Redox-responsive core cross-linked prodrug micelles prepared by click chemistry for pH-triggered doxorubicin delivery

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Abstract. A pH-triggered drug delivery system of degradable core cross-linked (CCL) prodrug micelles was prepared by click chemistry. Doxorubicin conjugated block copolymers of azido functional poly(ethylene oxide)-*b*-poly(glycidyl methacrylate) were synthesized by the combination of RAFT polymerization, epoxide ring-opening reaction, and acid-cleavable hydrazone linkages. The CCL prodrug micelles were produced by the reaction of dipropargyl 3,3'-dithiodipropionate and dipropargyl adipate cross-linking agents with the azido groups of the micellar core *via* alkyne-azide click reaction, which were denoted as CCL/SS and CCL/noSS, respectively. The TEM images of CCL/SS prodrug micelles showed a spherical shape with the average diameter of 61.0 nm from water, and the shape was maintained with an increased diameter upon dilution with 5-fold DMF. The high DOX conjugation efficiency was 88.4%. In contrast to a very slow DOX release from CCL/SS prodrug micelles under the physiological condition (pH 7.4), the drug release is much faster (90%) at pH 5.0 and 10 mM of GSH after 96 h. The cytotoxicity test and confocal laser scanning microscopy analysis revealed that CCL/SS prodrug micelles had much enhanced intracellular drug release capability in HepG2 cells than CCL/noSS prodrug micelles.

Keywords: smart polymers, cross-linking, prodrug, doxorubicin, HepG2

1. Introduction

Polymeric core-shell architectures have attracted great attention due to potential applications in drug delivery [1–4]. In aqueous media, amphiphilic block copolymers can self-assemble into micelles which consist of hydrophilic corona and hydrophobic core. Drugs can be loaded into these micelles which not only avoid the metabolization and rapid clearance from the body but also reduce toxicity of drug towards healthy tissues [5–8]. The drug of interest is either dissolved, entrapped, attached or encapsulated into the polymeric micelles depending on the method of preparation [9–11]. However, the *in vivo* stability of polymeric micelles, following intravenous administration

still leaves a challenge due to dissociation into unimers under certain factors such as low concentration, high shearing force in the blood circulation [12]. To stabilize polymeric micelles for *in vivo* applications, hydrophobic blocks can be cross-linked by various strategies including dimethylmaleide photo-cross-linking [13], phototriggered disulfide cross-link [14], and metal-coordination complexes [9, 15], resulting in core cross-linked micelles. Nevertheless, conventional antitumor drugs are quickly distributed at administration in the body leading to fast renal clearance, short durations of action, and significant dissipation, with only a fraction reaching targeted side [16]. Therefore, drug molecules may be incorporated

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to high molecular weight polymer to increase their solubility and bioavailability, reduce renal elimination as well as improve pharmacokinetic profile [17]. In recent years, a covalent linkage has been designed between a drug and a polymer to prepare a polymer-based prodrug [18]. Drugs can be stably entrapped in prodrugs by chemical bonds to provide a long-term retention during the circulation time and be released effectively at tumor cells. Prodrugs are inactive derivatives of drug molecules and undergo chemical transformation to regenerate the active forms. Indeed, polymeric prodrugs can overcome inherent drawbacks of drug such as poor water solubility, chemical instability, and rapid metabolism. There are many routes for preparation of polymeric prodrugs which showed high drug loading efficiency [19, 20]. They can be prepared by preformed polymers [21, 22], polymerization reaction of drug-bearing monomers [23–25], or ring-opening polymerization of a drug-initiator [26, 27]. More recently cross-linked prodrug micelles have been suggested, which can provide dramatic improvements in pharmacokinetics and biodistribution of drugs [28]. Prodrug micelles can be cross-linked at micellar corona [29], or micellar core [30, 31]. The conjugated drugs could be cleaved from polymeric prodrugs by external stimuli, such as enzymatic [32], passive hydrolysis [33–36], pH [37], light [38], or reduction [39]. In particular, pH and redox-responsive prodrug systems have gained tremendous attention because there are different pH conditions between normal cells (pH~7.4) and cancer cells (pH~5.0–5.5) as well as redox potential gradient between the extracellular (~2 μ M) and intracellular environment (1–10 mM) [40, 41]. Additionally, the incorporation of drug containing hydrazone bonds into polymeric micelles could provide an opportunity for designing an intracellular specific drug delivery system. Even though both the drug conjugated polymer backbone and drug contained cross-linking agent using a hydrazone bond have obtained high drug uploading capacity, the polymer-drug isolation from drug-free polymers was complicated [42, 43]. In this light, we have recently reported the synthesis of nanogels and dual-responsive cross-linked micelles based on pH-sensitive hydrazone bond by using azide-alkyne click chemistry [44, 45]. The drug, prednisolone, was loaded into the micelles by the click chemistry during the cross-linking reaction. A part of azide groups of the poly(glycidyl methacrylate) (PGMA- N_3) block was used to core cross-link-

