (A) and TEM (B); empty CCL micelles in DMF by DLS (C) and TEM (D); PTX-loaded CCL micelles in water by DLS (E) and TEM (F).

Preparation and Reduction-responsive Drug Release of PTX-loaded CCL Micelles

Herein, PTX was used as a model anticancer drug to *in situ* prepare PTX-loaded CCL micelles. The initial weight ratio of PTX to polymer was 1:10. The DLC and DLE of PTX in CCL micelles are 4.43 % and 59.8 %, respectively. Interestingly, the size and distribution of the PTX-loaded CCL micelles detected by DLS and TEM (Fig. 3E and F) were analogous to those before drug loading (Fig. 3A and B), indicating that the core crosslinking may be beneficial for the micelles to maintain stable size and morphology after drug encapsulation.

For tumor cells, there exist a significant difference in the redox potentials, ranging from the mildly oxidizing extracellular milieu to the reductive intracellular fluids (cytosol) and organelles owing to different concentrations of glutathione (GSH), resulting in the selectively cleavage of the disulfide bonds.[45, 46] Herein, 10 mM DTT was chosen to mimic the reducing agent level in cytoplasmic environment, and the reduction-induced PTX release from disulfide CCL micelles was investigated by dialysis. The solutions taken out at predetermined intervals were lyophilized, followed by dissolving in certain ethanol and characterized by UV-vis spectrometry at 227 nm depending on the calibration curves, which were established from known concentrations of PTX in corresponding medium. As a control, the PTX release from non-disulfide CCL micelles and non-crosslinked micelles were also carried out. As shown in Fig. 4, rapid release of PTX from the non-crosslinked micelles was presented, approximately 80% of the PTX was released within 20 h. While the release of PTX from disulfide CCL micelles without DTT was largely inhibited. Less than 10 % drug was released even after 48 h. These results indicate that core-crosslinking can not only increase the micelle stability, but also form an excellent physical barrier to impede PTX burst release. Remarkably, after treating with 10 mM DTT, rapid drug release was observed for disulfide CCL micelles, 65 % of PTX was released after 48 h. But for non-disulfide CCL micelles, minimized release was presented during the same period. It can be assumed that, under the stimulus of DTT, the disulfide linkage of micelles are reduced and cleaved, and the core crosslinking structures of micelles are destructed, resulting in unstable non-crosslinked micelles, which leads to accelerated release of PTX.

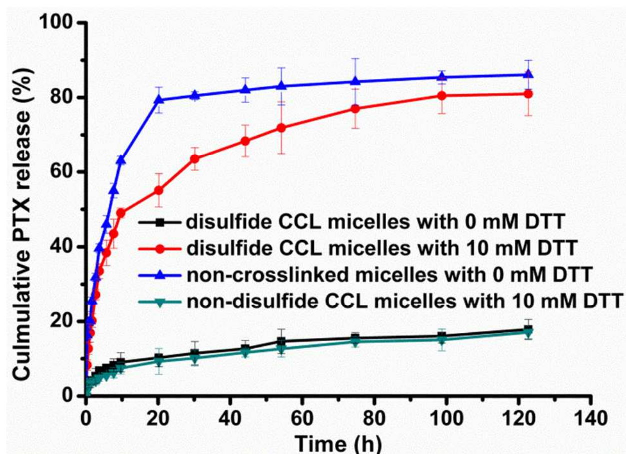

Fig. 4 *In vitro* PTX release profiles from PTX-loaded disulfide CCL micelles, non-disulfide CCL micelles with 10 mM DTT, and disulfide CCL micelles, non-crosslinked micelles without DTT.

In vitro cytotoxicity of empty and PTX-loaded CCL micelles

For drug delivery applications, it is meaningful to evaluate the potential toxicity of polymeric carriers. So the cytotoxicity of empty disulfide CCL micelles against Hela cells was investigated by CCK-8 assay. The cells were incubated with micelles for 48 h at varying concentrations from 0.1 to 100 mg/L. As shown in Fig. 5, the cell viabilities were almost around 100 % at the test concentrations up to 100 mg/L, clearly revealed that, disulfide CCL micelles showed excellent biocompatibility, and could be safely used as nanocarriers for efficient intracellular drug delivery.

