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Redox-Sensitive Nanoparticles from Amphiphilic Cholesterol-Based Block Copolymers for Enhanced Tumor Intracellular Release of Doxorubicin

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Abstract

A novel amphiphilic cholesterol-based block copolymer comprised of a polymethacrylate bearing cholesterol block and a polyethylene glycol block with reducible disulfide bonds (PC5MA-SS-PEO) was synthesized and evaluated as a redox-sensitive nanoparticulate delivery system. The self-assembled PC5MA-SS-PEO nanoparticles (SS-NPs) encapsulated the anticancer drug doxorubicin (DOX) with high drug loading (18.2% w/w) and high encapsulation efficiency (94.9%). DOX-encapsulated PC5MA-SS-PEO self-assembled nanoparticles (DOX-encapsulated SS-NPs) showed excellent stability and exhibited a rapid DOX release in response to dithiothreitol (DTT) reductive condition. Importantly, following internalization by lung cancer cells, the reducible DOX-encapsulated SS-NPs achieved higher cytotoxicity than the non-reducible thioester NPs whereas blank nanoparticles were non-cytotoxic. Furthermore, *in vivo* imaging studies in tumor-bearing severe combined immunodeficiency (SCID) mice showed that the nanoparticles preferentially accumulated in tumor tissue with remarkably reduced accumulation in the healthy non-target organs. The results indicated that the SS-NPs may be a promising platform for cancer-cell specific delivery of hydrophobic anticancer drugs.

Graphical abstract

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Supplementary Material: Polymer synthesis and characterization are available in the Supplementary Material section.

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A novel redox-sensitive cholesterol-based block copolymer (PC5MA-SS-PEO) is synthesized for cancer-cell specific delivery of an anticancer drug, doxorubicin (DOX). The DOX-encapsulated PC5MA-SS-PEO nanoparticles exhibit an accelerated drug release under a reductive condition and rapid nucleus accumulation of DOX. The nanoparticles mainly distribute in tumor tissues after an intravenous administration in mice.

Keywords

Redox-Sensitive nanoparticles; block copolymer; cholesterol; intracellular release; cancer therapy

In recent years, self-assembled nanoparticles from amphiphilic block copolymer have emerged as one of the most promising platforms for targeted cancer therapy due to their ability to encapsulate and stabilize hydrophobic anticancer drugs, and accumulate in tumor tissues through the enhanced permeability and retention (EPR) effect.¹⁻⁴ Furthermore, the polymeric nanoparticles allowed conjugation with different targeting ligands to achieve active tumor targeting.^{5, 6} Unfortunately, translational potential of current polymeric nanoparticle delivery systems is limited due to low drug loading levels, poor *in vivo* stability, and slow drug release in tumor tissue and/or inside the tumor cells. Many anticancer drugs such as DOX and camptothecin can only induce therapeutic effects when they are in the cell nuclei.⁷ The slow and incomplete release of the drugs from the nanoparticles may retard the drug influx into the nuclei along with the increased risk of drug deactivation in the endosome/lysosomes where the nanoparticles are retained after endocytosis.

To increase drug release in the tumor tissue or tumor cells, stimuli-responsive nanocarriers have been developed that release a therapeutic payload in response to a microenvironment trigger such as pH, redox, and enzyme.⁸⁻¹⁵ Redox-Sensitive nanocarriers containing disulfide bonds have received much attention for intracellular drug delivery due to the existence of a high glutathione (GSH) concentration in the tumor microenvironment and cancer cells.¹⁶⁻¹⁸ For instance, several groups have reported redox-sensitive polymer/DNA complexes,¹⁹ polyion complex micelles for siRNA delivery,^{20, 21} crosslinked micelles,²² and degradable nanogels²³ with good stability under the physiological conditions that, rapidly released encapsulated drugs in the intracellular reductive environment. However,

many synthetic copolymers upon erosion *in vivo* yield non-endogenous oligomers and monomers that might adversely interact with the surrounding tissue.²⁴ Therefore, reducible block copolymers based on biologically-compatible components may be promising nanocarriers for intracellular drug delivery with minimal toxicity of the polymer carrier and its degradation products.

