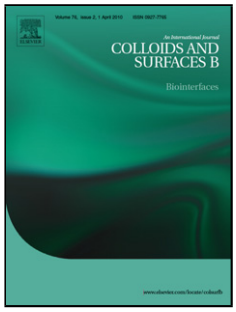

Preparation of ROS-responsive Core Crosslinked Polycarbonate Micelles with thioketal linkage

Deqi Wang (Methodology) (Investigation) (Formal analysis) (Writing - original draft), Song Wang (Investigation) (Formal analysis), Yingchun Xia (Methodology) (Writing - review and editing), Simeng Liu (Validation), Ruixin Jia (Investigation) (Formal analysis), Gege Xu (Formal analysis), Junjie Zhan (Investigation) (Validation), Yanbing Lu (Conceptualization) (Methodology) (Supervision) (Writing - review and editing)

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Preparation of ROS-responsive Core Crosslinked Polycarbonate Micelles with thioketal linkage

Deqi Wang [a, #], Song Wang [b, #], Yingchun Xia [a], Simeng Liu [a], Ruixin Jia [a], Gege Xu [a], Junjie Zhan [a], Yanbing Lu*[a, b]

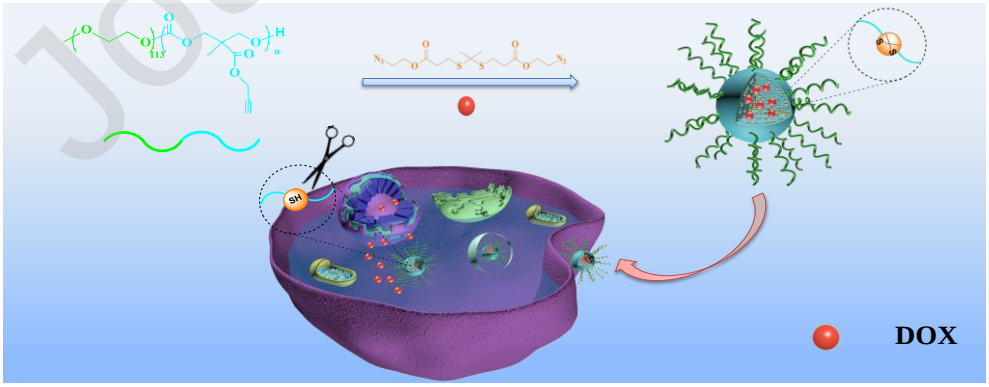
[a] College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, P. R. China.

[b] State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan Provincial Key Laboratory of Biomacromolecular Chemical Biology, Hunan University, Changsha 410082, P. R. China

[#] Contributed equally.

[*] To whom correspondence should be addressed. E-mail: yanbinglu@hnu.edu.cn.

Graphic abstract



Highlights

- ROS-responsive core crosslinked micelles were conveniently prepared.
- The obtained CCL/TK micelles exhibited good biocompatibility and low toxicity.
- DOX-loaded CCL/TK micelles had higher toxicity effects for HeLa and MCF-7 cells.

Abstract:

Herein, we prepared novel reactive oxygen species (ROS) responsive core crosslinked (CCL/TK) polycarbonate micelles conveniently by click reaction between amphiphilic diblock copolymer poly(ethylene glycol)-poly(5-methyl-5-propargylxycarbonyl-1,3-dioxane-2-one) (PEG-PMPC) with pendant alkynyl group and thioketal containing azide derivative bis (2-azidoethyl) 3, 3'- (propane-2, 2-diylbis (sulfanediyl)) dipropanoate (TK-N_3). The CCL/TK micelles were obtained with small size of 146.4 nm, showing excellent stability against dilution and high doxorubicin (DOX) loading. In vitro toxicity tests demonstrated that the obtained CCL/TK micelles have good biocompatibility and low toxicity with cell viability above 95%. Furthermore, DOX-loaded CCL/TK micelles showed significantly superior toxicity with IC_{50} values for HeLa and MCF-7 cells about 3.74 μg/mL and 3.91 μg/mL, respectively. Confocal laser scanning microscope (CLSM) and flow cytometry showed excellent internalization efficiency and intracellular drug release of DOX-loaded CCL/TK micelles. The obtained ROS-responsive CCL/TK micelles showed great potential for anticancer drug delivery.

Keywords: polycarbonate; core crosslinked micelles; drug delivery; ROS responsive

materials; thioketal

1. Introduction

Nanocarriers have wide application prospects in the field of biomedicine, and have unique advantages in drug delivery, biosensors and bioimaging [1-3]. Great progress has been made in targeted delivery of different types of active agents such as anticancer drugs, genetic agents, and proteins, which are based on nanocarriers represented by liposomes, polymer nanoparticles, amphiphilic polymer micelles and dendrimers [4, 5]. Nanocarriers self-assembled by amphiphilic polymer micelles have several distinct merits in the delivery of anticancer drugs. For example, it improves the stability of the drug during transportation, increases the bioavailability of the drug, and extends the circulation time [6-8]. In addition, nano-sized polymer micelles can improve tumor accumulation through the enhanced permeability and retention (EPR) effect [9]. Polymer micelles are promising as a platform for specific targeted delivery of anticancer drugs. In the past decades, various types of nanocarriers had emerged [10]. However, many nanocarriers have the problem of premature drug leakage during transportation, which severely limits their clinical applications [11]. Undesired disintegration occurs prematurely because nanocarriers are diluted by blood when injected into a blood vessel. This not only reduces the therapeutic effect, but also causes a large number of aggregates in normal cells or tissues which cause serious side effects [12, 13]. Therefore, addressing the stability of nanocarriers during transportation and achieving drug release at diseased sites play an extremely

important role in cancer treatment.

