Published in final edited form as:

Biomaterials. 2011 September ; 32(27): 6633–6645. doi:10.1016/j.biomaterials.2011.05.050.

Well-defined, Reversible Disulfide Cross-linked Micelles for On-demand Paclitaxel Delivery

Yuanpei Li^{a,1}, Kai Xiao^{a,f,1}, Juntao Luo^{b,*}, Wenwu Xiao^a, Joyce S. Lee^{a,e}, Abby M. Gonik^c, Jason Kato^d, Tiffany Dong^g, and Kit S. Lam^{a,d,**}

^aDepartment of Biochemistry & Molecular Medicine, UC Davis Cancer Center, University of California Davis, Sacramento, CA 95817, USA

^bDepartment of Pharmacology, SUNY Upstate Cancer Research Institute, SUNY Upstate Medical University, Syracuse, NY, 13210

^cDivision of Gynecologic Oncology, Department of Obstetrics and Gynecology, UCD Cancer Center, University of California Davis, Sacramento, CA 95817, USA

^dDivision of Hematology and Oncology, Department of Internal Medicine, University of California Davis, Sacramento, CA 95817, USA

^eDepartment of Pharmacy, University of California, Davis Medical Center, Sacramento, CA 95817, USA

^fNational Chengdu Center for Safety Evaluation of Drugs, West China Hospital, Sichuan University, Chengdu 610041, China

^gPrinceton University, Princeton, New Jersey 08544, USA

Abstract

To minimize premature release of drugs from their carriers during circulation in the blood stream, we have recently developed reversible disulfide cross-linked micelles (DCMs) that can be triggered to release drug at the tumor site or in cancer cells. We designed and synthesized thiolated linear-dendritic polymers (telodendrimers) by introducing cysteines to the dendritic oligo-lysine backbone of our previously reported telodendrimers comprised of linear polyethylene glycol (PEG) and a dendritic cluster of cholic acids. Reversibly cross-linked micelles were then prepared by the oxidation of thiol groups to disulfide bond in the core of micelles after the self-assembly of thiolated telodendrimers. The DCMs were spherical with a uniform size of 28 nm, and were able to load paclitaxel (PTX) in the core with superior loading capacity up to 35.5% (w/w, drug/micelle). Cross-linking of the micelles within the core reduced their apparent critical micelle concentration and greatly enhanced their stability in non-reductive physiological conditions as well as severe micelle-disrupting conditions. The release of PTX from the DCMs was significantly slower than that from non-cross-linked micelles (NCMs), but can be gradually facilitated by increasing the concentration of reducing agent (glutathione) to an intracellular reductive level. The DCMs demonstrated a longer *in vivo* blood circulation time, less hemolytic activities, and superior toxicity profiles in nude mice, when compared to NCMs. DCMs were found to be able to

© 2011 Elsevier Ltd. All rights reserved.

*Corresponding author. Tel.: +1 315 464 7965; fax: +1 315 464 8014. **Corresponding author. Tel.: +1 916 734 0910; fax: +1 916 734 4418.

¹Authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

preferentially accumulate at the tumor site in nude mice bearing SKOV-3 ovarian cancer xenograft. We also demonstrated that the disulfide cross-linked micellar formulation of PTX (PTX-DCMs) was more efficacious than both free drug and the non-cross-linked formulation of PTX at equivalent doses of PTX in the ovarian cancer xenograft mouse model. The anti-tumor effect of PTX-DCMs can be further enhanced by triggering the release of PTX on-demand by the administration of the FDA approved reducing agent, N-acetylcysteine, after PTX-DCMs have reached the tumor site.

Keywords

paclitaxel; polymeric micelles; disulfide cross-linking; tumor targeting; on-demand drug release

1. Introduction

Over the past decade, polymeric micelles have been extensively investigated as carriers to deliver conventional anticancer drugs, such as paclitaxel (PTX) and doxorubicin (DOX) [1–6]. Polymeric micelles offer several distinct advantages for these drugs, such as improved solubility, prolonged *in vivo* circulation time and preferential accumulation at tumor site via the enhanced permeability and retention (EPR) effect [7, 8]. The optimal particle size of micelles for passive tumor targeting has been reported to range from 10 to 100 nm [9]. However, polymeric micelles are a thermo-dynamic system, and there is a well-known equilibrium that exhibits between micelles and unimers in aqueous condition. Conventional self-assembled polymeric micelles, after intravenous administration, are susceptible to be diluted below the critical micelle concentration (CMC), which may lead to dissociation into unimers. In addition, the interactions with blood cells and adsorption of unimers to plasma proteins, such as high density lipoprotein (HDL) and low density lipoprotein (LDL), may also disrupt the equilibrium between micelles and unimers [10]. The above factors may lead to premature drug release and early disintegration of the micelles before reaching the tumor target. Burt et al demonstrated that the biodistribution of tritium-labeled paclitaxel was different from ¹⁴C-labeled monomethoxy poly(ethylene glycol)-*block*-poly(D,L-lactide) (PEG-PDLLA) micelles after intravenous administration in rats [11]. Another study indicated that the loss of integrity of poly(ϵ -caprolactone)-*b*-poly(ethylene oxide) (PCL-PEO) micelles was observed at 1 h after intramuscular or subcutaneous injection [12].

Following the pioneering work by Wooley and co-workers, there has been increasing interest in utilizing the cross-linking approach to improve the stability of polymeric micelles for drug delivery [10, 13–22]. Thus far, the reported approaches include shell-cross-linking [13, 19–21, 23–25], core-cross-linking [10, 15–17] and cross-linking at the core-shell interface [18, 26]. Cross-linking not only can improve the structural stability of micelles, but also can control the release rate of the entrapped drugs [10, 15–21]. However, in some cases, excessively stabilized micelles may prevent the drug from releasing to target sites, thus reducing the therapeutic efficacy [27]. In order to render the cross-linking reversible, several degradable linkages, such as reducible disulfide bonds [14, 26–29], pH cleavable [15] or hydrolysable ester bonds [10, 30], have been utilized to design drug delivery systems in response to redox or external stimulus.

The intracellular concentration of glutathione (GSH), a thiol-containing tripeptide generated in cell cytoplasm, is known to be substantially higher than that in the cellular exterior (~10 mM vs ~2 μ M) [26, 27]. An elevated intracellular GSH level has been reported in many human and murine tumor cell lines that are resistant to platinum agents and some other anticancer drugs when compared to normal cell lines [31, 32]. Chemical reactions involving the reduction of disulfide bonds by glutathione has been utilized to design intracellular

specific drug, protein and gene delivery systems [14, 26–29, 33–36]. For example, disulfide cross-linked micelles have been used to encapsulate DOX and methotrexate for cancer therapy [26, 27]. Although promising, these methods require additional cross-linking reagents and processing steps after drug loading into the micelles, and therefore may affect the loading efficiency, particle size and uniformity of the micelles. Furthermore, most of these studies are still at a proof-of-concept stage using cultured cancer cells. None of the disulfide cross-linked delivery systems reported thus far have been evaluated *in vivo*. Therefore, substantial efforts are needed toward the convenient formulation of cross-linked micelles with superior drug loading and narrow size distribution coupled with further *in vivo* evaluation of these nanocarrier systems for future clinical application.

We previously reported the development of several drug delivery systems based on micelles formed by a class of well-defined amphiphilic PEGylated oligomer of cholic acids synthesized via peptide chemistry. We have already demonstrated that such PTX-loaded micelles exhibit superior toxicity and efficacy profile in xenograft models when compared to the FDA approved free drug (Taxol®) or albumin bounded PTX (Abraxane®) [37–40]. The modular design enables us to assemble different components in a step-wise fashion with high flexibility. For example, nontoxic building blocks, such as hydrophilic spacers (e.g. Ebes [37]), and amino acids, can be easily introduced into the telodendrimers at the desired position and at the optimal numbers. In the present study, we aimed to develop a reversibly cross-linked micelle system for specific delivery of paclitaxel to tumor sites. To this end, we introduced cysteines into the telodendrimers and developed a self-assembling disulfide-crosslinking system so that micelles can be further stabilized to avoid premature release of the loaded drugs during circulation (schemes shown in Fig 1). After reaching the tumor sites, the intra-micellar disulfide bonds will be cleaved under reductive conditions, such as the intracellular environment (glutathione) or the enhanced reductive extracellular conditions by the additional administration of the reducing agent (N-acetylcysteine (NAC)), and the drug payload will be released. The formulation of paclitaxel into the DCMs was optimized with respect to drug loading efficiency, size and clinical feasibility. The DCMs were further characterized *in vitro* for the drug release profile, cellular uptake and cytotoxicity. Then, the biodistribution and blood elimination kinetics of near infrared fluorescence (NIRF) dye labeled DCMs were evaluated *in vivo* via optical imaging and fluorescence spectrometry, respectively. Finally, the therapeutic efficacy and toxicity profiles of the DCMs entrapped with PTX were investigated in SKOV-3 tumor xenograft bearing mice.