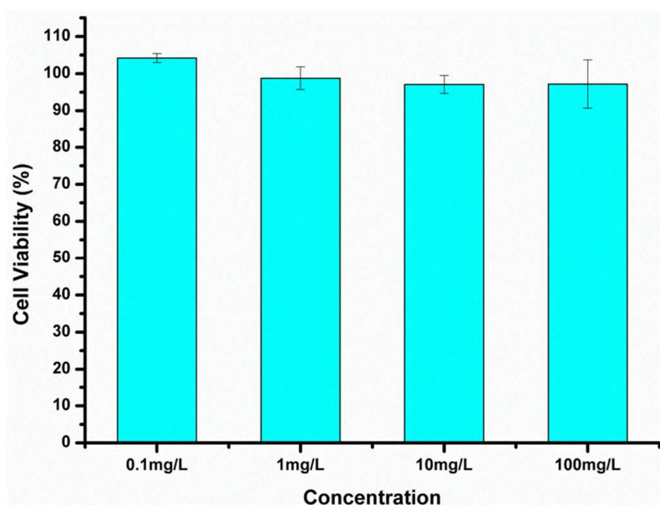

Fig. 5 The cytotoxicity of empty disulfide CCL micelles with different concentrations after incubated with Hela cells for 48 h.

The cytotoxicities of PTX-loaded CCL micelles (disulfide and non-disulfide) and free PTX were also detected and compared via CCK-8 assay in Fig. 6 with different PTX concentrations (0.005 to 10 mg/L). Clearly, the cytotoxicity of free PTX increased with the increase of dose, and a similar trend was also observed for the PTX-loaded CCL micelles. However, PTX-loaded CCL micelles exhibited lower cytotoxicity than free PTX, which is probably ascribed to the fact that the loaded PTX were released after micelles were endocytosed to enter the cells, which was a delayed process

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compared to the rapidly diffusion of free PTX. Importantly, compared with PTX-loaded non-disulfide CCL micelles, cells treated with PTX-loaded disulfide CCL micelles exhibited higher inhibition efficacy, which might be ascribed to the fact that disulfide bonds in micelles can be rapidly split in response to high intracellular GSH concentrations in cytoplasm of the cells, causing PTX to burst-release from de-crosslinked micelles. Whereas, for PTX-loaded non-disulfide CCL micelles, the encapsulated PTX was release by passive diffusion and presented a slow release. These results further confirming that introduction of reduction-labile disulfide crosslinker is beneficial in improving anti-tumor activity of PTX-loaded CCL micelles.

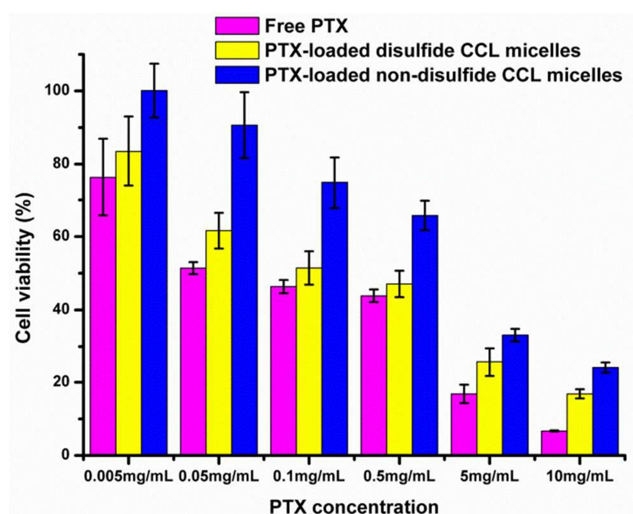

Fig. 6 Cytotoxicity studies of PTX-loaded CCL micelles and free PTX against HeLa cells after incubation for 48 h.