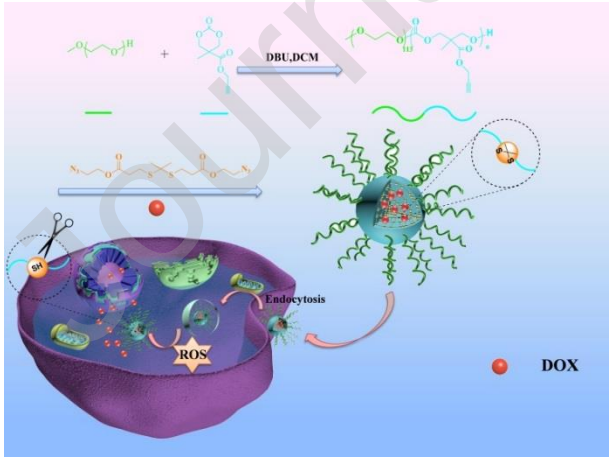
Stimuli-responsive core crosslinked (CCL) micelles have been extensively studied as a strategy to improve the stability of nanocarriers [14-16]. Drugs encapsulated by stimulus-responsive CCL micelles can be released by the triggers such as pH [17], redox [18], reactive oxygen species (ROS) and enzymes [19], in appropriate cellular microenvironment. Compared with uncrosslinked (UCL) micelles, CCL micelles have the obvious superiority to improved drug loading and prevented the disintegration of the drug carrier by the extreme dilution of biological fluids [20]. However, while the crosslinked micelles prevent dilution, they also restrain the drug from being released in the hydrophobic inner core, which resulting in insufficient therapeutic effects [21]. In comparison with permanent CCL micelles, decrosslinked micelles can solve this limitation and achieve a strategy for enhance the effect of cancer treatment [22, 23]. ROS is a substance that contains oxygen and has high reactivity, including singlet oxygen, superoxide anion radicals, hydroxyl radicals and hydrogen peroxide [24]. Compared with normal tissues, the level of ROS in cancer cells is higher than that in normal tissues, the difference in ROS concentration provides a physiological and chemical basis for developing a novel stimulus-responsive nanocarrier which can be applied to cancer therapy [25, 26]. Currently, ROS-responsive functional groups are often used to construct and design anticancer drug carriers such as Se [27], Te [28], arylboronic acid [29], thioketal [30], and thioether [31]. The thioketal groups can be broken under the stimulation of a suitable tumor microenvironment, and have been widely explored to build stimuli-responsive drug carrier to achieve the triggered

release of drugs at the target site [25, 32-37]. Li [38] synthesized a diblock copolymer (PEG-TK-PLA) by using thioketal as a connecting bridge between PEG and PLA, which was used to encapsulate the anti-cancer drug PTX to achieve on-demand drug delivery. What's more, Xu [39] designed a ROS-responsive prodrug micelle based on PEG and anticancer drug DOX in combination with ROS-cleavable thioketal linkage between PEG and DOX, and Li [40] synthesized polyphosphoester-based, core crosslinked ROS responsive micelles for delivery of anticancer drugs.

Aliphatic polyesters and polycarbonates have the advantages of low toxicity, biodegradability and excellent biocompatibility, and have been widely studied for used in medical devices, genes, and drug delivery [41]. Based on biodegradability and biocompatibility, polycarbonates that used to design amphiphilic polymer micelles with different stimuli responsiveness has been continuously explored for the delivery of anticancer drugs [42]. Polycarbonates as the hydrophobic core can not only improved the solubility of hydrophobic drugs but also enhanced the biocompatibility of polymer micelles. In addition, polycarbonates had the adjustable side chain structure, which showed attractive advantages in the construction and design of CCL micelles [43]. Wu [44] prepared pH-responsive CCL PEG-b-poly(mono-2, 4, 6-trimethoxy pentaerythritol benzylidene-carbonate) (PEG-b-P(TMBPEC-co-AC)) based micelles for intracellular release of paclitaxel (PTX). Li [45] prepared of pH and redox dual-sensitive CCL micelles though the reaction of bis-(azidoethyl) disulfide with the alkynyl group on the side chain of a pH responsive PEG-p (TMBPEC-co-MPMC) for DOX delivery. Our group [46] also synthesized redox sensitive CCL micelles based on

amphiphilic diblock copolymer PEG-b-polycarbonate (p(MPC)), in which a-lipoic acid derivative (LA) was introduced to form crosslinked network structure under the catalysis of DTT, enabling release of encapsulated drugs in the existence of 10 mM GSH.

Based on self-assembly of amphiphilic diblock polyethylene glycol-polycarbonate micelles (PEG-PMPC), ROS responsive core crosslinked polymer micelle was synthesized by the azide-alkyne click method for delivery of anticancer drug DOX (Scheme 1). The shell composing of PEG could prolong the circulation time in the blood, polycarbonate block as the inner core of micelles could improve the biocompatibility of CCL/TK micelles and reduce biotoxicity. The thioketal containing crosslinker TK-N_3, not only imparted ROS responsiveness to the amphiphilic micelles, but also improved the stability and drug loading of the micelles. The cytotoxicity of micelles were investigated in HeLa and MCF-7 cells. The anticancer effect and internalization efficiency of DOX-loaded CCL/TK micelles were also investigated. Results showed that the crosslinked micelles have low biotoxicity, and the DOX-loaded crosslinked micelles have excellent anti-cancer effects and internalization efficiency.



Scheme 1 Illustration of ROS responsive CCL/TK micelles for DOX delivery in vitro. DOX loaded CCL/TK micelles with high DOX loading efficiently accumulate and trigger encapsulated DOX release.

2. Experimental

2.1 Materials

2-Bromoethanol, 2,2-dimethylolpropionic acid, 3-mercaptopropionic acid, 3-bromopropyne, 4-dimethylaminopyridine (DMAP), 1,8-diazabic-yclo[5.4.0]undec-7-ene (DBU), N,N'-Dicyclohexylcarbodiimide (DCC), 1,6-dibromohexane and doxorubicin hydrochloride (DOX·HCl) were purchased from Energy Chemical. Monomethoxy poly(ethylene glycol) (PEG_{5k}) was purchased from Sigma-Aldrich and L-sodium ascorbate was purchased from Shanghai Titan Scientific Co., Ltd. Ethyl chloroformate was purchased from Beijing Xiya Chemistry Co., Ltd. Cyclic carbonate monomer 5-Methyl-5-propargylxycarbonyl-1,3-dioxane-2-one (MPC) was prepared as reported before [47]. HeLa and MCF-7 cells were obtained from the cell bank of the central laboratory at Hunan Xiangya hospital. DAPI was purchased from USA Solarbio. RPMI1640, DMEM medium was obtained from Beijing neuronbc. Fetal bovine serum (FBS) was purchased from Biological Industries, USA. Penicillin-Streptomycin, 0.25% Trypsin-EDTA was obtained from Suzhou NCM Biotech. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd.