Cholesterol is an important component of cell membranes involved in lipid organization, signal transduction, cell adhesion, and cell migration.²⁵ Therefore, cholesterol-based block copolymers are interesting materials for cell attachment and proliferation, forming the basis of polymeric scaffolds, and drug delivery.²⁶⁻³⁰ Our previous studies have shown that amphiphilic brush block copolymers containing cholesterol blocks formed long circulating self-assembled nanoparticles for improved DOX delivery to tumor.^{31, 32} However, these nanoparticles showed a very slow drug release pattern that reduced their anti-tumor effect.³²

In this study, we designed cholesterol-based block copolymers containing disulfide bonds for triggered release of DOX in cancer cells. The block copolymer was comprised of a polymethacrylate bearing cholesterol block and a polyethylene glycol block with reducible disulfide bonds (PC5MA-SS-PEO) as shown in Figure 1a. As a non-reducible control, PC5MA-PEO-thioester block copolymer was also synthesized and compared *in vitro* and *in vivo*. The synthesis and characterization of the both copolymers are described in the Supplementary Material section. Both amphiphilic block copolymers self-assembled in aqueous media to form reducible and non-reducible nanoparticles PC5MA-SS-PEO NPs (SS-NPs) and PC5MA-PEO-thioester NPs (thioester-NPs), respectively. The resulting PC5MA-SS-PEO nanoparticles while being stable under physiological conditions are expected to rapidly release DOX by cleavage of disulfide linkage under a cytosolic reducing environment following cellular entry, resulting in effective cytotoxicity to cancer cells (Figure 1b). The *in vitro* cytotoxicity and cellular internalization behavior of the nanoparticles were evaluated in cancer cells (A549) and normal cells (NIH3T3). Finally, the biodistribution of PC5MA-SS-PEO NPs was evaluated in tumor-bearing severe combined immunodeficiency (SCID) mice using near infrared (NIR) imaging.

Methods

Preparation and characterization of DOX-encapsulated self-assembled NPs

Blank self-assembled NPs were prepared by a dialysis method. Briefly, PC5MA-SS-PEO or PC5MA-PEO-thioester was dissolved in DMF with the aid of sonication. The solution was then transferred to a dialysis bag (MWCO: 10,000 Da) and dialyzed against distilled water for 48 h. To prepare DOX-loaded SS-NPs and thioester-NPs, DOX·HCl was first dissolved in DMF containing 2 equivalents of triethylamine (TEA) and stirred overnight in the dark to form hydrophobic DOX and TEA·HCl. Each copolymer was added, and then the solution was stirred for another 1 h in the dark. The solution was then dialyzed against distilled water for 48 h to remove solvents. Since DOX is hydrophobic with limited solubility, un-encapsulated DOX beyond the solubility will precipitate in water. The precipitated DOX was removed by centrifugation at 8000 rpm for 10 min, followed by filtration through 0.45 µm syringe to collect clear red solution of DOX-encapsulated nanoparticles. The final products were collected after lyophilization.

The average particle size, size distribution and zeta-potential of the DOX-loaded SS or thioester NPs (1 mg/mL) were measured using a dynamic light scattering (DLS) instrument (Malvern Zetasizer). The morphologies of DOX-loaded SS or thioester NPs were imaged by TEM (FEI Tecnai Biotwin, Eindhoven, Netherlands). Specimens were prepared by adding a suspension of the nanoparticles dropwise to a Formvar/carbon film grid followed by air-drying.

The Critical Aggregation Concentration (CAC) of PC5MA-SS-PEO or PC5MA-PEO-thioester copolymers was determined by fluorescence measurements using pyrene as a hydrophobic probe. Pyrene solutions (3×10^{-4} M) in acetone were added to glass tubes and were subsequently evaporated to remove the organic solvent. Various concentrations of copolymer solutions (10 mL) were added to the tubes and sonicated for 3 h at 60 °C to equilibrate the pyrene and the nanoparticles. The copolymer concentrations ranged from 0.005 to 0.5 mg/mL and the final concentration of pyrene was 6.0×10^{-7} M. The emission spectra of pyrene were recorded from 350–450 nm using a fluorescence spectrophotometer (Perkin Elmer LS-55B, USA) at an excitation wavelength of 336 nm. For the measurement of the intensity ratio of the first (374.5 nm) and the third highest energy bands (386 nm) in the pyrene emission spectra, the slit opening for the excitation and emission spectra was set at 5 nm.