2. Materials and Methods

2.1 Materials

Monomethylterminated poly(ethylene glycol) monoamine (MeO-PEG-NH₂, M_w: 5000 Da) was purchased from Rapp Polymere (Germany). PTX was purchased from AK Scientific Inc. (Mountain View, CA). Taxol® (Mayne Pharma, Paramus, NJ) was obtained from the Cancer Center of University of California, Davis. (Fmoc)lys(Boc)-OH, (Fmoc)Lys(Dde)-OH, (Fmoc)Lys(Fmoc)-OH, (Fmoc)Cys(Trt)-OH and (Fmoc)Ebes-OH were obtained from AnaSpec Inc. (San Jose, CA). 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD), BODIPY650/665 and 4, 6-diamidino-2-phenylindole (DAPI, blue) were purchased from Invitrogen. Cholic acid, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide], Ellman's reagent [DTNB, 5,5'-dithiobis(2-nitrobenzoic acid)] and all other chemicals were purchased from Sigma-Aldrich (St. Louis).

2.2 Synthesis of telodendrimers

The thiolated telodendrimer (named as PEG^{5k}-Cys₄-L₈-CA₈, Fig1, Fig S-1) was synthesized via solution-phase condensation reactions from MeO-PEG-NH₂ utilizing stepwise peptide chemistry [38]. The typical procedure for synthesis of PEG^{5k}-Cys₄-L₈-CA₈ was as follows: (Fmoc)Lys(Dde)-OH (3 eq.) was coupled onto the N terminus of PEG using DIC and HOBt as coupling reagents until a negative Kaiser test result was obtained, thereby indicating completion of the coupling reaction. PEGylated molecules were precipitated by adding cold ether and then washed with cold ether twice. Fmoc groups were removed by the treatment with 20% (v/v) 4-methylpiperidine in dimethylformamide (DMF), and the PEGylated molecules were precipitated and washed three times by cold ether. White powder precipitate was dried under vacuum and two coupling of (Fmoc)Lys(Fmoc)-OH and one coupling of (Fmoc)lys(Boc)-OH were carried out respectively to generate a third generation of dendritic polylysine terminated with four Boc and Fmoc groups on one end of PEG. After the removal of Boc groups with 50% (v/v) trifluoroacetic acid (TFA) in dichloromethane (DCM), (Fmoc)Cys(Trt)-OH, (Fmoc)Ebes-OH and Cholic acid NHS ester [38] were coupled step by step to the terminal end of dendritic polylysine. The Trt groups on cysteines were removed by TFA/H₂O/ethanedithiol (EDT)/triethylsilane (TIS) (94:2.5:2.5:1, v/v) resulting in PEG^{5k}-Cys₄-L₈-CA₈ thiolated telodendrimer (Fig S-1). The thiolated telodendrimer was recovered from the mixture by three cycles of dissolution/precipitation with DMF and ether, respectively. Finally, the thiolated telodendrimer was dissolved in acetonitrile/water and lyophilized. The PEG^{5k}-CA₈ thiol free telodendrimer was synthesized to prepare the non-cross-linked micelles according to our previously reported method [38, 39]. BODIPY650/665 (NIRF dye) labeled telodendrimers were synthesized by coupling BODIPY NHS ester to the amino group of the proximal lysine between PEG and cholic acid after the removal of 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting group by 2% (v/v) hydrazine in DMF.

Ellman's test was used to determine the number of cysteines conjugated to telodendrimers by free thiol groups. After adding Ellman reagents to a standard thiol (cysteine) for 15 min, a calibration curve was prepared by plotting the absorbance at 412 nm as function of cysteine concentrations (Fig S-2). Based on the calibration curve, the number of cysteines on the telodendrimers was calculated from the absorbance of samples in Ellman's test. The mass spectra of the telodendrimers were collected on ABI 4700 MALDI TOF/TOF mass spectrometer (linear mode) using R-cyano-4-hydroxycinnamic acid as a matrix. ¹H NMR spectra of the polymers were recorded on an Avance 500 Nuclear Magnetic Resonance Spectrometer using CDCl₃ and D₂O as solvents. The concentration of the polymers was kept at 5 × 10⁻⁴ M for NMR measurements.

2.3 Preparation of disulfide cross-linked micelles

20 mg PEG^{5k}-Cys₄-L₈-CA₈ telodendrimer was dissolved in 1 mL phosphate buffered saline (PBS) to form micelles and then sonicated for 10 min. The thiol groups on the telodendrimer were oxidized to form disulfide linkages by purging oxygen into the micelle solution. The level of free thiol groups were monitored by Ellman's test over time. The micelle solution was used for further characterizations without dialysis after the level of free thiol groups remained at a constant low value.

2.4 Preparation of PTX and DiD loaded micelles

PTX was loaded into the micelles by the solvent evaporation method as described in our previous studies. Briefly, PTX (1, 2, 3, 5, 7.5, 9 mg) and PEG^{5k}-Cys₄-L₈-CA₈ telodendrimers (20 mg) were first dissolved in chloroform in a 10 mL round bottom flask. The chloroform was evaporated under vacuum to form a thin film. PBS buffer (1 mL) was added to re-hydrate the thin film, followed by 30 min of sonication. The PTX-loaded

micelles were then cross-linked via O₂-mediated oxidization as described above. The amount of drug loaded in the micelles was analyzed on a HPLC system (Waters) after releasing the drugs from the micelles by adding 9 times of acetonitrile and 10 min sonication. The drug loading was calculated according to the calibration curve between the HPLC area values and concentrations of drug standard. The loading capacity is defined as the highest drug concentration that can be achieved by the micelles in aqueous solution while the loading efficiency is defined as the ratio of drug loaded into micelles to the initial drug content. One part of the PTX-loaded micelle solutions was stored at 4°C for characterizations and the rest was lyophilized. The PTX loaded non-cross-linked micelles were prepared by using PEG^{5k}-CA₈ thiol free telodendrimer as reported previously[38]. DiD (hydrophobic NIRF dye) was loaded into the micelles using the same method as described above. The micelle solution was filtered with 0.22 µm filter to sterilize the sample.

2.5 Characterizations of micelles

The size and size distribution of the micelles were measured by dynamic light scattering (DLS) instruments (Microtrac). The micelle concentrations were kept at 1.0 mg/mL for DLS measurements. The zeta potential of these micelles was measured by DLS using the function of Zetatrac (Microtrac). All measurements were performed at 25 °C, and data were analyzed by Microtrac FLEX Software 10.5.3. The morphology of micelles was observed on a Philips CM-120 transmission electron microscope (TEM). The aqueous micelle solution (1.0 mg/mL) was deposited onto copper grids, stained with phosphotungstic acid, and measured at room temperature. The critical micelle concentration (CMC) of the PEG^{5k}-CA₈ micelles and PEG^{5k}-Cys₄-L₈-CA₈ micelles before and after cross-linking was measured through fluorescence spectra by using pyrene as a hydrophobic fluorescent probe as described previously [6, 25, 38, 41]. Briefly, micelles were serially diluted in PBS to give the concentrations ranging from 5×10^{-7} to 5×10^{-4} M. The stock solution of pyrene in methanol was added into the micelle solution to make a final concentration of pyrene of 2×10^{-6} M. The solution was mildly shaken over night. Excitation spectra were recorded ranging from 300 to 360 nm with a fixed emission at 390 nm. The ratios of the intensity at 337 to 332 nm from the excitation spectra of pyrene were plotted against the concentration of the micelles. The CMC was determined from the threshold concentration, where the intensity ratio I₃₃₇/I₃₃₂ begins to increase markedly.

2.6 Stability of micelles in SDS and human plasma

The stability study was performed to monitor the change in particle size of the DCMs and NCMs in the presence of sodium dodecyl sulfate (SDS), which was reported to be able to efficiently break down polymeric micelles [26]. An SDS solution (7.5 mg/mL) was added to aqueous solutions of micelles (1.5 mg/mL). The final SDS concentration was 2.5 mg/mL and the micelle concentration was kept at 1.0 mg/mL. The size and size distribution of the micelle solutions was monitored at predetermined time intervals. The stability of the micelles was also evaluated in the presence of GSH and NAC (20 mM) together with SDS. The lyophilized PTX-loaded micelle powder was re-hydrated with PBS and tested under the same conditions. At the end of the stability study, the samples were further observed under TEM. The stability of PTX-loaded NCMs and DCMs was further studied in 50% (v/v) plasma from healthy human volunteers. The mixture was incubated at physiological body temperature (37 °C) followed by size measurements at predetermined time intervals up to 96 h.