2.2 Characterization

^{1}H NMR and ^{13}C NMR spectra were tested by a 400 MHz spectrometer (Bruker, USA). The number molecular weight (M_n) and polydispersity index ($Đ$) of the obtained copolymer were performed on a Waters 1515 gel permeation chromatography (GPC) instrument (Waters, USA). The sizes of micelles were measured by Nano ZS90 zeta-potential and particle analyzer (Malvern, UK). Transmission electron microscopy (TEM,

JEM-2100Plus) was used to observe the morphology of micelles. Fluorescent spectra were tested using a Hitachi F-4600 fluorescence spectrophotometer (Hitachi, Japan). Cell viability assay was measured on a SynergyTM Mx multi-mode microplate reader (BioTek, USA). Confocal fluorescence imaging was obtained on a confocal laser scanning microscope (CLSM, C1-Si, Nikon, Japan). Flow cytometric analysis was measured on a Gallios flow cytometer (Beckman-Coulter, USA).

2.3 Synthesis of bis (2-azidoethyl) 3, 3'-(propane-2, 2-diylbis (sulfanediyl)) Dipropanoate (TK-N_3)

As shown in Scheme S1, the thioketal containing crosslinker TK-N_3 was prepared in three steps. 3, 3'-(propane-2, 2-diylbis (sulfanediyl)) dipropionic acid (TK) was firstly synthesized according to previous reports [39]. 3-Mercaptopropionic acid (10 g, 94.2 mmol) and anhydrous acetone (10.9 g, 188.4 mmol) were mixed in a flask and dry hydrogen chloride atmosphere (HCl (g)) was continuously introduced for 4 h. Then, the reaction solution was crystallized in an ice-salt bath. The obtained product was washed three times with hexane (20 mL) and cold water (20 mL) respectively. TK was collected as a white solid. Yield: 13.1 g (55%).

TK (2.00 g, 7.94 mmol) and DCC (3.60 g, 17.46 mmol) were dissolved in anhydrous DCM (60 mL), and the mixture was stirred at 0^{o}C for 30 min. Then, 2-bromoethanol (1.89 g, 15.12 mmol) and DMAP (0.20 g, 1.59 mmol) were added. After keeping incubated at 0^{o}C for 1 h, the reaction was stirred at room temperature for 24 h. The mixture was filtered, and the filtrate was washed with saturated $NaHCO_3$ and saturated NaCl aqueous solution respectively. The organic phase was then dried over anhydrous

$MgSO_4$, filtered, and evaporated to remove the solvent. The residue was further purified by silica column chromatography with petroleum ether/ethyl acetate (4/1, v/v) as eluent. TK-Br was obtained as colorless oil. Yield: 1.79 g (51%).

TK-Br (1.14 g, 2.46 mmol) and NaN_3 (0.48 g, 7.42 mmol) were dissolved in DMF (25 mL), and the mixture was stirred at 80°C for 24 h. The reaction was quenched with deionized water (50 mL) and extracted with ethyl ether (3×30 mL). The organic phase was then washed with DI water (3×50 mL), dried over anhydrous $MgSO_4$, filtered, the solvent was removed by evaporation. TK-N_3 was obtained as yellow oil. Yield: 0.62 g (65%).

The non-responsive cross-linker, 1,6-diazidohexane was prepared according to the method reported previously[45].

2.4 Synthesis of diblock copolymer PEG-PMPC

The diblock copolymer PEG-PMPC was synthesized via ring-opening polymerization (ROP) of cyclic carbonate monomer MPC using PEG_{5k} as a macro-initiator and DBU as the catalyst. MPC (0.2 g, 1.01 mmol), PEG_{5k} (0.202 g, 0.0404 mmol), DBU (0.77 mg, 1.01×10^{-2} mmol) and anhydrous DCM (2 mL) were put into a dried schlenk flask, and degassed by three freeze–pump–thaw cycles. Subsequently, the mixture was keep stirring at room temperature for 7 h. After that, the solution was precipitated by cold ether and centrifugation. The product was dried in vacuum and a white powder was obtained. Yield: 288 mg (71.3%).

2.5 Preparation and characterization of UCL micelles

UCL micelles were prepared via a dialysis method. PEG-PMPC (5 mg) was dissolved in DMF (1 mL). Then, 10 mL of DI water was slowly dropwise added under vigorous stirring. After keeping stirring at room temperature for 2 h, the micelles were prepared via further dialysis against deionized water for 24 h (MWCO=3.5 kDa).

Nile Red (NR) was used as a fluorescent probe to measure the critical micelle concentration (CMC) of PEG-PMPC. NR dissolved in THF (0.12 mg/L, 30 μL) was put in the vials. After THF was evaporated, a series of micelles of different concentrations (2×10^{-4}-0.2 mg/mL) were added to the vials. Subsequently, the solution was kept stirring overnight. The fluorescence intensity value of micelles were measured by a fluorescence spectrometer (λ_{ex}=550 nm, λ_{em}=560-720 nm).

2.6 Synthesis of CCL micelles

Taking CCL/TK micelles as an example. PEG-PMPC (5.0 mg, 0.5×10^{-3} mmol, 1 eq. of alkyne groups) and TK-N_3 (3.9 mg, 10.24×10^{-3} mmol, 0.8 eq. of alkyne groups) were dissolved in 1 mL DMF and stirred for 1h. Then, 10 mL of DI water was added dropwise under vigorous stirring. After stirred for 2 h, $CuSO_4{\cdot}5H_2O$ (0.78 mg, 3.125×10^{-3} mmol, 0.4 eq. of alkyne groups) and sodium ascorbate (0.62 mg, 3.125×10^{-3} mmol, 0.4 eq. of alkyne groups) were added into the mixed solution. After stirred at room temperature for 24 h, the CCL/TK micelles were prepared by dialysis against deionized water for 24 h (MWCO=3.5 kDa). Non-responsive cross-linked (CCL/CC) micelles were synthesized using 1,6-diazidohexane as a crosslinker according to the same method.