The amount of DOX-encapsulated into nanoparticles was determined by a colorimetric method. The lyophilized DOX-loaded SS or thioester NPs (0.5 mg) were dissolved in DMF (2 mL) to obtain clear solutions. The absorbance at 480 nm was detected with a UV-VIS spectrophotometer (Shimadzu, Japan). DOX solutions were prepared at various concentrations and the absorbance at 480 nm was measured to generate a calibration curve for calculating drug-loading content. The drug-loading content (DLC) and encapsulation efficiency (EE) were calculated using the following equations:

$$\text{DLC} = \frac{\text{Amount of DOX in nanoparticles}}{\text{Amount of DOX-loaded nanoparticles}} \times 100$$

$$\text{EE} = \frac{\text{Amount of DOX in nanoparticles}}{\text{Amount of DOX used for nanoparticle preparation}} \times 100$$

Stability of DOX-encapsulated NPs

Lyophilized DOX-loaded SS or thioester NPs (1 mg/mL) were suspended in the serum-containing phosphate-buffered saline (PBS) solution (50% FBS), followed by sonication for 10 min and filtration through a 0.45 µm syringe filter membrane. The particle size of the nanoparticles stored at 4 °C was monitored over the storage time using a Malvern Zetasizer. Reduction-triggered destabilization of DOX-encapsulated SS-NPs was observed by detecting the change in size of the NPs in response to 10 mM DTT in aqueous medium. Briefly, DOX-encapsulated SS-NPs were dissolved in 10 mM DTT containing PBS at a

concentration of 1 mg/mL and then kept in a shaking bath at 37 °C. The particle size was measured by DLS at a pre-determined time.

Reduction-triggered release of DOX from the SS or thioester NPs

In vitro release of DOX from the nanoparticles was studied using a dialysis method. Briefly, lyophilized DOX-loaded NPs (6 mg) were suspended in 3 mL of PBS (0.01 M, pH 7.4), followed by sonication for 10 min to yield an optically clear suspension. The suspensions were introduced into 5 mL-dialyzers (MWCO: 10,000 Da) and immersed in 20 mL of PBS or PBS containing 10 mM DTT at 37 °C in a shaking bath at 100 rpm. At selected time intervals, aliquots (10 mL) were removed from the dissolution medium and an equivalent volume of fresh medium was compensated. The concentration of DOX was immediately measured by UV at 480 nm. The percentage of DOX released was calculated based on a standard curve of known DOX concentrations.

Intracellular uptake and release behavior of DOX-encapsulated NPs

To observe the cellular uptake, A549 and NIH3T3 cells were seeded at a density of 1.0×10^5 cells/well in an 8-well chamber of a Lab-Tek II chamber slide and preincubated for 24 h at 37 °C and 5% CO₂. Serum-free DMEM containing free DOX and DOX-NPs at equivalent doses (10 µg/mL) was added to each well, followed by incubation for 30 min, 2 h and 4 h at 37 °C. The cells were then rinsed with PBS, and fixed with 4% formaldehyde solution for 10 min. Cover glasses were then placed on glass slides. The cellular uptake and release behavior of DOX-encapsulated SS or thioester NPs were imaged by confocal laser scanning microscopy (Leica, England) at an excitation wavelength of 488 nm for DOX.

Cytotoxicity of DOX-encapsulated NPs

A549 and NIH3T3 cells (7500 cells/well) were seeded on 96-well plates and cultured in 200 µL of DMEM supplemented with 10% FBS, 1% antibiotics, and 1% L-glutamine for 24 h at 37 °C and 5% CO₂. After incubation, various concentrations of DOX-encapsulated SS or thioester NPs and free DOX (1-50 µg/mL of DOX equivalents) dissolved in DMEM without supplements were added. After 24 h of incubation, cytotoxicity was determined using 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide dye (MTT dye, final concentration of 0.5 mg/mL) uptake at 540 nm on a microplate reader (Tecan group Ltd., Männedorf, Switzerland).

In vivo imaging of PC5MA-SS-PEONPs

All animal studies were performed according to an approved protocol by Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. Humane use and care of vertebrate animals were ensured in the research.

The biodistribution of PC5MA-SS-PEO NPs was assessed by *in-vivo* near-infrared (NIR) imaging. A NIR fluorophore, DiR, was loaded into the nanoparticles by a dialysis method. Briefly, the PC5MA-SS-PEO (10 mg) and DiR (0.6 mg) were dissolved in DMF (3 mL). The resulting solution was dialyzed against distilled water for 48 h, and then filtered through a 0.45 µm membrane before lyophilization. The loading content of DiR was determined spectrophotometrically at a wavelength of 750 nm.