2.7 Drug release study

PTX-loaded cross-linked micelle solution was prepared to determine the *in vitro* drug release profile. The initial PTX concentration was 4.6 mg/mL. Aliquots of PTX-loaded cross-linked micelle solution were injected into dialysis cartridges (Pierce Chemical Inc.)

with a 3.5 kDa MWCO. The cartridges were dialyzed against 1 L PBS with various GSH concentrations (0, 2 μ M, 1 mM, and 10 mM) at 37 °C. In order to make an ideal sink condition, 10 g charcoal was added in the release medium. The concentration of PTX remaining in the dialysis cartridge at various time points was measured by HPLC. The drug release profiles of Taxol® and PTX loaded non-cross-linked micelles (PTX concentration: 5.0 mg/mL) were determined under identical condition for comparison. In some experiments, GSH or NAC (10 mM) were added to the release medium at a specific release time (5 h). The PTX release profiles of the lyophilized and rehydrated micelle solution were evaluated under the same conditions. Values were reported as the means for each triplicate sample.

2.8 Cell uptake and MTT assay

SKOV-3 ovarian cancer cells were seeded at a density of 50000 cells per well in eight-well tissue culture chamber slides (BD Biosciences, Bedford, MA, USA), followed by 24 h of incubation in McCoy's 5a Medium containing 10% FBS. The medium was replaced, and DiD labeled micelles (100 μ g/mL) were added to each well. After 30 min, 1h, 2h and 3h, the cells were washed three times with PBS, fixed with 4% paraformaldehyde and the cell nuclei were stained with DAPI. The slides were mounted with cover slips and observed under confocal laser scanning microscope (Olympus, FV1000).

SKOV-3 cells were seeded in 96-well plates at a density of 10000 cells/well 24 h prior to the treatment. The cells were first treated with or without GSH-OEt (20 mM) for 2 h and then washed 3 times with PBS. Empty micelles and various formulations of PTX with different dilutions were added to the plate and then incubated for 2 h. The cells were washed with PBS and incubated for another 22 h in a humidified 37 °C, 5% CO₂ incubator. MTT was added to each well and further incubated for 4 h. The absorbance at 570 nm and 660 nm was detected using a micro-plate ELISA reader (SpectraMax M2, Molecular Devices, USA). Untreated cells served as a control. Results were shown as the average cell viability $[(OD_{\text{treat}} - OD_{\text{blank}})/(OD_{\text{control}} - OD_{\text{blank}}) \times 100\%]$ of triplicate wells.

2.9 Hemolysis assay

The hemolysis of NCMs and DCMs was investigated using fresh citrated blood from healthy human volunteers. The red blood cells (RBCs) were collected by centrifugation at 1000 rpm for 10 min, washed three times with PBS, and then brought to a final concentration of 2% in PBS. 200 μ L of erythrocyte suspension was mixed with different concentrations (0.2 and 1.0 mg/mL) of NCMs and DCMs, respectively, and incubated for 4 h at 37°C in an incubator shaker. The mixtures were centrifuged at 1000 rpm for 5 min, and 100 μ L of supernatant of all samples was transferred to a 96-well plate. Free hemoglobin in the supernatant was measured by the absorbance at 540 nm using a micro-plate reader (SpectraMax M2, Molecular Devices, USA). RBC incubation with Triton-100 (2%) and PBS were used as the positive and negative controls, respectively. The percent hemolysis of RBCs was calculated using the following formula: $\text{RBCs hemolysis} = (OD_{\text{sample}} - OD_{\text{negative control}})/(OD_{\text{positive control}} - OD_{\text{negative control}}) \times 100\%$.

2.10 Animal and tumor xenograft model

Female athymic nude mice (Nu/Nu strain), 6–8 weeks age, were purchased from Harlan (Livermore, CA). All animals were kept under pathogen-free conditions according to AAALAC guidelines and were allowed to acclimatize for at least 4 days prior to any experiments. All animal experiments were performed in compliance with institutional guidelines and according to protocol No. 07-13119 and No. 09-15584 approved by the Animal Use and Care Administrative Advisory Committee at the University of California, Davis. The subcutaneous xenograft model of ovarian cancer was established by injecting

7×10^6 SKOV-3 ovarian cells in a 100 μ L of mixture of PBS and Matrigel (1:1 v/v) subcutaneously into the right flank of female nude mice.

2.11 In vivo blood elimination kinetics and biodistribution

DiD or BODIPY labeled NCMs and DCMs were prepared for the blood elimination study as described in Section 2.2 and 2.4. The concentration of BODIPY conjugated micelles was 5 mg/mL. The concentration of DiD loaded micelles was 20 mg/mL with DiD loading at 0.5 mg/mL. The fluorescence spectra of these fluorescently labeled micelles diluted 20 times by PBS were characterized by fluorescence spectrometry (SpectraMax M2, Molecular Devices, USA). 100 μ L of BODIPY conjugated or DiD loaded NCMs and DCMs were injected into tumor free nude mice via tail vein. 50 μ L blood was collected at different time points post-injection to measure the fluorescence signal of DiD or BODIPY.

Nude mice with subcutaneous SKOV-3 tumors of an approximate 8~10 mm diameter were subjected to *in vivo* NIRF optical imaging. At different time points post injection of DiD and PTX co-loaded cross-linked micelles (the concentrations of DiD and PTX were both 0.5 mg/mL), mice were scanned using a Kodak multimodal imaging system IS2000MM with an excitation bandpass filter at 625 nm and an emission at 700 nm. The mice were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg) before each imaging. After *in vivo* imaging, animals were euthanized by CO₂ overdose at 24 h after injection. Tumors and major organs were excised and imaged with the Kodak imaging station.

2.12 In vivo toxicity of empty micelles

In order to investigate for telodendrimer related toxicity, both empty non-cross-linked and cross-linked micelles were injected in tumor free nude mice at the single dose of 200 mg/kg and 400 mg/kg via tail vein. Mice were checked for possible signs of toxicity and the survival situation was monitored daily for two weeks.

2.13 In vivo therapeutic study

Nude mice bearing SKOV-3 ovarian cancer xenografts were used to evaluate the therapeutic efficacy of the different formulations of PTX. The treatments were initiated when tumor xenograft reached a tumor volume of 100–200 mm³ and this day was designated as day 0. On day 0, these mice were randomly divided into seven groups and injected intravenously via the tail vein with the formulations and repeated every 3 days for total 6 doses. Injection volume was 0.1 mL for each 10 g of mouse body weight. The seven groups (n=8–10) are shown in Table 1. Taxol® was given at a dose of 10 mg/kg which is close to its maximum tolerated dose (MTD) [39, 42]. PTX loaded NCMs and DCMs were administered at the same PTX dose (10 mg/kg) for comparison. Because the micellar formulations of PTX are much more tolerated as we reported previously (MTD 75 mg/kg) [39], The PTX loaded micelles were also administered at a higher dose (30 mg/kg) to determine if the anti-tumor effect could be enhanced. N-acetylcysteine (NAC) is a reducing agent and has been approved by FDA for mucolytic therapy (brand name: Mucomyst®) and the treatment of acetoaminophen overdose. In the seventh group, NAC was injected at a dose of 100 mg/kg into the mice via tail vein at 24 h after the administration of every dose of PTX loaded DCMs. Tumor size was measured with a digital caliper twice per week. Tumor volume was calculated by the formula $(L \times W^2)/2$, where L is the longest and W is the shortest in tumor diameters (mm). To compare between groups, relative tumor volume (RTV) was calculated at each measurement time point (where RTV equals the tumor volume at given time point divided by the tumor volume prior to initial treatment). To monitor potential toxicity, the body weight of each mouse was measured every 3 days. For humane reasons, animals were euthanized when the implanted tumor volume reached 1500 mm³, which was considered as the end point of survival data.

2.14 Statistical analysis

Statistical analysis was performed by Student's t-test for two groups, and one-way ANOVA for multiple groups. All results were expressed as the mean $\pm$ standard error (SEM) unless otherwise noted. A value of $P < 0.05$ was considered statistically significant.