2.7 DOX-loaded and ROS responsive release of DOX

DOX-loaded CCL micelles were prepared by the similar step as for blank CCL micelles. Taken 20% of theoretical drug loading contents as an example, PEG-PMPC (10 mg/mL in DMF, 0.2 mL), TK-N_3 (1.56 mg) and DOX (5 mg/mL in DMSO, 100 μL) were mixed together. Then, DI water (3 mL) was added dropwise into the mixture under vigorous stirring. After 2 h, $CuSO_4 \cdot 5H_2O$ (0.312 mg, 1.25×10^{-3} mmol, 0.4 eq of alkyne groups) and sodium ascorbate (0.248 mg, 1.25×10^{-3} mmol, 0.4 eq of alkyne groups) were added into the mixed solution. After stirred at room temperature for 24 h, the DOX-loaded CCL/TK micelles were obtained by dialysis against deionized water for 24 h. The whole process was maintained in dark. DOX-loaded CCL/CC micelles were synthesized by replacing TK-N_3 crosslinker with 1,6-diazidehexane according to the same method. In the absence of crosslinker, the DOX loaded UCL micelles were prepared via the same procedure.

The drug loading capacity (DLC) and drug loading efficiency (DLE) of the micelles were determined via calculating the fluorescence intensity of DOX in the medium collected outside the dialysis bag. The content of loaded drug was measured by spectrofluorimetry (DOX: λ_{ex}=485 nm, λ_{em}=592 nm). The DLC and DLE were calculated via the following formulation.

DLC (%) = (weight of loaded drug/total weight of polymer and loaded drug) × 100%

DLE (%) = (weight of loaded drug/weight of feeding drug) × 100%

The in vitro drug release profiles from DOX-loaded CCL/TK micelles were studied in different media (PBS (10 mM, pH=7.4), 5 mg/mL and 10 mg/mL H_2O_2). 1 mL of DOX-loaded CCL/TK micelles (CCL/TK micelles: 0.4 mg/mL, DOX: 88 μg) were placed in a

dialysis bag (MWCO=8.0-14.0 kDa), which was immersed in 25 mL of various media and followed by shaking at 37°C. At certain time intervals, 3 mL of release medium was removed to determine the DOX concentration by fluorescence, and replenished with an equal volume of same solution. The release results were performed in three parallel experiments.

2.8 Cytotoxicity assay in vitro

In vitro cytoxicities of micelles were evaluated in HeLa and MCF-7 cells. Briefly, cells were seeded on 96-well plates (6000 cells per well) and incubated in fresh RPMI-1640 medium containing 10% FBS for 24 h. Then, micelle solutions with different concentrations were added into the wells, which had been replaced with fresh culture media. In absent of micelle solution, cells were treated as control. After 24 h, MTT (5 mg/mL) was added and further incubated for another 4 h. After that, the unreacted MTT was removed carefully and replaced with DMSO to dissolve the crystals formed by the living cells. The absorbance of the each well at the wavelength of 570 nm was measured using a microplate reader to determine cell relative viability. The cytotoxicities of DOX-loaded CCL/TK, CCL/CC, UCL micelles and free DOX were tested in the same way. In addition, the cytoxicities of DOX-loaded CCL/TK micelles and free DOX after being cultured for 48 h were also investigated. The cytoxicities of CCL/TK micelles with exogenous H_2O_2 was also investigated in HeLa cells for 24 h, according to the method reported previously [48].

2.9 Cellular uptake in vitro

HeLa cells were planted on 24-well plates (2×10^4 cells per well) and incubated in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin for 24 h (37°C, 5% CO_2) and then cultured with DOX-loaded CCL/TK, CCL/CC, UCL micelles and free DOX for 4 h and 8 h respectively (37°C, 5% CO_2) at a final DOX concentration of 2 μg/mL. The cells were fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI. Fluorescent images were observed in a confocal laser scanning microscope (C1-si, Nikon, Japan). The cell uptake was further detected using a flow cytometer. HeLa cells were seeded on 24-well plates (2×10^4 cells per well) containing fresh cell culture medium for 24 h and then cultured with DOX-loaded CCL/TK, CCL/CC, UCL micelles and free DOX (DOX dose: 5 μg/mL) for 4 h and 8 h, respectively. The cells were then digested by trypsin and collected by centrifugation. Subsequently, the sample was analysis by flow cytometry.

3. Results and discussion

3.1 Preparation and characterization of ROS responsive core crosslinked micelles

Amphiphilic diblock copolymer PEG-PMPC was obtained by ring-opening polymerization of cyclic MPC using PEG as initiator and DBU as catalyst (Scheme S1) according to the method described previously [47]. PEG-PMPC was successful synthesized and confirmed by ^{1}H NMR analysis (Fig. 1a). The theoretical degree of PEG-PMPC of the polymer was set at 25 and calculated to be 23, comparing the integrals of peaks at δ = 3.58 (methylene protons of PEG) and δ = 2.48 (the alkynyl proton of PMPC). GPC curve of MPC-PMPC showed that the number average molecular weights

and polydispersity index (*Đ*) of the obtained PEG-PMPC was 8.9 kDa and 1.11 (Table S1, Fig. S1). The thioketal containing crosslinker TK-N_3 and non-responsive crosslinker 1,6-diazidohexane were synthesized according the route shown in Scheme S1. The successful synthesis of the TK-N_3 and 1,6-diazidohexane were confirmed via ^{1}H NMR spectra (Fig. S2-S4), ^{13}C NMR spectra (Fig. S5) and FT-IR spectra (Fig. S6).

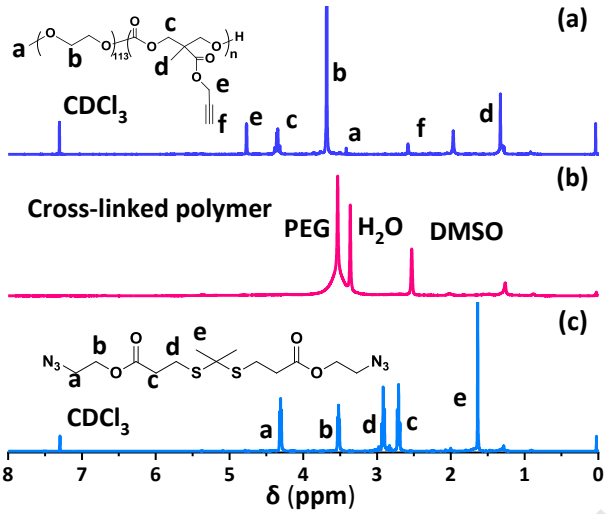


Fig.1. ^{1}H NMR spectra of PEG-PMPC (a), crosslinked polymer (b) and crosslinker TK-N_3 (c).