ing and the other part was used simultaneously to load the drug. In this work, the doxorubicin (DOX) conjugated block copolymer was prepared first, followed by core cross-linking, in order to achieve very high-efficiency of drug loading. In addition, we investigated their *in vitro* cytotoxicity study towards cancer cells. So far it is desirable that DOX is efficiently loaded into micelles by a stimuli-responsive linkage and then cross-linking takes place using a facile method.

Herein, we describe a facile route for synthesis of dual-responsive core cross-linked (CCL) prodrug micelles based on DOX conjugation using an acid-sensitive hydrazone bond and a cross-linking agent containing a thiol-reducible disulfide bond to produce a biocompatible, robust, and smart nanocarrier (Figure 1). DOX was previously conjugated to the PGMA segment of azido functional poly(ethylene oxide)-*b*-poly(glycidyl methacrylate) (PEO-*b*-PGMA- N_3) by a pH-sensitive hydrazone linkage affording DOX functional block polymers (PEO-*b*-PGMA- N_3)-DOX). The Huisgens cycloaddition was then allowed to take place between azido groups located along the PGMA- N_3 -DOX and alkyne groups of dipropargyl 3,3'-dithiodipropionate and dipropargyl adipate cross-linkers to form redox-responsive CCL prodrug micelles, which were denoted as CCL/SS and CCL/noSS, respectively. The drug controlled release was investigated in the presence of glutathione as a reducing agent at different pH values. *In vitro* cytotoxicity studies of CCL prodrug micelles against HepG2 cells indicated the stimuli-responsive controlled release of enhanced efficiency.

2. Experimental

2.1. Materials

Poly(ethylene oxide) methyl ether (PEO, M_n = 5000 g/mol, M_w/M_n = 1.10, Aldrich) were dried by azeotropic distillation using anhydrous toluene. Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 99.99%), and (+)-Sodium L-ascorbate ($\geq 99\%$) were purchased from Sigma-Aldrich (Korea). Doxorubicin hydrochloride (DOX·HCl) was purchased from Boryung Pharmaceutical Co., Ltd. (Seoul, Korea). *p*-Nitrophenyl chloroformate (*p*-NPCl, 98%) was purchased from TCI (Japan). *L*-Glutathione (GSH, reduced, 97%) was purchased from Alfa Aesar (Korea). *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), and acetone were dried over CaH_2 and distilled prior to use. Block copolymers of PEO-*b*-PGMA- N_3) and

Figure 1. CCL prodrug micelles of the PEO-*b*-PGMA-(N₃)-DOX block polymer, followed by degradation and DOX release in cancer cell

cross-linking agent dipropargyl adipate and dipropargyl 3,3'-dithiodipropionate were prepared as described in our previous work [45].