3 Results and Discussion

3.1 Physico-chemical characterizations of micelles

Because the well-established stepwise Fmoc peptide chemistry method was employed in the preparation of the polymer, the resulting thiolated telodendrimers have well-defined polymer structure (Fig 1, Fig S-1) [37–39]. Telodendrimer is designated as PEG^{5k}-Cys₄-L₈-CA₈ corresponding to length of PEG and the number of cysteines, hydrophilic spacers and cholic acids in the structure. As shown in Fig 1 and Fig S-1A, 2B, PEG^{5k}-Cys₄-L₈-CA₈ consists of a dendritic oligomer of cholic acids attached to one terminus of the linear PEG through a poly(lysine-cysteine-Ebes) backbone. The thiol free telodendrimer, PEG^{5k}-CA₈ was also synthesized for comparison as described previously [39]. Fluorescent dyes such as BODIPY can be attached to the ϵ -amino group of the lysine at the junction between the PEG and the oligo-cholic acid chains after removal of Dde protecting group. As determined by quantitative Ellman's test by using free cysteine to create a standard curve (Fig S-2), the number of covalently attached cysteines in PEG^{5k}-Cys₄-L₈-CA₈ was 3.97, which was consistent with the molecular formula of the target telodendrimer. The molecular weight of PEG^{5k}-Cys₄-L₈-CA₈ was determined with MALDI-TOF Mass Spectrometry comparing with the starting PEG and PEG^{5k}-CA₈. The mono-dispersed mass traces were detected for the starting PEG and the telodendrimers, and the molecular weights of the telodendrimers from MALDI-TOF MS (Fig S-3) were almost identical to the theoretical value (Table 2). The chemical shift of PEG chains (3.5–3.7 ppm) and cholic acid (0.6–2.4 ppm) could be observed in the ¹H NMR spectra of the PEG^{5k}-Cys₄-L₈-CA₈ in CDCl₃ (Fig S-4). The integration of these peaks can be used to calculate the chemical compositions of the telodendrimers. The number of cholic acids determined by ¹H-NMR for the telodendrimers was consistent with the molecular formula of the target telodendrimers (Table 1). These results demonstrate the well-defined structure of telodendrimers. When the NMR spectrum of PEG^{5k}-Cys₄-L₈-CA₈ was recorded in D₂O, the cholic acid proton peaks were highly suppressed (Fig S-4), indicating the entanglement of cholanes by the formation of core-shell micellar structure in the aqueous environment. The CMC of PEG^{5k}-CA₈ micelles and PEG^{5k}-Cys₄-L₈-CA₈ micelles before cross-linking were measured using pyrene as a hydrophobic fluorescent probe and found to be 5.53 μ M and 5.96 μ M, respectively.

The PEG^{5k}-Cys₄-L₈-CA₈ micelles exhibited a size of 26 nm before cross-linking, which is also similar to PEG^{5k}-CA₈ micelles (Table 2, Fig S-5A). These results indicate that PEG^{5k}-CA₈ micelles and PEG^{5k}-Cys₄-L₈-CA₈ micelles have similar physical properties. The PEG^{5k}-Cys₄-L₈-CA₈ micelles without drug loading (empty micelles) were further characterized with respect to particle size, apparent CMC and zeta potential following disulfide cross-linking. After the instant formation of micelles upon dispersion in aqueous solution, the free thiol groups of PEG^{5k}-Cys₄-L₈-CA₈ were oxidized by oxygen to form disulfide linkages. The O₂-mediated oxidation was monitored by Ellman's test. As shown in Fig 2A and Fig 2B, the free thiol groups were oxidized over time and more than 85% of thiol groups were reacted to form disulfide after 48 h of oxidation. Interestingly, the PEG^{5k}-Cys₄-L₈-CA₈ micelles retained a similar particle size of around 27 nm with the narrow distribution following disulfide cross-linking (Fig S-5B). This result suggests that the disulfide bond formation is an event that occurs within micelles. The PEG outer corona confines the cross-linking reaction intra-micellarly, preventing the formation of inter-micellar aggregates. After cross-linking, the apparent CMC of PEG^{5k}-Cys₄-L₈-CA₈ micelles

decreased significantly to 0.67 μM , which is 9 times lower than that of the non-cross-linked micelles (Table 2). This observation for the cross-linked micelles is consistent with the reported cross-linked Pluronic L121 micelles [25]. The zeta potential of the micelles was measured to be nearly neutral since these micelles were composed of uncharged PEG^{5k}-Cys₄-L₈-CA₈. PEG^{5k}-CA₈ micelles were selected as absolute non-cross-linked micelles (NCMs) in the following *in vitro* and *in vivo* evaluations, instead of the PEG^{5k}-Cys₄-L₈-CA₈ micelles prior to oxidation, because the latter will undoubtedly be partially cross-linked by oxygen in air upon storage. Furthermore, we want to directly compare the current cross-linked nanoformulation with our previously published non-cross-linked PEG^{5k}-CA₈ micelle [38, 39].

3.2 Loading paclitaxel into DCMs

We have previously demonstrated that PTX, a wide-spectrum anti-tumor agent, can be efficiently encapsulated into PEG^{5k}CA₈ non-cross-linked micelles via solvent evaporation method [39]. PEG^{5k}-Cys₄-L₈-CA₈ micelles exhibited similar excellent PTX loading capacity before and after cross-linking via O₂-mediated oxidation. Before cross-linking, the PTX loading capacity in PEG^{5k}-Cys₄-L₈-CA₈ micelles was able to reach a very high level of 8.6 mg/mL (8.6 mg PTX loaded in 20 mg micelles in 1 mL PBS) (Fig 2C). The loading efficiencies were almost 100% (Fig S-6) and the final particle sizes remained in the range of 25–50 nm (Fig 2D) for all the loadings prior to cross-linking. After cross-linking via oxygen, the PTX loading capacity of the micelles decreased slightly from 8.6 mg/mL to 7.1 mg/mL, which is equivalent to 35.5% (w/w) of drug/micelle ratio (Table 2). It should be mentioned that the micelles retained the similar particle size and 100% PTX loading efficiency at a PTX loading of 5.0 mg/mL and lower after cross-linking. However, beyond 5.0 mg/mL, the particle sizes of the cross-linked micelles increased significantly (Fig 2D) while the loading efficiency decreased to 81% (Fig S-6). The changes in the particle size and loading efficiency after cross-linking could be explained by the dynamic process of micelles encapsulating hydrophobic drugs. For the micelles with low to medium drug loading (e.g. PTX loading < 5.0 mg/mL), polymers have large extent of inter-molecular overlapping, thereby allowing the high level inter-polymer disulfide formation after oxidation. The disulfide bond formation is restricted within micelles, which is similar to that of empty micelles as described in Section 3.1. When the drug loading is high (e.g. PTX loading > 5.0 mg/mL), the micelles may re-assemble into bigger micelles in order for overlapping polymers to form disulfide bonds and at the same time accommodating the large amount of drug. A small portion of drug (< 19%) may leak out from the micelles during the re-assembly.

The morphology of the PTX loaded cross-linked micelles was observed to be spherical with uniform sizes under a TEM after staining with phosphotungstic acid (Fig 2F). The size of the micelles observed under TEM were consistent with those measured by DLS (Fig 2E, 2F). In order to make a more convenient formulation for clinical application, the PTX loaded cross-linked micelles prepared in water were lyophilized. Following re-hydration with PBS and vortexing, the lyophilized PTX-DCMs yielded a clear micelle solution with similar PTX content and uniform size around 27 nm (Fig 3E). This size range enables these PTX loaded micelles to take full advantage of the EPR effect and accumulate at tumor sites [9, 38].

3.3 Stability study of the micelles

Both PTX loaded non-cross-linked micelles (PTX-NCMs) and disulfide cross-linked micelles (PTX-DCMs) have been found to be very stable at 4 °C, showing no significant changes in average particle size and drug contents over 8 months (data not shown). We further investigated whether the intra-micellar disulfide cross-linking can enhance micellar

stability against physiological conditions and severe micelle-disrupting conditions. To demonstrate the stability of the micelles in physiological conditions including blood, the PTX-NCMs and PTX-DCMs were incubated with 50% human plasma, and the particle sizes of micelles were monitored by DLS over time. Both of the DCMs and NCMs micelles with similar PTX loading retained the average particle size around 30 nm in human plasma for 24 hours (Fig 3). However, the PTX-DCMs still kept the uniformity and narrow distribution in size while the PTX-NCMs showed significantly broader size distribution and population of size over 100 nm, indicating the formation of large aggregates (Fig 3). Sodium dodecyl sulfate (SDS), a strong ionic detergent, has been reported to be able to efficiently break down polymeric micelles [26]. The exchange rate between polymeric micelles and unimers is accelerated by low concentrations of SDS while at higher concentrations, the presence of SDS micelles solubilize the amphiphilic block copolymers resulting in destabilization of the polymeric micelles [10]. The stability of NCMs and DCMs was also tested in the presence of the reported micelle-disrupting SDS concentration of 2.5 mg/mL [26]. The size of SDS background is below the detection limit of DLS analysis, showing a 0.9 nm population in the spectra (Fig S-7). After each micelle solution (1.0 mg/mL) was mixed with an aqueous solution of SDS (2.5 mg/mL), the particle size was monitored at various time points. The immediate disappearance of particle size signal of the NCMs reflects the distinct dynamic association-dissociation property of non-cross-linked micelles (Fig 4A, Fig S-8A, 8B). The constant particle size of the DCMs under similar condition over time indicated that such cross-linked micelles remained intact (Fig 4A, Fig S-8C, 8D). The re-hydrated lyophilized PTX-DCMs also retained the particle size at around 26 nm in the presence of SDS (Fig 3E, 3F). The GSH concentration inside cells (~10 mM) is known to be substantially higher than the extracellular level (~2 μ M) [26]. As shown in Fig S-8E, the DCMs were stable in SDS solution with a cellular exterior level of GSH (~2 μ M). However, in the presence of SDS and an intracellular reductive GSH level (10 mM), the disulfide cross-linked micelle particle size signal remained unchanged for 30 min until it decreased suddenly (within 10 sec), indicating that rapid dissociation of the micelle when a critical number of disulfide bonds were reduced (Fig 4A, Fig S-8F). The responses of PTX-NCMs and PTX-DCMs to SDS and different levels of GSH were similar to those of empty NCMs and DCMs, respectively. We also found that N-acetyl cysteine (NAC) could efficiently cleave the disulfide bonds of the DCMs, as evidenced by the complete disappearance of particle size of DCMs after 40 min in the presence of SDS and NAC (10 mM) (data not shown). The samples of DCMs and NCMs were further examined by TEM at the end point of the stability study. It was further confirmed that the micellar structure of NCMs was destroyed in SDS solution (Fig 4B). The TEM images also demonstrated the micellar structure of DCMs were well retained in the presence of SDS (Fig 4C) but efficiently broken down in the presence of SDS and 10 mM of GSH (Fig 4D).