ROS-responsive core crosslinked micelles were prepared via click reaction between thioketal containing crosslinker TK-N_3 and pendant alkynyl groups of hydrophobic polycarbonates block of PEG-PMPC. As shown in Fig. 1b, the proton peaks of the hydrophobic inner core of the core crosslinked polymer and TK-N_3 were not observed. It was probably because the crosslinked structure hindered the dissolution of the inner core and shielded the proton peak of the hydrophobic inner core [18].

The critical micelle concentration (CMC) of PEG-PMPC was determined to be approximately 0.029 mg/mL using NR as a fluorescent probe (Fig. 2a). The size and morphology of CCL/TK, CCL/CC and UCL micelles were determined with DLS and TEM. The particle size of CCL/TK, CCL/CC and UCL micelles were 146.4 nm, 144.1 nm and

98.8 nm measured by DLS (Fig. 2b). The increased size of micelles after crosslinking may due to the crosslinked network structure formed by the hydrophobic core, which made the core of the micelles swell [18, 49]. TEM images of CCL/TK, CCL/CC and UCL micelles showed a spherical morphology formed in the aqueous solution with a size of 35.6 nm, 33.1 nm and 28.7 nm (Fig. 2e). The smaller particle size obtained by TEM than DLS was due to the shrinkage of the hydrophilic shell after drying [17, 20]. Consistent with the results obtained by DLS, TEM images also showed that the particle size of CCL/TK micelles was larger than those of CCL/CC and UCL micelles.

In order to evaluate the stability of CCL/TK, CCL/CC and UCL micelles, micelles against extensive dilution were investigated and their changes in diameters were determined by DLS. When diluted with 10-fold volume of DMF, the CCL/TK and CCL/CC micelles still maintained their structures while the sizes only increased slightly, but the sizes of UCL micelles became chaotic and smaller sizes were observed (Fig. 2c). Moreover, after 100-fold dilution with water to let the micelle concentration below the CMC of PEG-PMPC, the sizes of the CCL/TK and CCL/CC micelles increased slightly. It may be due to the presence of crosslinked network structure of the CCL micelles that only increased the sizes because of swelling. In contrast, the UCL micelles disintegrated under 100-fold dilution of water (Fig. 2d). Additionally, the stability of the micelles in PBS (10 mM, pH 7.4) and plasma were also evaluated. Compared with UCL micelles, the particle sizes of CCL/TK and CCL/CC micelles were not significantly different in PBS and plasma, while the particle sizes of UCL micelles increased slightly (Fig. S6). These results indicated that CCL/TK and CCL/CC micelles had excellent stability due to the

core cross-linked network.

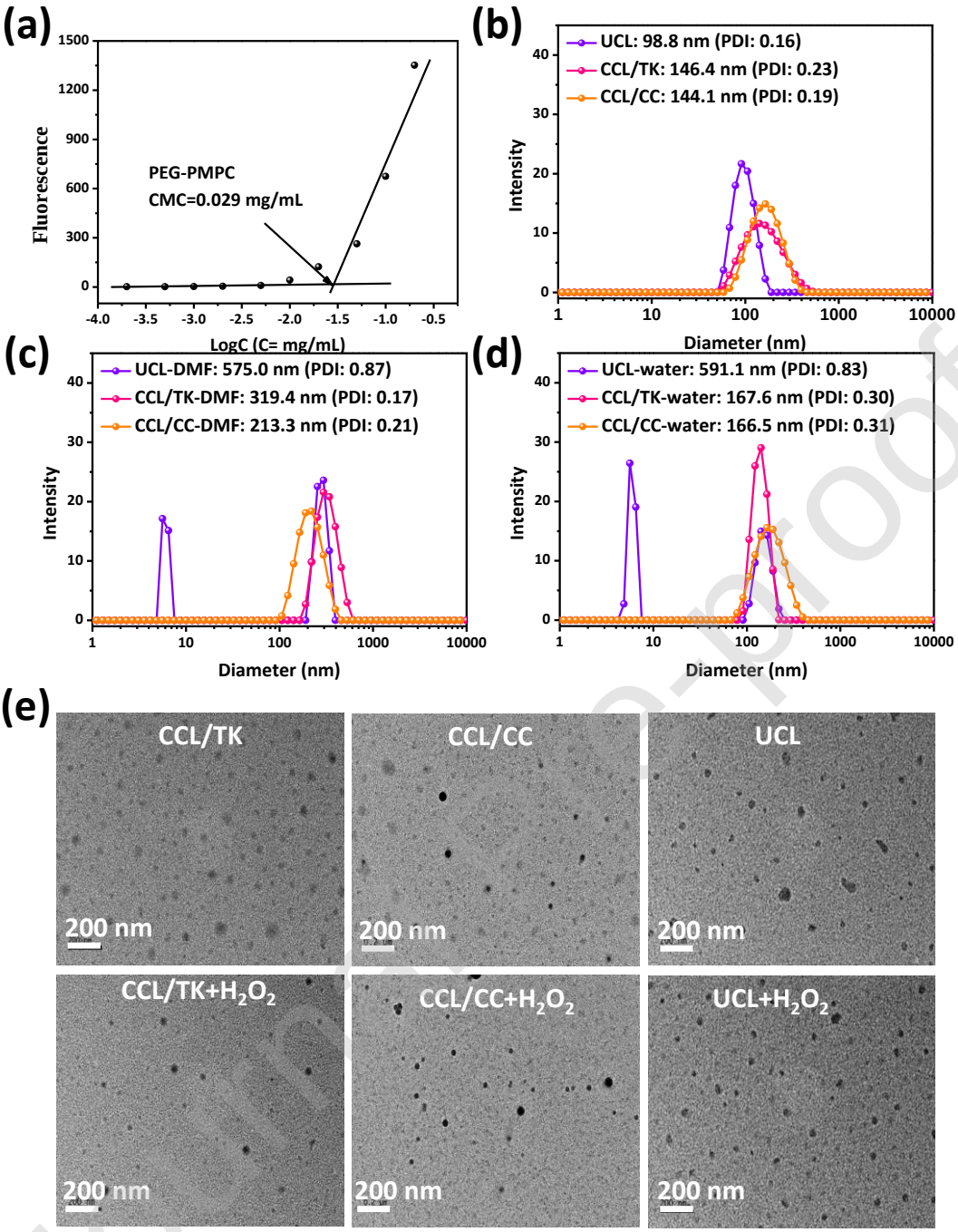


Fig. 2. The CMC value of UCL micelles (a). The size distributions of UCL, CCL/TK and CCL/CC micelles in water (b), after 10-fold dilution with DMF (c), after 100-fold dilution with water (d). TEM images of CCL/TK, CCL/CC and UCL micelles before and after treatment with 10 mM H_2O_2 (e).