2.2. Preparation of PEO-*b*-PGMA-(N₃)-DOX prodrug polymer

PEO-*b*-PGMA-(N₃) copolymer (200 mg, 0.644 mmol of OH moieties) and triethylamine (0.306 mL, 3 mmol) were dissolved in 5 mL of DMF homogeneously. The copolymer solution was dropped into *p*-NPC (350 mg, 1.73 mmol, 2.7 equiv) solution in 5 mL of DMF for 1 h, and reaction was kept for 48 h in dark with stirring. An excess amount of methanol was added to remove unreacted *p*-NPC, and then the solvent was removed by a rotary evaporator. The product was precipitated in cool ether and dried. PEO-*b*-PGMA-(N₃)-pNPC (300 mg, 1.09 mmol) dissolved in 7 mL of DMF was added dropwise slowly into hydrazine monohydrate solution (22.3 mmol, 2.0 mL) in 2 mL of DMF and stirred in dark overnight. The product was dialyzed against water. After removal of deionized water, the residue and 4 mg (0.007 mmol) of DOX were dissolved in 10 mL of

DMF. The mixture was kept stirring for 48 h at 60 °C. The product was dialyzed against deionized water pH-adjusted to 9 for 12 h to remove any residual solvent and unreacted DOX [46]. The purified PEO-*b*-PGMA-(N₃)-DOX was freeze-dried and conjugation percentage of DOX was determined by testing the UV-absorption at 485 nm on a UV-Vis Optizen POP spectrophotometer. Serially diluted concentrations of free DOX solutions were used to construct a calibration curve.

2.3. CCL prodrug micelles of PEO-*b*-PGMA-(N₃)-DOX

PEO-*b*-PGMA-(N₃)-DOX (8.0 mg, 7.86 μmol of azide group) and dipropargyl 3,3'-dithiodipropionate (0.36 mg, 1.26 μmol) or dipropargyl adipate (0.28 mg, 1.26 μmol) were dissolved in anhydrous DMF (2.0 mL) homogeneously. Next, 30 mL of deionized water was adjusted to pH 8.5 with borate buffer solution in order to avoid hydrolysis of the hydrozone bond, and added dropwise to the solution under vigorous stirring for one day. CuSO₄·5H₂O (2.2 mg, 8.8 μmol) in water (100 μL) and sodium ascorbate

(1.75 mg, 8.8 μmol) in water (100 μL) were added in order and the solution was stirred at room temperature for 24 h. After that CCL solution was transferred into a dialysis bag (MW cutoff, 1 kDa), and subjected to dialysis against 2 L of distilled water at pH 8.5 which was renewed every 4 h during the course of 2 days. The CCL micelle solution was passed through 0.45 μm filter and then freeze-dried.

2.4. *In vitro* drug release

The release of DOX from CCL prodrug micelles in times was followed by dispersion of CCL prodrug micelles (3.0 mg) in 5.0 mL of either phosphate buffered saline (PBS, 10 mM, pH 7.4) solution or acetate buffer solution (10 mM, pH 5.0). Then, solution was transferred to a dialysis membrane tube (MW cutoff, 1 kDa) which was incubated in 40 mL of buffer solution with or without GSH at 37 °C in a bath with shaking rate of 80 rpm. At selected time intervals, 2.0 mL of incubated solution was taken out and 2.0 mL of fresh buffer at the same condition was added to refill the incubation solution to 40 mL. The amount of DOX release was measured using UV absorbance at 485 nm in aqueous solution based on the standard calibration curve. The experiment was carried out in triplicate and average values were taken.

2.5. Cell culture

HepG2 (human hepatocellular carcinoma) and HEK293 (non-cancerous human embryonic kidney) cells were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Cells were cultured in MEM (HyClone Laboratories, Logan, UT, USA) medium supplemented with 10% heat inactivated fetal bovine serum (FBS, HyClone Laboratories) and 1% penicillin-streptomycin (PAA Laboratories GmbH, Austria) at 37 °C and 5% CO_2 .

2.6. Cell viability assay

Exponential phases of HepG2 and HEK293 cells ($5 \cdot 10^3$ cells/well) were seeded on 96-well plates (SPL Lifesciences, Gyeonggi, Korea) in triplicate. The cytotoxicity of CCL prodrug micelles using the HepG2 cell line and blank CCL micelles using HEK293 was measured by MTT method. Following overnight incubation, various concentrations (0.1, 0.5, 1, 5, 10, and 15 $\mu\text{g/mL}$) of free DOX or CCL prodrug micelles were introduced to HepG2 cells and 5, 10, 50, 100, 150, 250, 500 $\mu\text{g/mL}$ of blank CCL micelle solution were supplied on HEK293 cells, then incubated for

24 h. After treatment, 10 μL of EZ-Cytox Cell Viability Assay Solution WST-1[®] (Daeil Lab Service, Jong-No, Korea) was added to each well and incubated for an additional 3 h. The absorbance of reaction was measured using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 460 nm and inhibitory rates were calculated.