3.4 PTX release profiles of micelles

The PTX release profiles from Taxol®, NCMs and DCMs were compared by using the dialysis method. PTX release from Taxol® was rapid and about 60% of PTX was released within the first 5 h. In contrast, PTX release from NCMs and DCMs was significantly slower (Fig S-9). In the presence of GSH at its extracellular level (2 μ M), the PTX release profile from DCMs was similar to that in the release media without GSH. It was noted that the PTX release was gradually facilitated as the GSH concentration increased up to the intracellular level (10 mM) (Fig 5A). This drug release study also indicated that PTX release from DCMs was significantly slower than that from NCMs (Fig S-9, Fig 5B). When 10 mM GSH was added at the 5 h time point, there was a burst of drug release from the DCMs but not the NCMs (Fig 5B). Subsequent release curves (after 6 h) of both preparations were identical (Fig 5B). This two-stage drug release strategy can be exploited such that premature drug release during circulation *in vivo* can be minimized, but with accelerated release upon

internalization of the micelles into cancer cells. The PTX release profiles of the re-hydrated lyophilized PTX-DCMs were found to be very similar to the fresh sample and can be greatly facilitated by GSH (Fig 5C). NAC, a FDA approved reducing agent, was demonstrated to have the same effect as GSH in triggering the PTX release from the disulfide cross-linked micelles (Fig 5D). Therefore, NAC can be applied *in vivo* as an on-demand cleavage reagent via systemic i.v. injection to trigger drug release after nanotherapeutics have accumulated in tumor sites.

3.5 In vitro cellular uptake and cytotoxicity studies

Since the GSH-mediated drug release from DCMs should occur inside cells, it is essential to investigate the internalization of these micelles into cancer cells. The cellular uptake of DiD-labeled DCMs was observed in SKOV-3 ovarian cancer cells (Fig S-10). As the incubation time increased from 0.5 h to 3 h, the DiD fluorescence intensity increased gradually. Confocal microscopy images showed that DCMs internalized within cells were mainly localized in the cytoplasmic region. All the building blocks for the telodendrimers, including PEG, lysine, cysteine, Ebes and cholic acid are nontoxic. Both empty NCMs and DCMs showed no observable *in vitro* cytotoxicity up to 1.0 mg/mL against SKOV-3 ovarian cancer cells by MTT assay (Fig 6A). The *in vitro* anticancer activity of PTX loaded disulfide cross-linked micelles (PTX-DCMs) and PTX loaded non-cross-linked micelles (PTX-NCMs) was evaluated on SKOV-3 cells and compared with Taxol®. The SKOV-3 cells were incubated with different formulations of PTX for 2 h, washed to remove the unbound drugs and then further incubated for 22 h. PTX-NCMs showed comparable *in vitro* anti-tumor effects against SKOV-3 cells as Taxol® (Fig 6B). However, PTX-DCMs were found to be less cytotoxic than Taxol and PTX-NCMs, which was expected due to the slower release of PTX within the cell culture media as well as after the cellular uptake of PTX-DCMs (Fig 6B). Because of its anionic nature, GSH itself is not effectively transported into cells. It was reported that glutathione monoethyl ester (GSH-OEt), a neutralized form of GSH, is able to penetrate cellular membranes and can be rapidly hydrolyzed in the cytoplasm to generate GSH [29, 43]. The *in vitro* anticancer activity study was further performed in SKOV-3 cells with an enriched GSH level to investigate whether the GSH-responsive PTX-DCMs could display enhanced intracellular drug release. In this experiment, the intracellular GSH concentration was manipulated by pre-treating the cells with 20 mM GSH-OEt for 2 h. Pre-incubation of cells with GSH-OEt enhances the inhibition effect of PTX-DCMs when the concentration of PTX was higher than 10 ng/mL (Fig 6B). In contrast, the toxicity profile of PTX-NCMs was not affected by the GSH-OEt pre-treatment. As described above, the addition of GSH-OEt increases the intracellular GSH concentration, and facilitates intracellular drug release because of the cleavage of intra-micellar disulfide bridges of DCMs, which results in enhanced cytotoxicity.

3.6 Hemolysis study

Detrimental interaction of polymeric micelles with blood constituents, such as red blood cells, must be avoided when these particles are injected into the blood stream as vehicles for drug delivery. Therefore, the hemolysis study provides information on micelles concerning their hemato-compatibility in the case of *in vivo* application [44]. The NCMs were formed by amphiphilic telodendrimers and have the potential to solubilize lipids or insert into phospholipid membranes, causing disruption of plasma membranes similar to small molecular surfactants. As shown in Fig 6C, empty NCMs were found to have dose dependent RBC lysis. The percentage of hemolysis increased from 9.0% to 16.3% with the increasing NCMs concentrations from 0.2 mg/mL to 1.0 mg/mL. In contrast, empty DCMs showed no observable hemolytic activities (<5%) in the RBCs at the same experimental concentrations. The intra-micellar disulfide bridges prevent DCMs from dissociation to form amphiphilic telodendrimers, thus minimizing the hemolytic activities.

3.7 In vivo blood elimination kinetics and biodistribution

NIRF dyes enable deep tissue imaging with high penetration, low tissue absorption and scattering [39]. In order to track and compare blood elimination kinetics and biodistribution of the micelles, we prepared two separate types of near infrared dye-labeled NCMs and DCMs. BODIPY 650/665, an organic near infrared dye, was conjugated to telodendrimers to track the *in vivo* fate of the carriers. A hydrophobic near infrared dye, DiD, was physically encapsulated into the core of the micelles as a drug surrogate to monitor the *in vivo* distribution of payloads. The blood elimination of BODIPY 650/665 or DiD labeled NCMs and DCMs were measured, respectively, by monitoring the fluorescence intensity of BODIPY or DiD in the collected blood samples from tumor free nude mice at different time points post intravenous injection. As shown in Fig S-11A, the NIRF signal of blood background was found to be very low. DiD or BODIPY 650/665 labeled NCMs and DCMs had comparable *in vitro* near infrared fluorescence signals at an estimated *in vivo* concentration (Fig S-11B). After i.v. injection into mice, BODIPY signal of NCMs was rapidly eliminated from circulation and fell into the background level within 8 hours post injection. It should be mentioned that BODIPY signal of DCMs in blood was 8 times higher than that of NCMs at 8 hours post injection and sustained up to 24 hours (Fig 7A). The overall micelle concentrations injected for DiD loaded NCMs or DCMs were 20 mg/mL, 4 times higher than that for BODIPY labeled NCMs or DCMs (5 mg/mL). Nevertheless, a similar trend of circulation kinetics was observed for the DiD loaded NCMs and DCMs. DiD signal of NCMs decreased faster in spite of the initial increase while that of the DCMs sustained in blood up to 30 h (Fig 7B). The above profiles of elimination kinetics for both vehicle and payload indicated that the cross-linked micelles have longer blood circulation time than the non-cross-linked micelles.

Noninvasive NIRF optical imaging approach was utilized to monitor the biodistribution and tumor targeting ability of DiD-labeled micelles in mice bearing human SKOV-3 ovarian cancer xenograft. We have previously demonstrated that non-cross-linked PEG^{5k}-CA₈ micelles could preferentially accumulate in tumor due to the EPR effect. In contrast, fluorescence uptake of injected free DiD dye into the tumor was not seen [39]. In the present study, we demonstrated DiD and PTX co-loaded DCMs could also preferentially accumulate in SKOV-3 ovarian tumor. The particle size of the DiD and PTX co-loaded DCMs was 26±4 nm as determined by DLS (data not shown). A significant contrast of fluorescence signal was observed between tumor and background at 4 h after administration and sustained up to 72 h (Fig 8). *Ex vivo* imaging at 72 h post injection further confirmed the preferential uptake of DCMs in tumor compared to normal organs (Fig 8). This is due to the prolonged *in vivo* circulation time of the micelles and the size-mediated EPR effect. A relatively high uptake in the liver was observed compared to other organs, which is likely attributed to the nonspecific clearance of nanoparticles by the reticuloendothelial system (RES). Similar biodistribution and tumor uptake of the DiD and PTX co-loaded DCMs and NCMs were observed both via EPR effects. However, NIRF optical imaging is not quantitative enough to distinguish the difference in the tumor uptake levels of DCMs and NCMs (comparison data was not shown).