The stability of CCL/TK micelles against reactive oxygen species H_2O_2 was also evaluated. After 48 h of incubation with 5 mM, 10 mM and 20 mM H_2O_2, the sizes of CCL/TK micelles decreased gradually to 137.0 nm, 127.3 nm and 120.9 nm respectively

(Fig. S7). However, the sizes of CCL/CC and UCL micelles remained unchanged. It was possibly due to decrosslinking of CCL/TK micelles caused by thioketal cleavage. It can also be obtained from the TEM images that the diameters of CCL/TK micelles became smaller after cultured with H_2O_2, while CCL/CC and UCL micelles maintained unchanged morphology (Fig. 2e).

3.2 DOX loading and the vitro drug release of CCL micelles

DOX-loaded micelles were formed by hydrophobic interactions. The theoretical DOX loading contents were set at 10, 20 and 30 wt.%. The DLC and DLE values of DOX-loaded CCL/TK and CCL/CC micelles were remarkably higher than those of DOX-loaded UCL micelles under the same theoretical drug loading (Table 1). It demonstrated that the crosslinking network of the CCL/TK and CCL/CC micelles could prevent the leakage of the loaded DOX, thereby extending the circulation time in the blood. Compared with unloaded micelles, the sizes of DOX-loaded CCL/TK, CCL/CC and UCL micelles increased significantly. It was due to the encapsulated DOX expanding the core of the micelles.

Table 1. Characterization of DOX-loaded micelles

DOX feed ratio (wt%)	UCL Micelles				CCL/CC Micelles				CCL/TK Micelles			
	DLC (%)	DLE (%)	Size (nm)	PDI	DLC (%)	DLE (%)	Size (nm)	PDI	DLC (%)	DLE (%)	Size (nm)	PDI
10	4.8	50.1	144.1	0.12	7.7	84.7	151.7	0.27	7.6	75.6	156.8	0.11
20	10.4	62.5	155.1	0.20	14.7	88.2	171.7	0.14	16.9	84.9	191.9	0.38
30	12.5	54.0	227.1	0.20	20.9	90.6	192.6	0.18	25.8	86.1	241.4	0.34

The in vitro ROS responsiveness of DOX-loaded CCL/TK micelles was performed at different concentrations of H_2O_2 (0, 5, 10 mM). In the absence of H_2O_2 (PBS, pH 7.4), DOX-loaded CCL/TK micelles had a cumulative release of only 20% in 48 h. When the concentration of H_2O_2 increased to 5 mM and 10 mM, the cumulative release of DOX

reached 51% and 65%, respectively (Fig. 3). At the same time, DOX cumulatively released 16% from DOX-loaded UCL micelles at pH 7.4, and 18% from DOX-loaded CCL/CC in the presence of 10 mM H_2O_2. It demonstrated that the DOX-loaded CCL/TK micelles showed ROS-responsive behavior and could undergo decrosslinking to release encapsulated DOX.

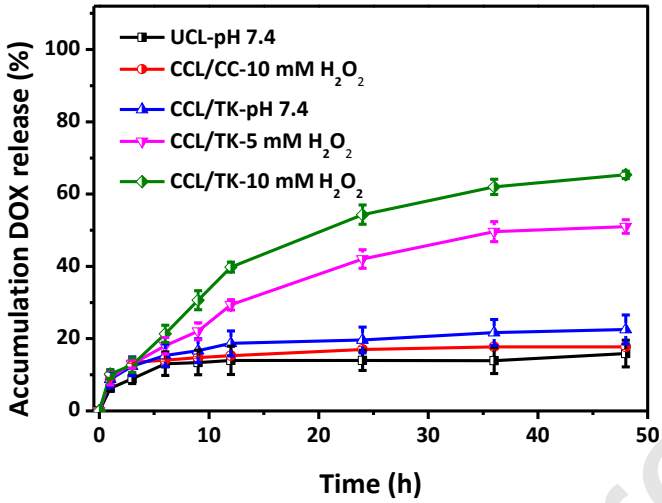


Fig. 3. In vitro DOX release profiles of DOX-loaded micelles in the presence and absence of H_2O_2.

3.3 In vitro cytotoxicity assay

The in vitro cytotoxicities of CCL/TK, CCL/CC, UCL micelles against HeLa and MCF-7 cells were evaluated by MTT assay. The cells were incubated for 48 h at varying concentrations of micelles from 0 to 500 μg/mL. Results showed that CCL/TK, CCL/CC and UCL micelles had excellent biocompatibility and low toxicity. HeLa and MCF-7 cells had a high viability more than 95% at all tested concentrations (Fig. 4a-4b).

The in vitro cytotoxicities of DOX loaded micelles also were evaluated in HeLa and MCF-7 cells using MTT assay (Fig. 4c-4d) with free DOX as control. The results showed that cell survival rate decreases with increasing DOX concentration. The half-maximal inhibitory concentration (IC_{50}) of free DOX (2.52 μg/mL for HeLa, 2.67 μg/mL for MCF-