2.7. DAPI staining

HepG2 cells were treated with the concentration of 10 and 15 $\mu\text{g/mL}$ of CCL prodrug micelles for 8 and 16 h. The cells were rinsed once with phosphate-buffered saline (PBS) solution (135 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate, 1.4 mM potassium dihydrogen phosphate) and stained by addition of 1 $\mu\text{g/mL}$ of DAPI solution (Roche Applied Science, Indianapolis, IN, USA) in methanol (Sigma-Aldrich, St. Louis, MO, USA). After incubation in the dark at 37 °C for 20 min, cells were washed twice with PBS solution, and then fixed with 4% paraformaldehyde (Junsei, Tokyo, Japan) for 15 min. The nuclear morphology of the cells was observed under a Laser Scanning Confocal Microscope (Carl Zeiss LSM 700, Jena, Germany).

2.8. Characterization

¹H NMR spectra were recorded on a JNM-ECP 400 (JEOL) instrument. Gel permeation chromatography (GPC) was performed using a HP 1100 apparatus, with THF as a solvent at 25 °C and an elution rate of 1 mL/min. The columns were calibrated with commercial polystyrene standards. Fourier transform infrared (FTIR) spectra were measured on a JASCO FT/IR-4100 spectrometer with DLATGS detector in the 4000–400 cm^{-1} spectral region. The samples were finely ground, mixed with spectroscopic grade KBr and pressed into pellets. For transmission electron microscopy (TEM; JEOL JEM-2010), the samples were prepared by dropping micellar solutions on copper grids. No staining was applied to the sample. Dynamic laser light scattering (DLS) measurements for determining the average hydrodynamic diameter of polymeric micelles were performed using an electrophoretic light scattering instrument (ELS-8000, Otsuka Electronics Corporation), equipped with an ELS controller, and a He-Ne laser at wavelength of 632.8 nm. The intensity of scattered light was detected at 90° to an incident beam. All the analyses were performed at 25 °C. The sample solutions (1 mg/mL) was prepared by dispersing the micelles in solvent

with ultrasonic oscillation for 30 minutes and the solution was allowed to settle down a small portion of agglomerate prior to analysis.

3. Results and discussion

3.1. Synthesis and characterization of CCL prodrug micelles

PEO-*b*-PGMA copolymers were prepared by the RAFT polymerization of GMA according to our previous work. The controlled molecular weight ($M_n = 10,600$ g/mol) and narrow polydispersity (PDI = 1.21) of the block copolymer indicated a good level of control in the RAFT-mediate reaction as evidenced by gel permeation chromatography (GPC) analysis [45]. The copolymer was functionalized with azido and hydroxyl groups by the epoxide ring-opening reaction resulting in PEO-*b*-PGMA-(N₃). The multiple hydroxyl groups on the PGMA-(N₃)(OH) part were first modified with *p*-NPC and hydrazine hydrate followed by the amine-ketone reaction with DOX to produce a pH-responsive feature of PEO-*b*-PGMA-(N₃)-DOX as described in the literature (Figure 2). PEO-*b*-PGMA-(N₃)-DOX and alkyne terminated cross-linkers were dissolved in DMF and a large amount of deionized water was added dropwise under vigorously stirring to form micelles. CuSO₄·5H₂O and sodium ascorbate were introduced to crosslink micelles via alkyne-azide click chemistry.