3.8 In vivo toxicity profile of empty NCMs and DCMs

PTX loaded NCMs have been safely applied for *in vivo* cancer treatment [39]. The single treatment MTD in mice was observed to be 75 mg PTX/kg, the corresponding telodendrimer dosage was 300 mg/kg. However, without the encapsulation of hydrophobic PTX inside NCMs to keep the telodendrimers together, the micelles tend to be more dynamic and dissociate more easily upon dilution. The *in vivo* toxicity profiles of both empty NCMs and DCMs were evaluated in tumor free nude mice via tail vein injection. At a single dose of 200 mg/kg, all the mice in NCMs group showed significant body weight loss and 1 of 4

mice died within 2 days post injection. All the mice in the group treated with a higher NCMs dose of 400 mg/kg died within 2 hours post injection. Bloody urine was observed for some mice, indicating the hemolytic potential caused by NCMs at high dosage. On the contrary, none of the mice treated with DCMs were dead at the single dose of 400 mg/kg and no obvious signs of toxicity were observed within two weeks post-injection.

3.9 In vivo anti-tumor efficacy in ovarian cancer xenograft mouse model

For the *in vivo* therapeutic study, the PTX-DCMs and PTX-NCMs were prepared according to the procedure described in Section 2.4. The PTX loading of PTX-DCMs was 4.6 mg/mL while that of PTX-NCMs was 5.0 mg/mL. Both of the PTX-DCMs and PTX-NCMs have similar particle sizes in the range of 25–30 nm (Table 2), which is suitable for passive tumor targeting via EPR effect [9, 38]. The anti-tumor effects of PTX-DCMs and PTX-NCMs were evaluated in the subcutaneous SKOV-3 tumor bearing mice in comparison with the clinical formulation of PTX (Taxol®). The seven groups (n=8–10) are shown in Table 1. The tumor growth inhibition and survival rate of SKOV-3 tumor bearing mice treated with PBS, Taxol®, PTX-NCMs, and PTX-DCMs were compared and the results are shown in Fig 9. Compared with the control group, mice in all the treatment groups showed significant inhibition of tumor growth ($P < 0.05$). However, at the dose of 10 mg PTX/kg (MTD of Taxol®), PTX-NCMs and PTX-DCMs exhibited superior tumor growth inhibition (Fig 9A) and longer survival time compared to Taxol® (Fig 9B). The median survival time was 21 days for PBS, 27 days for 10 mg/kg Taxol®, 28.5 days for 10 mg/kg PTX-NCMs, and 32.5 days for 10 mg/kg PTX-DCMs, respectively. The superior growth inhibition exhibited by the micelle formulations can be attributed to the higher amount of PTX that reached the tumor site via the EPR effect. Importantly, the tumor growth rate of mice treated with 10 mg PTX/kg PTX-DCMs was lower compared to those treated with PTX-NCMs at 10 mg PTX/kg ($P < 0.05$). PTX-DCMs showed increased *in vivo* therapeutic efficacy, regardless of their lower *in vitro* cytotoxicity against SKOV-3 cancer cells, which may due to the higher amount of PTX that reached the tumor site via their prolonged circulation time as discussed in Section 3.7. At the tumor site and particularly inside the tumor cells, the high glutathione level is expected to facilitate drug release from micelles and increase cytotoxicity (Fig 10A). In order to achieve a better therapeutic efficacy, three groups of mice were administrated at higher dose level: 30 mg PTX/kg PTX-NCMs, 30 mg PTX/kg PTX-DCMs and 30 mg PTX/kg PTX-DCMs followed by 100 mg/kg NAC 24 h later, respectively. It is important to realize that 30 mg/kg is more than double the maximum tolerated dose (MTD) for mice if it were given in the standard Cremophor EL/ethanol formulation of PTX (Taxol®). Tumor growth was totally inhibited in mice treated with all three micellar PTX formulations at 30 mg PTX/kg (Fig 9A). The median relative tumor volume (RTV) for all the three high dose groups was less than 1.0 before day 36. However, tumor progression was subsequently noted for all these high dose groups. PTX-DCMs were demonstrated to be more efficacious in tumor inhibition than PTX-NCMs after day 36. It should be noted that the treatment of 30 mg PTX/kg PTX-DCMs followed by 100 mg/kg of NAC was the most efficacious for inhibiting tumor growth. No palpable tumors were detected in 6 of the 8 mice by day 93. The highest complete tumor response rate of 75% was achieved with the combination treatment of PTX-DCMs and NAC (Fig 9C). NAC is commonly used in clinic as a reducing agent and has been approved by FDA for mucolytic therapy and for the treatment of acetaminophen overdose. NAC has also been employed with a number of chemotherapy agents (e.g. cisplatin, carboplatin) as a means of reducing systemic toxicity. However, in some cases, the administration of NAC also reduced the therapeutic efficacy of these chemotherapy agents [45–47]. In this study, the treatment of 30 mg PTX/kg PTX-DCMs followed by 100 mg/kg NAC exhibited a better anti-tumor effect than the treatment without NAC. This result indicated that NAC mainly played the role of a reducing agent when administered 24 hours after nanotherapeutic treatment to cleave the intra-micellar disulfide

bridges and release the drug on-demand (Fig 10B). This important *in vivo* observation has great translational potential and can be easily tested in clinical trials in the future.

Toxicities were assessed by analyzing effects on animal behavior and body weight change. The group of mice treated with 10 mg PTX/kg Taxol® frequently demonstrated decreased overall activity over 10 min post injection. This is likely due to the use of Cremophor EL and ethanol as vehicle for paclitaxel and the rapid high peak level of PTX in the blood [42]. No noticeable change in activity was observed after administration of 10 mg PTX/kg PTX-NCMs and 10 mg PTX/kg PTX-DCMs. The group of mice receiving 30 mg PTX/kg PTX-DCMs followed with 100 mg/kg of NAC exhibited slight more body weight loss (12.2%) during the treatment cycle compared to other micellar PTX groups (Fig S-12). One possible reason is the injection of high dose NAC. On the other hand, there may be still a small portion of PTX-DCMs in the blood circulation at 24 h post-injection. The administration of NAC may trigger the release of drug from circulating PTX-DCMs into blood stream, therefore resulting in toxicity to the mice. The systemic toxicity can probably be minimized by optimizing the dose and injection time of NAC.

We have identified several high affinity cancer targeting peptide ligands from the one-bead one-compound (OBOC) combinatorial library method in our group [48–51]. It is expected that the cross-linked micelles decorated with these cancer targeting ligands will achieve enhanced tumor accumulation while spare normal organs. We are currently investigating the anti-tumor efficacy and cytotoxicity of actively targeted DCMs nanocarrier system in related tumor models.

4. Conclusions

We have developed a reversible disulfide cross-linked micelle system for targeted PTX delivery by using cysteine containing telodendrimers. The system has the characteristics of superior drug loading capacity, enhanced micellar stability, lack of hemolytic activity, prolonged *in vivo* circulation time and preferential tumor targeting. The release of PTX from the cross-linked micelles was significantly slower than that from non-cross-linked micelles and can be gradually facilitated in a reducing environment. The cross-linked micelle preparation of PTX was demonstrated to be more efficacious in nude mice bearing ovarian cancer xenografts than the equivalent dose of non-cross-linked micelle preparations and Taxol®. The therapeutic efficacy of PTX loaded cross-linked micelles can be further enhanced by administering a reducing agent (N-acetylcysteine) to trigger the release of the drug on-demand. This new class of nanotherapeutics shows great promise in future cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the financial support from NIH/NCI R01CA115483, R01CA140449 and US Department of Defense Breast Cancer Research Program Postdoctoral Training Award (W81XWH-10-1-0817).