7) was significantly lower than those of DOX-loaded CCL/TK (3.74 μg/mL for HeLa, 3.91 μg/mL for MCF-7), DOX-loaded CCL/CC (5.15 μg/mL for HeLa, 4.67 μg/mL for MCF-7) and DOX-loaded UCL (7.18 μg/mL for HeLa, 6.74 μg/mL for MCF-7) micelles. Compared with DOX-loaded CCL/CC and UCL micelles, DOX-loaded CCL/TK micelles had higher cytoxicity to HeLa and MCF-7 cells. DOX-loaded CCL/TK micelles exhibited similar cytotoxicity as free DOX, and could release loaded DOX to kill cancer cells. Compared with free DOX, DOX-loaded CCL/TK micelles had lower cytotoxicities. The possible reason was that the process of DOX release from the ROS-responsive DOX-loaded CCL/TK micelles was delayed after CCL micelles endocytosed into the cell [45]. When the incubation time extended to 48 h, DOX-loaded CCL/TK micelles (1.68 μg/mL for HeLa, 0.97 μg/mL for MCF-7) showed higher cytoxicity than free DOX (2.37 μg/mL for HeLa, 2.04 μg/mL for MCF-7) (Fig. S8). Because the H_2O_2 concentration of in vitro cancer cells was much lower than that of tumor tissue and the cell culture medium could consume H_2O_2 [48], exogenous H_2O_2 was added to explore whether the addition of exogenous H_2O_2 could enhance the cytoxicity of DOX-loaded CCL/TK in HeLa cells. The cytoxicity of H_2O_2 was evaluated by the MTT assay (Fig. S9) and the appropriate concentration of H_2O_2 was set to be 30 μM. As shown in Fig. S10, trace amount of exogenous H_2O_2 had significant effect on the toxicity of DOX-loaded micelles. With the addition of 30 μM exogenous H_2O_2, the IC_{50} of DOX loaded CCL/TK, CCL/CC and UCL micelles were 2.39, 3.37 and 3.79 μg/mL respectively. The addition of exogenous H_2O_2 could not only enhance the toxicity of DOX loaded CCL/TK micelles but also enhanced the toxicity of DOX loaded CCL/CC and UCL micelles. It should further be noted that

the cytotoxicity of DOX-loaded CCL/TK micelles might be enhanced via some ways to further increase ROS levels in cancer cells such as loaded photosensitizer [38, 40].

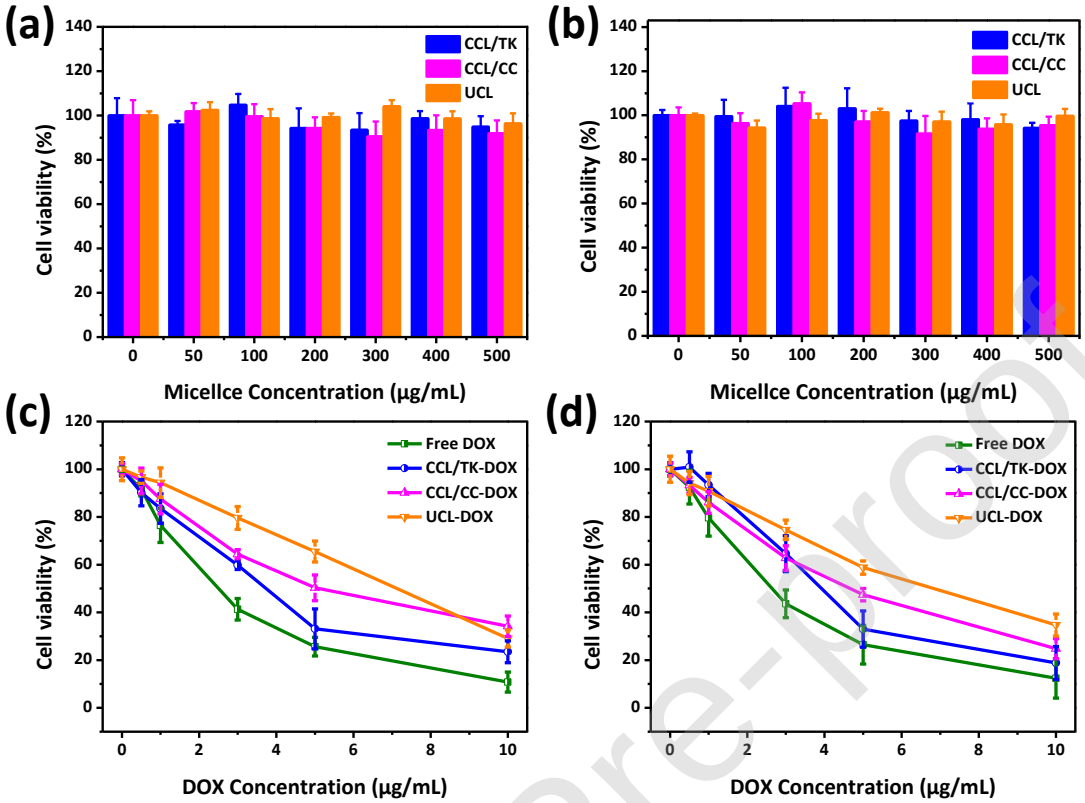


Fig. 4. Cytotoxicity of micelles in HeLa cells (a) and MCF-7 cells (b), free DOX and DOX loaded micelles in HeLa (c) and MCF-7 cells (d). The cells were incubated for 24 h. Data were expressed as mean ± standard deviation (n=3).

3.4 In vitro cellular uptake

The cellular internalization and intracellular distribution of DOX-loaded micelles were investigated in HeLa cells by CLSM and flow cytometry. From the CLSM image (Fig. 5 and Fig. S11), the brighter red DOX fluorescence appeared surrounding the nucleus after DOX-loaded CCL/TK micelles incubated for 4 h. When extending the incubation time to 8 h, stronger DOX fluorescence was observed around the nucleus. However, the fluorescence intensity of free DOX was relatively weak under same culture time (4 h, 8 h), which was possibly due to the fluorescence quenching of free

DOX after intercalation into nuclear DNA [50]. Another possible reason was the different ways to enter the cell. Free DOX entered the cell by diffusion, and it was easily effluxed from the cell [51]. DOX loaded micelles entered the cells through endocytosis, while the DOX released from loaded CCL/TK micelles may not be easily effluxed for a relatively long period [52]. Compared with DOX loaded CCL/CC and UCL micelles, DOX loaded CCL/TK micelles were observed to have stronger DOX fluorescence. This may be attributed to the fact that DOX loaded CCL/TK micelles could release more DOX in cancer cells, which were demonstrated by in vitro DOX release expriments. According to previous reports, the fluorescence of DOX was quenched when DOX was encapsulated in the core of micelles [53]. When DOX was released from the micelles, its fluorescence became stronger. Furthermore, flow cytometry results (Fig. 6 and Fig. S12) were consistent with those of CLSM. The fluorescence intensity of DOX-loaded CCL/TK micelles in HeLa cells was markedly higher than those of DOX loaded CCL/CC, DOX loaded UCL and free DOX after being cultured for 4 h or 8 h. The results proved that the DOX-loaded CCL/TK micelles had excellent internalization efficiency, which could effectively release the encapsulated DOX in cancer cells.