Cellular uptake and intracellular release of NR-loaded CCL micelles

NR was used as a fluorescent probe to prepare NR-loaded disulfide CCL micelles, which were used to investigate the cellular uptakes and intracellular release of hydrophobic agents from disulfide CCL micelles in response to GSH by CLSM. Free NR was used as a control, and non-disulfide CCL micelles were used as a comparison. As shown in Fig 7, apparent intracellular NR fluorescence was observed in the cells treated with the two NR-loaded CCL micelles, while almost no NR fluorescence signal was detected in the cells incubated with free NR even after 4 h incubation, which intelligently indicated that hydrophobic agent (free NR) alone could hardly be taken up by cells due to its poor solubility in water, and the micelles can transport hydrophobic agents into the cells by encapsulated them into the hydrophobic core. Meanwhile, the intensity of the NR fluorescence inside the cells incubated by disulfide CCL micelles is significantly stronger than that treated by non-disulfide CCL micelles. Furthermore, the intensity of the NR fluorescence inside the cells incubated by disulfide CCL micelles further increased and colocalized with nuclear Hoechst 33342 fluorescence by prolonging the incubation time from 1 h to 4 h, while no nuclear translocation was observed in Hela cells treated with non-disulfide CCL micelles even after 4 h. All of the CLSM results indicate that the high intracellular GSH concentration in cytoplasm could accelerate the cleavage of the disulfide crosslinker, resulting in a rapid release of NR from disulfide CCL micelles and its migration to cell nucleus.

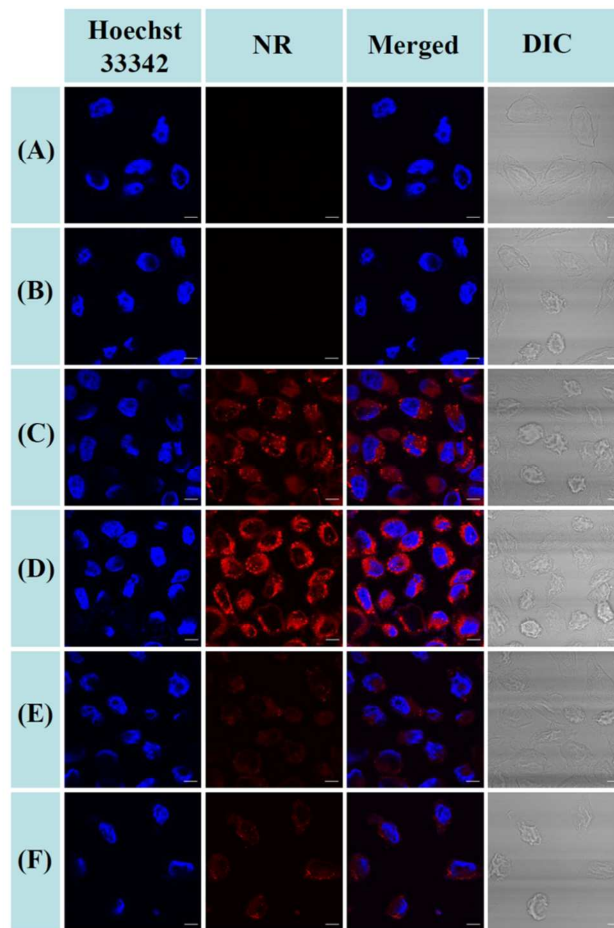

Fig. 7 CLSM images of Hela cells incubation with NR-loaded CCL micelles and free NR for 1 or 4 h. For each panel, the images from left to right showed cell nuclei stained by Hoechst 33342 (blue), NR in cells (red), overlays of two left images and bright field. The scale bars correspond to 10 mm in all the images. (A) free NR, 1 h incubation; (B) free NR, 4 h incubation; (C) NR-loaded disulfide CCL micelles, 1 h incubation; (D) NR-loaded disulfide CCL micelles, 4 h incubation; (E) NR-loaded non-disulfide CCL micelles, 1 h incubation; (F) NR-loaded non-disulfide CCL micelles, 4 h incubation.