Reference

1. Sutton D, Nasongkla N, Blanco E, Gao JM. Functionalized micellar systems for cancer targeted drug delivery. *Pharm Res.* 2007; 24:1029–1046. [PubMed: 17385025]
2. Torchilin VP. Micellar nanocarriers: Pharmaceutical perspectives. *Pharm Res.* 2007; 24:1–16. [PubMed: 17109211]

3. Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V, Langer R. Biodegradable long-circulating polymeric nanospheres. *Science*. 1994; 263:1600–1603. [PubMed: 8128245]
4. Liu J, Lee H, Allen C. Formulation of drugs in block copolymer micelles: Drug loading and release. *Curr Pharm Des*. 2006; 12:4685–4701. [PubMed: 17168772]
5. Lee ES, Na K, Bae YH. Super pH-sensitive multifunctional polymeric micelle. *Nano Lett*. 2005; 5(2):325–329. [PubMed: 15794620]
6. Li Y, Pan S, Zhang W, Du Z. Novel thermo-sensitive core-shell nanoparticles for targeted paclitaxel delivery. *Nanotechnology*. 2009; 20:65104.
7. Nie S, Xing Y, Kim GJ, Simons JW. Nanotechnology applications in cancer. *Annu Rev Biomed Eng*. 2007; 9:257–288. [PubMed: 17439359]
8. Gao ZG, Lukyanov AN, Singhal A, Torchilin VP. Diacyllipid-polymer micelles as nanocarriers for poorly soluble anticancer drugs. *Nano Lett*. 2002; 2:979–982.
9. Davis ME, Chen ZG, Shin DM. Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nat Rev Drug Discov*. 2008; 7:771–782. [PubMed: 18758474]
10. Rijcken CJ, Snel CJ, Schiffelers RM, van Nostrum CF, Hennink WE. Hydrolysable core-crosslinked thermosensitive polymeric micelles: Synthesis, characterisation and in vivo studies. *Biomaterials*. 2007; 28:5581–5593. [PubMed: 17915312]
11. Burt HM, Zhang XC, Toleikis P, Embree L, Hunter WL. Development of copolymers of poly(D,L-lactide) and methoxypolyethylene glycol as micellar carriers of paclitaxel. *Colloids Surf B Biointerfaces*. 1999; 16:161–171.
12. Savic R, Azzam T, Eisenberg A, Maysinger D. Assessment of the integrity of poly(caprolactone)-b-poly(ethylene oxide) micelles under biological conditions: A fluorogenic-based approach. *Langmuir*. 2006; 22:3570–3578. [PubMed: 16584228]
13. Thurmond KB, Kowalewski T, Wooley KL. Water-soluble knedel-like structures: The preparation of shell-cross-linked small particles. *J Am Chem Soc*. 1996; 118:7239–7240.
14. Kakizawa Y, Harada A, Kataoka K. Environment-sensitive stabilization of core-shell structured polyion complex micelle by reversible cross-linking of the core through disulfide bond. *J Am Chem Soc*. 1999; 121:11247–11248.
15. Chan Y, Wong T, Byrne F, Kavallaris M, Bulmus V. Acid-labile core cross-linked micelles for pH-triggered release of antitumor drugs. *Biomacromolecules*. 2008; 9:1826–1836. [PubMed: 18564874]
16. Jiang XZ, Liu SY, Narain R. Degradable Thermo responsive Core Cross-Linked Micelles: Fabrication, Surface Functionalization, and Biorecognition. *Langmuir*. 2009; 25:13344–13350. [PubMed: 19928937]
17. Zhang L, Liu WG, Lin L, Chen DY, Stenze MH. Degradable disulfide core-cross-linked micelles as a drug delivery system prepared from vinyl functionalized nucleosides via the RAFT process. *Biomacromolecules*. 2008; 9:3321–3331. [PubMed: 18842025]
18. Harada A, Kataoka K. Supramolecular assemblies of block copolymers in aqueous media as nanocontainers relevant to biological applications. *Prog Polym Sci*. 2006; 31:949–982.
19. Liu H, Jiang XZ, Fan J, Wang GH, Liu SY. Aldehyde surface-functionalized shell cross-linked micelles with pH-tunable core swellability and their bioconjugation with lysozyme. *Macromolecules*. 2007; 40:9074–9083.
20. Qi K, Ma QG, Remsen EE, Clark CG, Wooley KL. Determination of the bioavailability of biotin conjugated onto shell cross-linked (SCK) nanoparticles. *J Am Chem Soc*. 2004; 126:6599–6607. [PubMed: 15161288]
21. Zhang K, Rossin R, Hagooley A, Chen ZY, Welch MJ, Wooley KL. Folate-mediated cell uptake of shell-crosslinked spheres and cylinders. *J Polym Sci A Polym Chem*. 2008; 46:7578–7583. [PubMed: 19855851]
22. Cohn D, Sagiv H, Benyamin A, Lando G. Engineering thermoresponsive polymeric nanoshells. *Biomaterials*. 2009; 30:3289–3296. [PubMed: 19285720]
23. Liu S, Armes SP. The facile one-pot synthesis of shell cross-linked micelles in aqueous solution at high solids. *J Am Chem Soc*. 2001; 123:9910–9911. [PubMed: 11583561]

24. Zhang K, Fang H, Wang Z, Taylor JS, Wooley KL. Cationic shell-crosslinked knedel-like nanoparticles for highly efficient gene and oligonucleotide transfection of mammalian cells. *Biomaterials*. 2009; 30:968–977. [PubMed: 19038441]
25. Yang TF, Chen CN, Chen MC, Lai CH, Liang HF, Sung HW. Shell-crosslinked Pluronic L121 micelles as a drug delivery vehicle. *Biomaterials*. 2007; 28:725–734. [PubMed: 17055046]
26. Koo AN, Lee HJ, Kim SE, Chang JH, Park C, Kim C, et al. Disulfide-cross-linked PEG-poly(amino acid)s copolymer micelles for glutathione-mediated intracellular drug delivery. *Chem Commun*. 2008; 48:6570–6572.
27. Li YL, Zhu L, Liu ZZ, Cheng R, Meng FH, Cui JH, et al. Reversibly stabilized multifunctional dextran nanoparticles efficiently deliver doxorubicin into the nuclei of cancer cells. *Angew Chem Int Ed*. 2009; 48:9914–9918.
28. Miyata K, Kakizawa Y, Nishiyama N, Harada A, Yamasaki Y, Koyama H, et al. Block cationic polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression. *J Am Chem Soc*. 2004; 126:2355–2361. [PubMed: 14982439]
29. Wang YC, Li Y, Sun TM, Xiong MH, Wu JA, Yang YY, et al. Core-shell-corona micelle stabilized by reversible cross-linkage for intracellular drug delivery. *Macromol Rapid Commun*. 2010; 31:1201–1206. [PubMed: 21590876]
30. Talelli M, Iman M, Varkouhi AK, Rijcken CJ, Schiffelers RM, Etrych T, et al. Core-crosslinked polymeric micelles with controlled release of covalently entrapped doxorubicin. *Biomaterials*. 2010; 31:7797–7804. [PubMed: 20673684]
31. Black SM, Wolf CR. The role of glutathione-dependent enzymes in drug resistance. *Pharmacol Ther*. 1991; 51(1):139–154. [PubMed: 1685247]
32. Kartalou M, Essigmann JM. Mechanisms of resistance to cisplatin. *Mutat Res*. 2001; 478:23–43. [PubMed: 11406167]
33. Matsumoto S, Christie RJ, Nishiyama N, Miyata K, Ishii A, Oba M, et al. Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules*. 2009; 10:119–127. [PubMed: 19061333]
34. Sun Y, Yan X, Yuan T, Liang J, Fan Y, Gu Z, et al. Disassemblable micelles based on reduction-degradable amphiphilic graft copolymers for intracellular delivery of doxorubicin. *Biomaterials*. 2010; 31:7124–7131. [PubMed: 20580429]
35. Shu S, Zhang X, Wu Z, Wang Z, Li C. Gradient cross-linked biodegradable polyelectrolyte nanocapsules for intracellular protein drug delivery. *Biomaterials*. 2010; 31:6039–6049. [PubMed: 20471081]
36. Meng F, Hennink WE, Zhong Z. Reduction-sensitive polymers and bioconjugates for biomedical applications. *Biomaterials*. 2009; 30:2180–2198. [PubMed: 19200596]
37. Li Y, Xiao K, Luo J, Lee J, Pan S, Lam KS. A novel size-tunable nanocarrier system for targeted anticancer drug delivery. *J Control Release*. 2010; 144:314–323. [PubMed: 20211210]
38. Luo J, Xiao K, Li Y, Lee JS, Shi L, Tan YH, et al. Well-defined, size-tunable, multifunctional micelles for efficient paclitaxel delivery for cancer treatment. *Bioconjug Chem*. 2010; 21:1216–1224. [PubMed: 20536174]
39. Xiao K, Luo J, Fowler WL, Li Y, Lee JS, Xing L, et al. A self-assembling nanoparticle for paclitaxel delivery in ovarian cancer. *Biomaterials*. 2009; 30:6006–6016. [PubMed: 19660809]
40. Xiao K, Li Y, Luo J, Lee JS, Xiao W, Gonik AM, et al. The effect of surface charge on in vivo biodistribution of PEG-oligocholeic acid based micellar nanoparticles. *Biomaterials*. 2011; 32:3435–3446. [PubMed: 21295849]
41. Jones M, Leroux J. Polymeric micelles - a new generation of colloidal drug carriers. *Eur J Pharm Biopharm*. 1999; 48:101–111. [PubMed: 10469928]
42. Kim SC, Kim DW, Shim YH, Bang JS, Oh HS, Wan Kim S, et al. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. *J Control Release*. 2001; 72:191–202. [PubMed: 11389998]
43. Koo AN, Lee HJ, Kim SE, Chang JH, Park C, Kim C, et al. Disulfide-cross-linked PEG-poly(amino acid)s copolymer micelles for glutathione-mediated intracellular drug delivery. *Chem Commun*. 2008; 28:6570–6572.