The formation of block copolymers was confirmed by the FT-IR analysis. In Figure 3 curve a, the disappearance of epoxy signals at 844 and 993 cm⁻¹

demonstrated that the reaction between epoxy rings and NaN₃ took place in the presence of NH₄Cl. Additionally, the azido functionalized PEO-*b*-PGMA block was confirmed by the presence of a new peak at 2095 cm⁻¹ illustrating the stretching vibration of azido groups in PGMA-(N₃) segments. In Figure 3 curve b, the peak at about 1730 cm⁻¹ was attributed to *p*-NPC, and this peak was overlapped with the carbonyl group absorption peak. The peaks at 1594 and 1333 cm⁻¹ were identified by the carboxylation of PGMA-(N₃)(OH) moieties forming block copolymers-COO (C₆H₄)NO₂. However, these peaks disappeared after the amidation process (Figure 3 curve c). In addition, the occurrence of a new absorption peak at about 3228 cm⁻¹ could be ascribed to

Figure 3. FT-IR spectra of (a) PEO-*b*-PGMA-(N₃), (b) PEO-*b*-PGMA-(N₃)-*p*NPC, (c) PEO-*b*-PGMA-(N₃)-NH₂, (d) pure DOX, and (e) PEO-*b*-PGMA-(N₃)-DOX

Figure 2. Preparation of doxorubicin conjugated PEO-*b*-PGMA-(N₃)-DOX block copolymers

the -NH_2 group indicating the incorporation of hydrazide to the polymers. When the DOX was covalently attached to the polymers, the signals of the -NH_2 group were absent and a new shifted peak at 1603 cm^{-1} might be attributed to the conjugating of DOX (Figure 3 curve e).

^1H NMR spectra were used to demonstrate the structure of the block copolymer as well as DOX-polymer conjugation. Ring-opening reaction was confirmed by new peaks of the opened ring at 5.51 and 3.88 ppm whereas the epoxy ring protons at 4.31, 3.73, 3.21, 2.81, 2.67 ppm disappeared absolutely (Figure 4a). The result indicated that epoxy rings were successful opened to create azido and hydroxyl

groups on the backbone of PEO-*b*-PGMA. Then, the hydroxyl groups were further employed to conjugate the hydrophobic drug. The reaction between *p*-NPC and the hydroxyl group in the PGMA- (N_3) -OH segment produced PEO-*b*-PGMA- (N_3) -pNPC. The ^1H NMR spectrum presented the signals at 8.84 and 8.12 ppm corresponding to the aromatic protons of *p*-NPC (Figure 4b). The extent of the reaction was calculated to be 67.8% by comparing the signal intensity of *p*-NPC and PGMA group protons. Subsequently, the *p*-NPC groups were substituted with hydrazine. Figure 4c evidenced peaks at 8.21–7.68 ppm which were associated with -NH-NH_2 . After the formation of DOX conjugates, the new proton signals

Figure 4. ^1H NMR spectra of (a) PEO-*b*-PGMA- (N_3) , (b) PEO-*b*-PGMA- (N_3) -*p*NPC, (c) PEO-*b*-PGMA- (N_3) - NH_2 , and (d) PEO-*b*-PGMA- (N_3) -DOX in $\text{DMSO-}d_6$

appeared at 1.07, 7.08 to 7.40, 7.95, and 8.52 ppm, suggesting the anchoring of DOX onto the block copolymers (Figure 4d).

The block polymer of PEO-*b*-PGMA-(N₃)-DOX was dissolved in DMF and a large amount of deionized

water was added dropwise to the solution under vigorously stirring to form micelles. The core-shell structure consists of a PEO hydrophilic corona and a PGMA-(N₃)-DOX hydrophobic core. The cores contain azido groups which are used to react with