The tumor models were established by subcutaneous injection of A549 cells (2×10^6 cells in 100 μ L of PBS) into the flank of male SCID mice. When the tumor reached an acceptable size, the mice were treated with the DiR-loaded NPs (5 μ g/kg of equivalent DiR) via tail-vein injection. Whole body images were obtained at 1 h, 3 h, 6 h, 24 h, 48 h and 72 h after injection using an IVIS imaging system (PerkinElmer, Hopkinton, MA, USA). Images of various organs, including heart, kidney, liver, spleen, lung, and tumor, were also obtained after sacrifice of the mice at 72 h post-injection.

Results

Synthesis and characterization of PEO-SS-PC5MA

The detailed chemical structure of as-synthesized PEO-SS-PC5MA was confirmed by ^1H -NMR (Figure 2). The ^1H -NMR allowed the determination of molar composition and molecular weight of the obtained block copolymer. The signals at 5.3, 3.9 and 2.5-0.55 ppm were attributed to the protons of cholesterol. Additionally, monomer olefin peaks at 6.42, 6.09 and 5.54 ppm were absent in PEO-SS-PC5MA. The signals of the PEO block corresponding to the PEO repeating unit and the methylene end groups of PEO were observed at 3.6 ppm and 4.25 ppm, respectively. By comparing the integration of peaks in the ^1H -NMR spectra at 5.33 ppm (olefin group in cholesteryl moiety) and 3.64 ppm (PEO repeating unit), the weight fraction of the each block was determined.³¹ Gel permeation chromatography (GPC) was used to measure the number average molecular (M_n) and the polydispersity indices (PDI) of PEO-SS-PC5MA (Table 1). The PEO-SS-PC5MA sharply shifted to a higher molecular weight region with a narrow molecular weight distribution.

Preparation and characterization of self-assembled nanoparticles

The self-assembly behavior of the PC5MA-SS-PEO copolymers in aqueous solution was characterized by measuring the critical micelle concentration (CMC) using pyrene as a hydrophobic fluorescence probe.³³ The CMC value of the PC5MA-SS-PEO copolymer (11.4 ± 0.1 mg/L) was slightly lower than that of the PC5MA-PEO-thioester copolymer (13.1 ± 0.3 mg/L) (Table 2).

The hydrophobic DOX was successfully encapsulated into the PC5MA-SS-PEO and PC5MA-PEO-thioester self-assembled NPs through hydrophobic interactions between DOX and cholesterol moieties. With a DOX.HCl feed ratio of 20% (w/w), the drug loading content (DLC) in the PC5MA-PEO-thioester NPs was about 17.1% with encapsulation efficiency (EE) of 88.1%. The DLC and EE values were slightly higher in the PC5MA-SS-PEO copolymer (18.2% w/w and 94.9%, respectively) (Table 2). The size and morphology of blank PC5MA-SS-PEO NPs, blank PC5MA-PEO-thioester NPs, DOX-encapsulated PC5MA-SS-PEO NPs, and DOX-encapsulated PC5MA-PEO-thioester NPs were examined by TEM (Figure 3) and dynamic light scattering (DLS) (Table 2), respectively. The TEM images showed that all the self assembled nanoparticles were spherical in shape with sizes of 20-40 nm (Figure 3a-d). The average particle size of blank PC5MA-SS-PEONPs and blank PC5MA-PEO-thioester NPs measured by DLS was about 85.1 nm and 92.3 nm, respectively with narrow size distribution (PDI less than 0.2) (Table 2). These were larger than the sizes measured by TEM, which might be due to the existence of the self-assembled

NPs in a swollen state in aqueous media.³⁴ Encapsulation of DOX slightly increased the particle size of the nanoparticles to 89.4 nm in DOX-encapsulated PC5MA-SS-PEO NPs, and 101.3 nm in DOX-encapsulated PC5MA-PEO-thioester NPs. The SS-NPs, thioester-NPs, and DOX-loaded NPs were negatively charged at their surface as reflected by zeta potential values in the range of -22 mV to -26 mV. The physical loading of DOX slightly decreased the negative surface charge of the nanoparticles.