Moreover, the quantitative fluorescence intensities in the Hela cells were determined by fluorescence intensity with Spectramax M5. The results showed in Fig. 8 were consistent with the CLSM findings. The cells cultured in the NR-loaded CCL micelles all showed NR fluorescence intensity, while little fluorescence signal was detected in the cells treated with free NR. Remarkably, the fluorescence intensity of cells treating with NR-loaded disulfide CCL micelles was much higher than that incubated with NR-loaded non-disulfide CCL micelles for both 1 h and 4 h. And similarly, the fluorescence intensity of cells treated with NR-loaded disulfide crosslinked micelles increased correspondently with the incubation time, while slight increase was observed for NR-loaded non-disulfide CCL micelles. These results could be explained by the fact that disulfide bonds in micelles are rapidly cleaved in response to high intracellular GSH concentrations in cytoplasm of the cells, making NR to burst-release from de-crosslinking micelles, while NR-loaded non-disulfide CCL micelles still maintained high stability and sluggish release.

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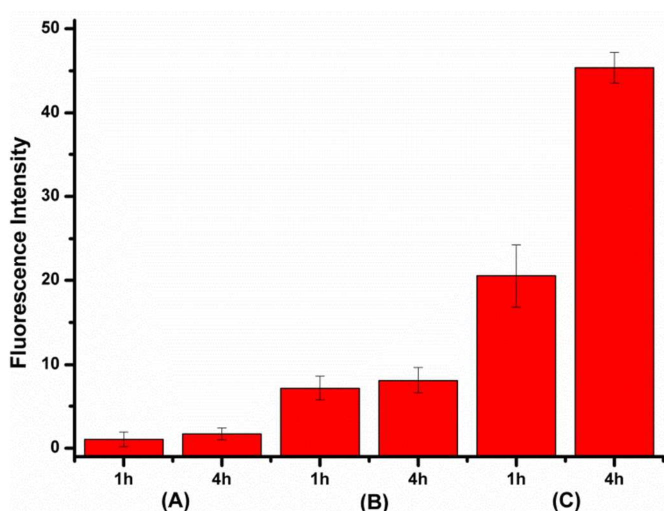

Fig. 8 Fluorescence intensity in Hela cells after incubation with free NR (A), NR-loaded non-disulfide CCL micelles (B) and NR-loaded disulfide CCL micelles (C) for 1 h, 4 h.

Conclusions

In this study, PTX-loaded reduction-sensitive CCL micelles were facilely prepared based on an amphiphilic block copolymer containing pendant azides and a dialkyne crosslinker with disulfide bond for tumoral-cell uptake and drug delivery. These smart micelles with superior stability in circulation are able to rapidly release loaded PTX in response to cytoplasmic glutathione, which leads to remarkably enhanced efficiency in killing cancer cells. This new type of efficient and smart drug delivery system with facilely preparation, reduction-response and great biocompatibility holds great potential for targeted drug delivery.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (21274121, 51173163 and 81100752), the Major State Basic Research Project (2011CB606001), the National Science-technology Support Plan Project of China (2012BAI07B01) and the Fundamental Research Funds for the Central Universities (2012QNA7043).

Notes and references

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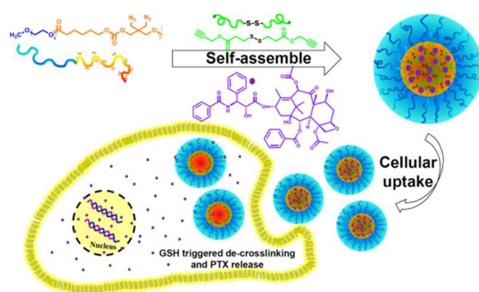
Graphic abstract

We provide a facile strategy to prepare redox-responsive core-crosslinked micelles for controlled release of paclitaxel.



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