Figure 5. DLS results of (a) non-CCL and CCL prodrug micelles, and the dilution with DMF. TEM images of (b) non-CCL, (c) CCL/noSS, and (d) CCL/SS prodrug micelles; TEM images after dilution with 5-fold DMF of (e) CCL/noSS and (f) CCL/SS prodrug micelles

cross-linkers to form CCL micelles with or without disulfide linkages. To evidence the cross-linked hydrophobic core, the dynamic laser light scattering (DLS) was used to evaluate the change in the hydrodynamic diameters of micelles before and after cross-linking. As shown in Figure 5a, the hydrodynamic sizes of non-CCL, CCL/noSS, and CCL/SS prodrug micelles were 83 ± 2.1 , 74.4 ± 1.5 , and 80.5 ± 2.5 nm, respectively. The cross-linked prodrug micelles were somewhat smaller than non-cross-linked prodrug micelles due to the tighter and compacter core-corona structure. Furthermore, the morphology and size of the particles were examined by transmission electron microscope (TEM) technique. In Figure 5b–5d, it can be seen that the particles were nanospheres with good dispersibility and clear boundary. The calculated diameters of the non-CCL, CCL/noSS, and CCL/SS prodrug micelles were ca. 70.8, 59.9, and 61.0 nm, respectively. The values of the hydrodynamic diameter measured by DLS are larger than those by TEM since the PEO coronas are swollen in water, while the micelles can be shrunk into a dried sphere during TEM observation.

The changes of size and morphology of cross-linked micelles were investigated upon addition of DMF as a good solvent for both the PEO and PGMA-(N₃)-DOX block. The non-cross-linked prodrug micelles showed no DLS signal after 5 times of DMF dilution due to the disruption of micelles. In contrast, the size of the cross-linked prodrug micelles increased from 74.4 to 155.0 nm and from 80.5 to 187.3 nm for CCL/noSS and CCL/SS prodrug micelles, respectively (Figure 5a). Meanwhile, TEM images illustrated that the spherical morphologies were still maintained after dilution of 5-fold DMF, with the approximate diameter of 145.8 and 179.3 nm corresponding

to CCL/noSS and CCL/SS prodrug micelles, respectively. It is considered that the CCL prodrug micelles possess insoluble swollen cores which still preserve the core-corona structure and prevent the disintegration from dilution. The results illustrated that the cross-linked micelles were much stable than non-cross-linked micelles. 10 mM of GSH was added to the phosphate buffered saline (PBS, 10 mM, pH 7.4) solution of cross-linked prodrug micelles as a reducing reagent and the size distributions were measured at different periods of time in order to examine whether the CCL prodrug micelles are stimuli-responsive in a redox environment. As shown in Figure 6, the size distribution of CCL/noSS prodrug micelles was nearly consistent after incubation for 24 h, whereas the hydrodynamic diameter of CCL/SS prodrug micelles increased significantly and showed a wider distribution of particle size indicating redox-sensitive cross-linking. The result could be explained by the fact that the CCL/SS prodrug micelles having S–S bonds in micellar cores were susceptible to a reducing reagent and cleaved by GSH.

3.2. DOX release

The DOX conjugation efficiency of PEO-*b*-PGMA-(N₃)-DOX copolymers with hydrazone linkages was 88.4% as determined by UV absorbance at a wavelength of 485 nm. This drug loading efficiency is higher than the one in our previous study, where prednisolone was loaded simultaneously with core cross-linking [45]. It is well-known that an acid-sensitive hydrazone bond can undergo hydrolysis under acidic conditions [47], which releases pristine DOX from PEO-*b*-PGMA-(N₃)-DOX. To verify whether CCL prodrug micelles could effectively protect DOX from premature release and exhibit pH and reduction

Figure 6. The de-cross-linked behavior of (a) CCL/noSS and (b) CCL/SS prodrug micelles in the presence of 10 mM of GSH over different time periods at 37 °C (the initial concentration of micelles is 0.094 mg/mL in PBS, 10 mM, pH 7.4)

Figure 7. *In vitro* DOX release at 37°C from the non-CCL and CCL prodrug micelles under different conditions of pH and GSH concentration. Data are presented as the mean $\pm$ standard deviation ($n = 3$)

responsive features, we investigated DOX-release profiles after CCL prodrug micelles were incubated in buffer solution at 37°C and various pH values in the presence and absence of GSH. As shown in Figure 7, there is an initial slight release of DOX at pH 7.4 owing to the DOX chemically linked PGMA core with the hydrazone bond which is relatively stable under basic conditions. After 96 h, the total release of DOX from CCL prodrug micelles was about 26% at pH 7.4. The DOX release was significantly improved at pH 5.0. For example, CCL prodrug micelles showed nearly 59% of DOX release at pH 5.0 for 96 h since a hydrazone linkage was known to be cleaved within a short period of time in an acidic circumstance. Interestingly, in the presence of 10 mM of GSH, the CCL/SS prodrug micelles presented a high rate of DOX release up to 65% after 96 h at