To observe reduction-triggered destabilization, the PC5MA-SS-PEO NPs were incubated with 10 mM DTT in PBS buffer (pH 7.4, 10 mM) at 37 °C with shaking, and the size change of the NPs was examined at different time intervals. An increase in the size of the PC5MA-SS-PEO NPs with the appearance of two peaks was observed at 30 min incubation with DTT (Figure 4). The initial size of PC5MA-SS-PEO NPs displayed a unimodal distribution with an average hydrodynamic diameter of 85.1 ± 3.1 nm. However, the particles size of the NPs dramatically increased to 235 nm after 30 min and to 540 nm after 2 h incubation with DTT. After 4 h of incubation with DTT, no particle size was measurable, indicating completed dissociation of the SS-NPs.

Stability of DOX-encapsulated NPs and in vitro DOX release

We evaluated the stability of DOX-encapsulated thioester NPs and SS-NPs in phosphate-buffered saline (PBS)/fetal bovine serum (FBS) solution using DLS to monitor the size change of the nanoparticles. The average particle size of the DOX-encapsulated PC5MA-SS-PEO NPs and PC5MA-PEO-thioester NPs did not change significantly after 1 week storage at 4 °C in 50% FBS (Figure 5a), and no precipitation or aggregation was observed.

To further assess the potential of the PC5MA-SS-PEO nanoparticles for triggered drug release, we performed a DOX release test in PBS (pH 7.4, 37 °C) with and without DTT using a dialysis method. Remarkably, the DOX-encapsulated SS-NPs released DOX rapidly in the presence of 10 mM DTT, a reductive environment analogous to that of the intracellular compartment, showing 50% DOX release in 5 h and about 70% drug releases within 24 h. In contrast, minimal drug release (~10%) was observed after 24 h for the non-reducible PC5MA-PEO-thioester NPs under the same conditions as well as for PC5MA-SS-PEO NPs in the absence of DTT (Figure 5b).

Cellular uptake and in vitro cytotoxicity

The cellular uptake and intracellular release behavior of DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs were monitored by confocal laser scanning microscopy (CLSM) in both cancer cells (A549) and normal cells (fibroblast NIH3T3). Since DOX itself is fluorescent, it was used directly to investigate cellular uptake without additional fluorescent labeling in of the nanoparticles. As shown in Figure 6, when incubating A549 cells with DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs for 30 min, the red fluorescent signal was observed mainly in the cytoplasm while free DOX entered the cellular nuclei. By increasing the incubation time to 2 h and 4 h, free DOX and DOX-encapsulated SS-NPs showed a strong red signal in the cellular nuclei (Figure 6a), whereas DOX-encapsulated thioester-NPs still remained in the cytoplasm (Figure 6b-c). In contrast, the intracellular drug release from non-reducible DOX-encapsulated thioester-NPs was

negligible under the same conditions. As a negative control, NIH3T3 cells were utilized to compare cellular uptake of DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs. When the cells were incubated with free DOX, red signal was observed in the cellular nuclei at all the time points from 30 min to 4 h. When the cells were exposed to DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs, the red fluorescent signal was visualized mainly in the cytoplasm up to 4 h (Figure 6 a-c). The results indicated that DOX release from the redox-sensitive nanoparticles was negligible in normal cells such as NIH3T3 during 4 h of incubation which might be due to low levels of GSH in the cells compared to cancer cells.

We next investigated *in vitro* cytotoxicity of DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs compared with that of free DOX in A549 and NIH3T3 cells using the MTT assay. Figure 7 show the viability of A549 and NIH3T3 cells treated with blank nanoparticles, free DOX, DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs at different equivalent DOX concentrations. Blank thioester-NPs and SS-NPs showed negligible toxicity to A549 cells even at a concentration of 1 mg/mL, with a cell viability >90% after 24 h treatment indicating good compatibility of the nanoparticles to A549 cells (Figure 7a). However, free DOX and DOX-encapsulated nanoparticles decreased the cell viability by 5-82% after 24 h of incubation in a dose-dependent manner (1-50 µg/mL DOX) (Figure 7b). By increasing DOX concentration from 1 µg/mL to 50 µg/mL, free DOX and DOX-encapsulated SS-NPs drastically decreased the cell viability while the DOX-encapsulated thioester-NPs gradually decreased the cell viability. At all DOX concentrations, the cytotoxicity of free DOX and DOX-encapsulated SS-NPs was significantly higher than that of DOX-encapsulated thioester-NPs. In NIH3T3 cells, the toxicity of DOX-encapsulated thioester-NPs was comparable to that of DOX-encapsulated SS-NPs at all tested DOX concentrations (Figure 7c).