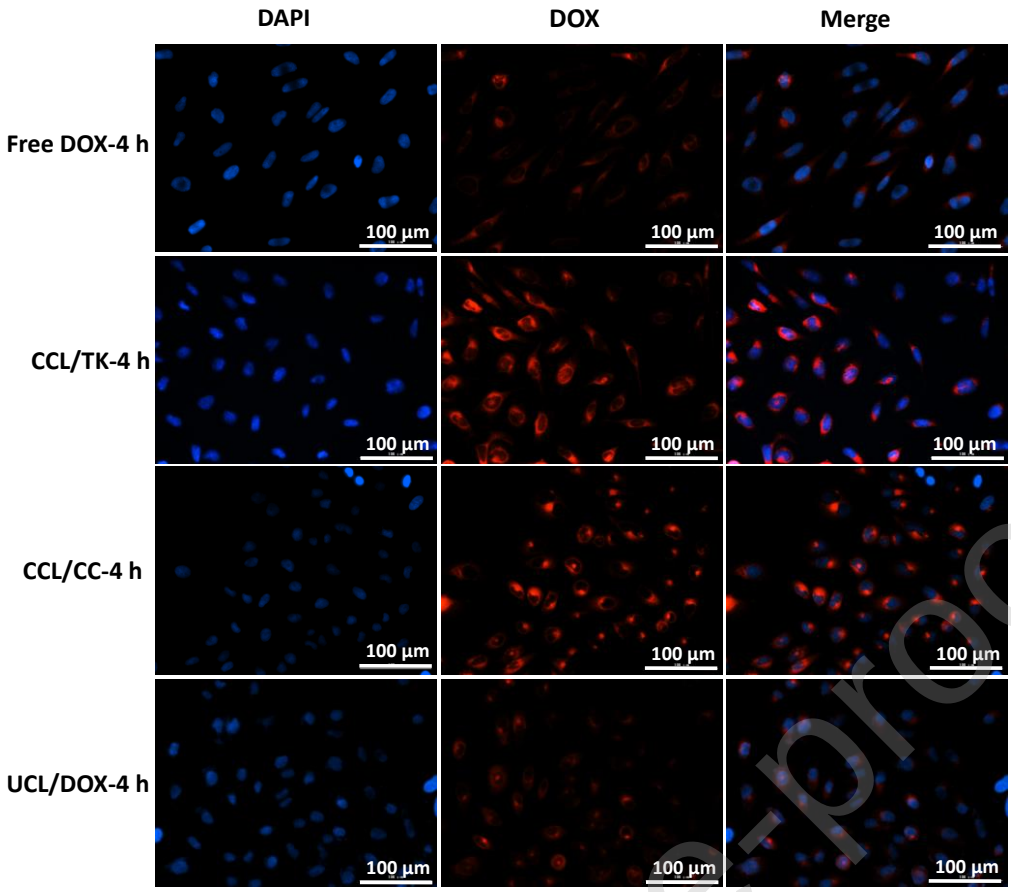


Fig. 5. CLSM images of HeLa cells after incubation with the free DOX, DOX loaded CCL/TK, CCL/CC and UCL micelles for 4 h (DOX dosage: 2 μg/mL).

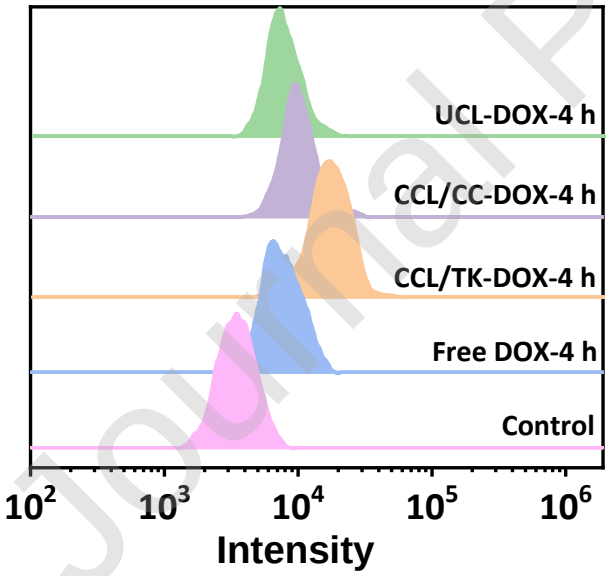


Fig. 6. Flow cytometry analysis of the HeLa cells incubated with free DOX and DOX loaded CCL/TK, CCL/CC and UCL micelles for 4h (DOX dosage: 5 μg/mL).

4. Conclusions

In conclusion, novel ROS-responsive core crosslinked polycarbonate micelles were

conveniently synthesized. The introduction of thioketal containing crosslinker TK-N_3 not only enhanced the stability of the micelles and improved the drug loading, but also endowed ROS-responsiveness for triggered intracellular drug release. The obtained CCL/TK micelles exhibited good biocompatibility and low toxicity for HeLa and MCF-7 cells. DOX-loaded CCL/TK micelles had significantly higher toxicity effects for HeLa and MCF-7 cells, and exhibited excellent internalization efficiency and drug release inside the cancer cells. As a result, polycarbonate based CCL/TK micelles are promising potential as a nanocarrier for drug delivery.

Credit Author Statement:

Deqi Wang: Methodology, Investigation, Formal analysis, Writing-Original draft preparation **Song Wang**: Investigation, Formal analysis **Yingchun Xia**: Methodology, Writing-Reviewing and Editing **Simeng Liu**: Validation, **Ruixin Jia**: Investigation, Formal analysis **Gege Xu**: Formal Analysis **Junjie Zhan**: Investigation, Validation **Yanbing Lu**: Conceptualization, Methodology, Supervision, Writing-Reviewing and Editing

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Yanbing Lu* contributed to design and guide of the research.

Deqi Wang# and Song Wang# contributed equally to this work.

Deqi Wang performed the synthesis and characterization of the CCL micelles and data analyses, and wrote the manuscript.

Song Wang contributed to the in vitro experiments.

Yingchun Xia, Simeng Liu, Ruixin Jia, Gege Xu and Junjie Zhan contributed to the conception of the study and discussion.

Yanbing Lu contributed to design of the research, data investigation, the conception of the study and discussion, and wrote the manuscript

Disclosure statement

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Appendix A. Supplementary data

Supplementary material in online version

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