pH 7.4. In this study, the 10 mM of GSH was selected to simulate the reducing environment in the cytoplasm in cancer cells whose GSH level is several times higher than that in healthy cells. Due to the disulfide-thiol exchange reaction in GSH environment, a significant breakage of disulfide cross-linkages within the CCL/SS prodrug micelles led to prominent escape of DOX from the micellar core. At pH 5.0 and in the presence of 10 mM of GSH, 90% of DOX release from the CCL/SS prodrug micelles was observed for 96 h, which was similar to non-cross-linked prodrug micelles (88%). Obviously, the DOX release of the cross-linked prodrug micelles was relatively very little at pH 7.4 and in the absence of GSH, indicating the dual-sensitive DOX release feature. The result demonstrated that the cross-linking and hydrazone linkages of CCL/SS prodrug micelles played an important role to prevent the undesirable release of DOX in physiological circumstance but burst release in cytoplasm environment of cancer cells.

3.3. *In vitro* cytotoxicity of CCL prodrug micelles on HepG2 and HEK293 cell growth

In vitro efficacy of CCL prodrug micelles was investigated by cell viability assay using a human liver cancer HepG2 cell line. As shown in Figure 8a, CCL prodrug micelles exhibited lower cytotoxicity than free DOX towards HepG2 cells after incubation for 24 h. This is because the CCL prodrug micelles release DOX by a slow manner after the endocytosis process of micelles into the cells. The CCL/SS prodrug micelles started to cause remarkable growth inhibition and a marked decrease in cell viability in HepG2 cells at a concentration of 10 $\mu\text{g/mL}$ of DOX

Figure 8. The cytotoxicity of (a) CCL prodrug micelles on HepG2 cells and (b) blank CCL micelles on HEK293 cells for 24 h incubation

equiv. The half maximal inhibitory concentration (IC_{50}) value of the CCL/SS prodrug micelles was approximate 15 $\mu\text{g/mL}$ after treatment for 24 h, and this concentration was used in subsequent experiments. The results initially indicated that the CCL/SS prodrug micelles induced significantly cytotoxicity in HepG2 cells and showed highly effective proliferation inhibition compared with CCL/noSS micelles. Contrary to HepG2 assay, the cytotoxicity of blank CCL micelles on the normal cell was also evaluated by the MTT method as shown in Figure 8b. It was demonstrated that blank CCL micelles at extremely high concentration (500 $\mu\text{g/mL}$) did not suppress the proliferation of HEK293 cells implying good biocompatibility of these polymeric prodrugs for drug delivery applications.

3.4. Effects of CCL prodrug micelles on the induction of apoptosis

The morphological change of HepG2 cells treated with CCL/SS prodrug micelles could be visualized under the inverted microscope ($\times 100$). Compared to non-treated cells, HepG2 cells treated with CCL/SS prodrug micelles (10–15 $\mu\text{g/mL}$ DOX equiv.) did exhibit morphological features of apoptosis, such as

cell membrane blebbing, cell shrinkage, increased cytoplasm granules and detachment from culture plates (Figure 9a). After DAPI staining of nucleus, the classical hallmark of apoptotic cells with chromatin condensation and apoptotic bodies was observed in HepG2 cells which were treated with the DOX equivalent concentration of 10 and 15 $\mu\text{g/mL}$ (Figure 9b), indicating that the HepG2 cell death stimulated by CCL/SS prodrug micelles was a typical apoptotic cell death.