In vivo fluorescence images of PC5MA-SS-PEO NPs in tumor-bearing mice

A hydrophobic NIRF dye, DiR was used as a model drug for encapsulation into PC5MA-SS-PEO NPs (5% w/w). We investigated DiR release from SS-NPs in simulated physiological condition and the result showed less than 7% of DiR release during 24 h without any burst release, indicating the stability of DiR in the nanoparticles. This dye emits strong NIR fluorescence that is minimally absorbed by water or hemoglobin. This enables less interference from background fluorescence during non-invasive animal imaging.³⁵ The DiR-loaded PC5MA-SS-PEO NPs were administrated to the A549 tumor-bearing SCID mice via tail-vein injection. At 1 h and 3 h post-injection, a fluorescence signal could be detected throughout the entire animal. The fluorescent signal in the liver was relatively weaker than the tumor signal (Figure 8a). Moreover, the contrast of the DiR signal in the tumor compared to the surrounding tissues of the animal was already apparent 6 h post-injection of the PC5MA-SS-PEO NPs in two mice, and even at 1 h post-injection in one mouse. At 24 h post-injection, a strong red signal was observed in tumor tissues and only a low signal was observed in the liver. This strong fluorescence signal in the tumors was maintained up to 72 h post-injection. After this time the mice were sacrificed and the major organs were isolated to analyze the tissue distribution of the DiR-loaded nanoparticles. As shown in Figure 8b, the highest NIRF intensity was observed in tumor tissues, while the

signal intensities were lower for other tissues such as liver, without any detectable fluorescence signal in the heart.

Discussion

We designed and synthesized amphiphilic PEO-SS-PC5MA comprised of polymethacrylate bearing a hydrophobic cholesterol block (poly(cholesteryl 6-methacryloyloxyhexanoate) (PC5MA)) and a hydrophilic PEO block with bio-reducible disulfide linkages between PC5MA and PEO. As a control, PC5MA-PEO-thioester was also synthesized. The absence of olefin peak in ¹H-NMR result indicates the complete conversion of monomer to polymer while the GPC results showed a monomodal symmetric distribution, indicating a well-controlled polymerization process.

The as-synthesized block copolymers readily self-assembled in aqueous solution to form nanoparticles due to the hydrophobic interaction between the cholesterol moieties. The CMC values in our experiments were in the typical range of PEG-based block copolymers,^{28, 36} suggesting that the PC5MA-SS-PEO copolymers may circulate as self-assembled nanoparticles *in vivo* for an extended period of time. These nanoparticles likely have a core/shell structure bearing a hydrophobic cholesterol inner core that could encapsulate hydrophobic drugs. DOX-encapsulated PC5MA-SS-PEO and PC5MA-PEO-thioester NPs had size less than 100 nm and negatively charged surface. It has been reported that the ideal size for self-assembled nanoparticles to evade renal clearance and liver capture is > 10 nm and < 100 nm, respectively.³⁷ Therefore, the reducible NPs have sizes and surface charges that are suitable for tumor targeting via the EPR effect.

In a reductive environment of DTT, particle size of DOX-encapsulated PC5MA-SS-PEO NPs significantly increased, indicating the nanoparticles were unstable. Furthermore, no particle size was detectable after 4 h of incubation, indicating a complete dissociation of the nanoparticles. This was due to the presence of disulfide bonds, which made NPs breakable in the reductive environment. These results suggest that PC5MA-SS-PEO NPs may remain stable during systematic blood circulation but rapidly dissociate and release DOX in a reductive intracellular environment due to the cleavage of disulfide linker between PC5MA and PEO blocks.

The stability of nanocarriers was found to be a key to a successful drug delivery system for systemic delivery.³⁸ The maintenance of nanoparticles size indicating that excellent stability was achieved by hydrophobic interactions between cholesterol molecules and/or between cholesterol, and DOX. This finding indicates that the NPs are stable and retain their structures in aqueous media. Such excellent stability of the PC5MA-SS-PEO nanoparticles will be beneficial for systemic drug delivery under diluted condition *in vivo*.