44. Li XR, Yang ZL, Yang KW, Zhou YX, Chen XW, Zhang YH, et al. Self-assembled polymeric micellar nanoparticles as nanocarriers for poorly soluble anticancer drug etaselen. *Nanoscale Res Lett*. 2009; 4:1502–1511. [PubMed: 20652138]
45. Neuwelt EA, Pagel MA, Hasler BP, Deloughery TG, Muldoon LL. Therapeutic efficacy of aortic administration of N-acetylcysteine as a chemoprotectant against bone marrow toxicity after intracarotid administration of alkylators, with or without glutathione depletion in a rat model. *Cancer Res*. 2001; 61:7868–7874. [PubMed: 11691805]
46. Wu JY, Muldoon LL, Neuwelt EA. The chemoprotective agent N-acetylcysteine blocks cisplatin-induced apoptosis through caspase signaling pathway. *J Pharmacol Exp Ther*. 2006; 312:424–431. [PubMed: 15496615]
47. Okur E, Kilinc M, Yildirim I, Kilic MA, Tolun FI. Effect of N-acetylcysteine on carboplatin-induced ototoxicity and nitric oxide levels in a rat model. *Laryngoscope*. 2007; 117:2183–2186. [PubMed: 17909450]
48. Peng L, Liu RW, Marik J, Wang XB, Takada Y, Lam KS. Combinatorial chemistry identifies high-affinity peptidomimetics against alpha(4)beta(1) integrin for in vivo tumor imaging. *Nat Chem Biol*. 2006; 2:381–389. [PubMed: 16767086]
49. Yao N, Xiao W, Wang X, Marik J, Park SH, Takada Y, et al. Discovery of targeting ligands for breast cancer cells using the one-bead one-compound combinatorial method. *J Med Chem*. 2009; 52:126–133. [PubMed: 19055415]
50. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. A new type of synthetic peptide library for identifying ligand-binding activity. *Nature*. 1991; 354:82–84. [PubMed: 1944576]
51. Xiao W, Yao N, Peng L, Liu R, Lam KS. Near-infrared optical imaging in glioblastoma xenograft with ligand-targeting alpha 3 integrin. *Eur J Nucl Med Mol Imaging*. 2009; 36:94–103. [PubMed: 18712382]

Fig.1.

Schematic representation of the disulfide cross-linked micelles formed by oxidation of thiolated telodendrimer $\text{PEG}^{5k}\text{-Cys}_4\text{-L}_8\text{-CA}_8$ after self-assemble.

Fig. 2.

The absorbance of PEG^{5k}-Cys₄-L₈-CA₈ micelle solutions in Ellman's test (A) and the thiol conversions (B) as a function of oxidation time. PTX loading (C) and size change (D) of PEG^{5k}-Cys₄-L₈-CA₈ micelles before and after cross-linking versus the level of drug added at initial loading. The volume of the final micelle solution was kept at 1 mL and the final concentration of the polymers at 20 mg/mL. DLS size distribution (E) and TEM image (F) of PTX-loaded cross-linked micelles (PTX loading was 4.6 mg/mL, TEM scale bar: 50 nm).

A. PTX-NCMs + plasma, 1 min

C. PTX-DCMs + plasma, 1 min

E. Re-hydrated PTX-DCMs

B. PTX-NCMs + plasma, 24 h

D. PTX-DCMs + plasma, 24 h

F. Re-hydrated PTX-DCMs + SDS

Fig. 3.

The particle size of PTX loaded NCMs (PTX loading: 5.0 mg/mL) in human plasma 50% (v/v) for 1 min (A), 24 h (B) and PTX loaded DCMs (PTX loading: 4.6 mg/mL) in plasma 50% (v/v) for 1 min (C) and 24 h (D) at 37 °C, respectively. The particle size of re-hydrated PTX-DCMs from lyophilized PTX-DCMs powder in the absence (E) and in the presence (F) of 2.5 mg/mL SDS.

Fig. 4.
 (A) The stability in particle size of NCMs and DCMs in the presence of 2.5 mg/mL SDS measured by DLS. TEM images of NCMs (B), DCMs (C) and DCMs treated with 10 mM GSH for 30 min (D) in the presence of 2.5 mg/mL SDS (scale bar: 50 nm).

Fig. 5.
 (A) PTX release profiles of DCMs at different GSH concentrations. GSH-responsive PTX release profiles of fresh prepared PTX-DCMs (B) and re-hydrated lyophilized PTX-DCMs (C) by adding GSH (10 mM) at a specific release time (5h) comparing with PTX-NCMs. NAC-responsive PTX release profiles of PTX-DCMs (D) by adding NAC (10 mM) at a specific release time (5h). Values reported are the mean diameter $\pm$ SD for triplicate samples.

Fig. 6.

MTT assays showing the viability of SKOV-3 cells after 2 h incubation with (A) different concentrations of empty NCMs and DCMs; and (B) Taxol®, PTX-NCMs and PTX-DCMs with and without pre-treatment of 20 mM GSH-OEt. (C) *In vitro* red blood cell (RBC) lysis of empty NCMs and DCMs. Values reported are the mean $\pm$ SD for triplicate samples.

Fig. 7.
The fluorescence signal of BODIPY labeled (A) and DiD loaded (B) DCMs and NCMs in the blood collected at different time points after i.v. injection in the nude mice.

Fig. 8.

In vivo and *ex vivo* near infra-red fluorescence (NIRF) optical imaging. Top: *In vivo* NIRF optical images of SKOV-3 xenograft bearing mouse were obtained with Kodak imaging system at different time points after i.v. injection of DCMs co-loaded with PTX and DiD; Bottom: *Ex vivo* NIR image of dissected organs and tumor was obtained at 72 h after injection.

Fig. 9.
 (A) *In vivo* anti-tumor efficacy after intravenous treatment of different PTX formulations in the subcutaneous mouse model of SKOV-3 ovarian cancer. Tumor bearing mice were administered i.v. with PBS (control) and different PTX formulations on days 0, 3, 6, 9, 12 and 15 when tumor volume reached about 100~200 mm³ (n=8–10). (B) Survival curve of mice in different treatment groups. (C) Complete tumor response rate of the three groups of mice treated with total six doses of PTX micellar formulations at the dose of 30 mg/kg.

Fig. 10. Schematic illustration of the hypothesized mechanisms for reducing agents (A: GSH, B: NAC) mediated drug release once the PTX-DCMs accumulated at tumor sites.

Table1

Treatment groups of nude mice bearing SKOV-3 ovarian cancer xenografts.

Group	Treatment ^a	Dose of PTX
1	PBS control	0
2	Taxol®	10 mg/kg
3	PTX-NCMs	10 mg/kg
4	PTX-DCMs	10 mg/kg
5	PTX-NCMs	30 mg/kg
6	PTX-DCMs	30 mg/kg
7	PTX-DCMs+ NAC (100 mg/kg) ^b	30 mg/kg

^a Administration started at the day when tumors reached a volume of 100–200 mm³ and then every three days for six doses.

^b In group 7, N-acetylcysteine (NAC) was administrated i.v. at a dose of 100 mg/kg 24 h after each dose of PTX-DCMs.

Physico-chemical properties of telodendrimers (PEG^{5k}-CA₈ and PEG^{5k}-Cys₄-L₈-CA₈) and the corresponding non-cross-linked and disulfide cross-linked micelles (NCMs and DCMs).

Table 2

Telodendrimers	M _w (theo.) ^a	M _w (MS) ^b	N _{cysteines} ^c	N _{CA} ^d	Micelles	CMC (μM) ^f	Size (nm) ^g	PTX loading capacity (mg/mL) ^h	Size with PTX (nm) ⁱ
PEG ^{5k} -CA ₈	9059	8918	0	7.5	NCMs	5.53	22±5	9.0	26±4
PEG ^{5k} -Cys ₄ -L ₈ -CA ₈	11313	11198	3.97	7.3	DCMs ^e	0.67	28±4	7.1	27±6

^aTheoretical molecular weight.

^bObtained via MALDI-TOF MS analysis (linear mode).

^cNumber of cysteines, obtained via Ellman's test.

^dNumber of cholic acids, calculated based on the average integration ratio of the peaks of methyl proton 18, 19, and 21 in cholic acid at 0.66, 0.87 and 1.01 ppm and methylene proton of PEG at 3.5–3.7 ppm in ¹H-NMR spectra in CDCl₃. The molecular weight of the starting PEG was 4912 (Fig S-3).

^eFormed by PEG^{5k}-Cys₄-L₈-CA₈ micelles after cross-linking.

^fMeasured via fluorescent method by using pyrene as a probe.

^gParticle size of NCMs and DCMs, measured by dynamic light scattering particle size (Microtrac).

^hPTX loading capacity of NCMs and DCMs, in the presence of 20 mg/mL of telodendrimers, measured by HPLC.

ⁱMeasured by dynamic light scattering particle size. The PTX loading of NCMs and DCMs was 5.0 mg/mL and 4.6 mg/mL, respectively.