The cellular uptake efficiency of CCL prodrug micelles was further investigated by using confocal laser fluorescence microscopy to determine whether the prodrug polymer inhibited the proliferation of HepG2 cells. Free DOX and CCL prodrug micelles were incubated with HepG2 cells for 8 and 16 h at 37°C with a DOX equivalent concentration of 15 $\mu\text{g/mL}$. The cancer cell apoptosis were confirmed by the DAPI staining of nuclei. The non-treated cells showed no DOX fluorescence as shown in Figure 10a. The DOX intracellular distribution was obviously different from the free DOX and CCL prodrug micelles after diffusion across the cell membranes. The free DOX presented a strong fluorescence of DOX in the HepG2 cell nuclei only after

Figure 9. Phase-contrast (a) and fluorescence (b) images of HepG2 cell upon CCL/SS prodrug micelles at different concentrations after treatment for 24 h. Scale bar 10 μm

Figure 10. Confocal microscopy images of HepG2 cells treated with CCL prodrug micelles (dosage: 15 μg DOX equiv. per mL). For each panel, the images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the two images. Scale bar 10 μm . (a) Non-treated cells, (b, d) free DOX, (c, e) CCL/SS prodrug micelles, and (f) CCL/noSS prodrug micelles.

8 h incubation period implying that the free DOX was rapidly uptaken into the intracellular organelles by the cell membrane and entered the nuclei (Figure 10b). The CCL/SS prodrug micelles exhibited moderate intracellular DOX fluorescence intensity in nuclei after incubation for 8 h (Figure 10c). It is likely that a part of DOX was released from CCL micelles for 8 h and subsequently entered into the nuclei and a part of DOX exists still inside the micelles. It is well-known that the cellular uptake of micelles is generally caused by endocytosis. The CCL prodrug micelles were exposed to GSH with a concentration up to 10 mM and an acidic pH (5.0) in the lysosomes [48], which could break disulfide bonds of the cross-linked core and the released DOX was slowly transferred into the nuclei. After 16 h incubation the CCL/SS prodrug micelles showed very strong fluorescence intensity comparing with 8 h incubation due to the fact that significant DOX was released from CCL/SS prodrug micelles with longer incubation time and afterwards diffused into the nuclei (Figures 10d and 10e). Interestingly, it should be noted that CCL/noSS prodrug micelles showed a much less red fluorescence intensity in the nuclei after incubation for 16 h compared to CCL/SS prodrug micelles because little DOX was released from the CCL/noSS micelles (Figure 10f). The DOX cleaved by the hydrolysis of hydrazone bonds was mostly confined in the cross-linked micellar core. On the basis of the above data, the profile for CCL/SS prodrug micelles caused apoptosis closely correlated with its growth suppressive effect. Thus, these phenomena demonstrated that CCL/SS prodrug micelles may be considered as an inducer of apoptosis on HepG2 cells.

4. Conclusions

A pH-triggered DOX delivery system based on redox dual-sensitive CCL prodrug micelles was proposed with a high DOX loading efficiency up to 88.4%. The core cross-linked prodrug micelles of PEO-*b*-PGMA-(N₃)-DOX were prepared with the block copolymer which bound DOX with acid sensitive hydrazone bonds, using facile alkyne-azide click reaction. The CCL/SS prodrug micelles were much more stable than non-cross-linked prodrug micelles under large volume dilution. The CCL/SS prodrug micelles having disulfide linkages could be de-cross-linked in a reducing environment, which facilitated drug controlled release. The release profile of the CCL/SS prodrug micelles showed a slight release at pH 7.4 but

a burst release (90%) in the presence of 10 mM of GSH at pH 5.0. The MTT assay in HepG2 of CCL/SS prodrug micelles illustrated remarkable growth inhibition and higher toxicity towards HepG2 compared to CCL/noSS prodrug micelles. The blank CCL micelles demonstrated to be non-toxic towards the normal HEK293 cell line at the concentration up to 500 µg/mL. The fluorescence images of the cancer cell experiment indicated that CCL/SS prodrug micelles could release DOXs in the mildly acidic endosomal compartment and highly reducing cytoplasm, which diffused into the nucleus and caused apoptosis on HepG2 cells. The combination between pH-sensitive loaded drug and redox-responsive CCL/SS prodrug micelles promises its prospects in drug delivery.

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