It is evident that the drug release from PC5MA-SS-PEO NPs is triggered by reduction of disulfide linkages. This result was consistent with the previous observation that PC5MA-SS-PEO NPs were destabilized in response to 10 mM DTT. It should be further noted that the DOX-encapsulated SS-NPs released DOX in a sustained release pattern without an initial burst release in PBS in the absence of DTT. The sustained release of DOX from the

nanoparticles may be due to diffusion of incorporated DOX molecules that interacted strongly with the hydrophobic cholesterol core of the nanoparticles. The release behavior of the PC5MA-SS-PEO NPs is useful for delivery of anticancer drugs, in which limited amounts of the drug are released in the blood stream until the nanoparticles reach the tumor tissues where the drug release is triggered inside cancer cells due to the dissociation of the nanoparticles in the presence of high intracellular GSH concentration.

The results of *in vitro* experiments indicated the fast dissociation of DOX-encapsulated SS-NPs in the intracellular reductive condition which triggered the release of DOX molecules resulting in the fast drug influx into the nuclei as evident by the cellular uptake study in cancer cells versus normal cells. Our previous study has also shown that non-reducible cholesterol-containing brush block copolymers slowly released DOX inside the cells and the drug signal was observed in the nuclei when increasing incubation time up to 24 h.³² The triggered DOX release from DOX-encapsulated SS-NPs resulted in significant higher cytotoxicity effect compared to non-reducible NP. It is well known that nanoparticles with ideal sizes accumulate within the tumor microenvironment by the EPR effect. However, the accumulation within tumor tissues may not always correlate with therapeutic outcome since cellular internalization is required for anticancer drugs to exert their biological function inside tumor cells.³⁹ The results suggest that the efficient cellular uptake and drug release of DOX-encapsulated SS-NPs may improve the therapeutic effect of DOX against cancers. In addition, the cytotoxicity results were further confirmed that DOX-encapsulated SS-NPs released DOX in the free form inside the cancer cells and induced its therapeutic effect. The results suggest that the PC5MA-SS-PEO NPs are effective for cancer-cell specific delivery of anticancer drugs.

To assess biodistribution of the PC5MA-SS-PEO NPs, *in vivo* fluorescence images of the PC5MA-SS-PEO NPs in tumor-bearing SCID mice were obtained using non-invasive near-infrared fluorescence (NIRF) imaging. The weak of fluorescent signal in the liver at 1 h and 3 h injection was consistent with our previously reported cholesterol-containing nanoparticles.³² The intense whole-body fluorescence was continuously observed at 6 h post-injection, indicating the prolong circulation time of the DiR-loaded PC5MA-SS-PEO NPs. Our previous *in vivo* imaging study showed that free hydrophobic DiR accumulated in liver from 1 h to 24 h with negligible tumor accumulation.³² The results demonstrated the effective accumulation of the PC5MA-SS-PEO NPs in tumor tissue. This appreciable tumor targeting ability of PC5MA-SS-PEO NPs may have resulted from the prolonged circulation time achieved by the stability of the nanoparticles and the EPR effect in tumor tissue due to their small size.⁴⁰ Compared to our previous cholesterol-containing nanoparticles,³² the smaller size of the current PC5MA-SS-PEO NPs favored tumor accumulation with reduced capture by the liver. Once accumulated in the tumor tissues, therapeutic effect of DOX may be enhanced by the triggering effect of the reducible NPs to release DOX from NPs and rapidly diffuse into tumor cell nuclei.

To summarize, in this study, a novel cholesterol-based block copolymer (PC5MA-SS-PEO) containing redox-sensitive linkage was synthesized and evaluated its potential for intracellular drug delivery. In aqueous solution, PC5MA-SS-PEO self-assembled to form nanoparticles. The DOX-encapsulated SS-NPs displayed a spherical shape with an average

size below 100 nm, high drug loading capacity, and excellent stability. Importantly, the reducible PC5MA-SS-PEO NPs are capable of rapidly releasing DOX inside cancer cells to induce higher cytotoxic effect compared to the non-reductive PC5MA-PEO-thioester nanoparticles due to the cleavage of disulfide bonds when exposed to an intracellular reductive environment. The *in vivo* imaging study on A549 lung tumor-bearing SCID mice demonstrated that PC5MA-SS-PEO NPs preferentially accumulated in the tumor tissues with low accumulation in the liver and non-target tissues. The results suggest that the redox-sensitive PC5MA-SS-PEO NPs is promising polymer platform for intracellular delivery of anticancer drugs with minimal toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
(a) Synthetic route of cholesterol-based block copolymer with disulfide linkage PC5MA-SS-PEO and (b) reduction-sensitive in aqueous media of PC5MA-SS-PEO NPs for intracellular drug release.

Figure 2.
¹H-NMR spectrum of as-synthesized PC5MA-SS-PEO.

Figure 3. TEM images of (a) blank thioester-NPs, (b) blank SS-NPs, (c) DOX-encapsulated thioester-NPs, and (d) DOX-encapsulated SS-NPs.

a

b

Figure 4. Particle size distribution of PC5MA-SS-PEO NPs after incubation with or without 10 mM of DTT solution.

c

Figure 5.

(a) Stability of DOX-encapsulated thioester-NPs and DOX-encapsulated SS-NPs in PBS/FBS (1:1) stored at 4 °C and (b) release profiles of DOX-encapsulated thioester NPs and DOX-encapsulated SS-NPs with and without DTT in PBS buffer (pH 7.4, 10 mM) at 37 °C.

a

b

c

Figure 6.

CLSM images of A549 and fibroblast (NIH3T3) cells incubated with free DOX, DOX-encapsulated thioester-NPs, and DOX-encapsulated SS-NPs for 30 min (a), 2 h (b) and 4 h (c) at 10 $\mu\text{g/mL}$ DOX equivalence. Scale bars are 10 μm . Blue-nuclei stained with DAPI; red-DOX.

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b

Figure 7.

Viability of A549 cells incubated with blank thioester-NPs and blank SS-NPs for 24 h (a), with free DOX, DOX-encapsulated thioester-NPs, and DOX-encapsulated SS-NPs (b), and cell viability of NIH3T3 cells incubated with free DOX, DOX-encapsulated thioester-NPs, and DOX-encapsulated SS-NPs (c) at different concentrations of DOX for 24 h.

Figure 8.

(a) *In vivo* fluorescence images of DiR-encapsulated SS-NPs in tumor-bearing SCID mice at 1 h, 3 h, 6 h, 24 h, 48 h, and 72 h-post intravenous injection. (b) *ex vivo* images of tumors and organs at 72 h-post injection.

Table 1
Molecular characterization of as-synthesized polymers

Polymer	M _n (g/mol)	Weight fraction ^b (%)		PDI ^a	Conversion ^c (%)
		GPC ^a	PEO	PC5MA	
PEO-SH	20 250	1.05	100	-	85
PEO macro chain transfer agent	20 500	1.12	100	-	92
PC5MA-SS-PEO	38 250	1.13	60	40	90
PC5MA-PEO-thioester	37 600	1.16	60	40	88

^a Determined by GPC calibrated at 40 °C with THF as the mobile phase with polystyrene standards.
^b The ratio of the integrals of peaks by ¹H-NMR spectra at 5.33 ppm (olefin group in cholesteryl moiety) and 3.64 ppm (PEO repeating unit) is used to calculate the weight fraction of the block copolymer.
^c Conversion of monomer to polymer was determined using ¹H- NMR analysis

Table 2
Characterization of self-assembled blank nanoparticles and DOX-encapsulated nanoparticles

Samples	CMC ^a (mg/L)	DOX.HCl feed ratio (%)	DLC ^b (%)	EE ^c (%)	Average size (nm)	PDI	Zeta potential (mV)
Blank SS-NPs	11.4 ± 0.1				85.1±3.1	0.16	-25.1±0.4
Blank thioester-NPs	13.1 ± 0.3				92.3±4.2	0.12	-26.3±0.2
DOX-encapsulated SS-NPs		20	18.2	94.5	89.4±5.4	0.17	-23.4±0.6
DOX-encapsulated thioester-NPs		20	17.1	88.1	101.3±6.9	0.19	-22.1±0.3

^aCMC: critical micelle concentration, measured by the probe fluorescence technique

^bDLC: drug loading content = (amount of DOX in nanoparticle/amount of DOX-loaded nanoparticle) × 100

^cEE: encapsulation efficiency = (amount of DOX in nanoparticle/amount of DOX used for nanoparticle preparation) × 100