

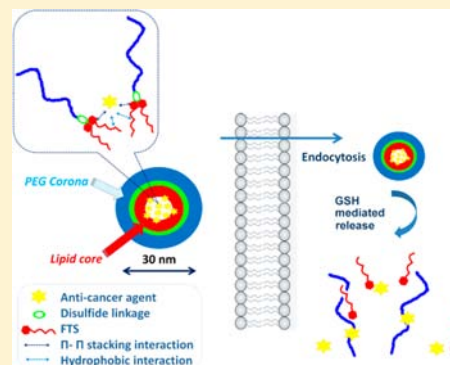
## Reduction-Sensitive Dual Functional Nanomicelles for Improved Delivery of Paclitaxel

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### Supporting Information

**ABSTRACT:** We have developed a dual-functional nanocarrier composed of a hydrophilic polyethylene glycol (PEG) and a hydrophobic farnesylthiosalicylate (FTS, a nontoxic Ras antagonist), which is effective in delivery of hydrophobic anticancer drug, paclitaxel (PTX). To facilitate the retention of the therapeutic activity of the carrier, FTS was coupled to PEG via a reduction-sensitive disulfide linkage (PEG<sub>5k</sub>-S-S-FTS<sub>2</sub>). PEG<sub>5k</sub>-S-S-FTS<sub>2</sub> conjugate formed uniform micelles with very small size (~30 nm) and the hydrophobic drug PTX could be readily incorporated into the micelles. Interestingly, inclusion of a disulfide linkage into the PEG<sub>5k</sub>-FTS<sub>2</sub> micellar system resulted in a 4-fold decrease in the critical micelle concentration (CMC). In addition, the PTX loading capacity and colloidal stability of PTX-loaded micelles were improved. HPLC-MS showed that parent FTS could be more effectively released from PEG<sub>5k</sub>-S-S-FTS<sub>2</sub> conjugate in tumor cells/tissues compared to PEG<sub>5k</sub>-FTS<sub>2</sub> conjugate *in vitro* and *in vivo*. PEG<sub>5k</sub>-S-S-FTS<sub>2</sub> exhibited a higher level of cytotoxicity toward tumor cells than PEG<sub>5k</sub>-FTS<sub>2</sub> without a disulfide linkage. Furthermore, PTX-loaded PEG<sub>5k</sub>-S-S-FTS<sub>2</sub> micelles were more effective in inhibiting the proliferation of cultured tumor cells compared to Taxol and PTX loaded in PEG<sub>5k</sub>-FTS<sub>2</sub> micelles. More importantly, PTX-loaded PEG<sub>5k</sub>-S-S-FTS<sub>2</sub> micelles demonstrated superior antitumor activity compared to Taxol and PTX formulated in PEG<sub>5k</sub>-FTS<sub>2</sub> micelles in an aggressive murine breast cancer model (4T1.2).



## INTRODUCTION

Polymeric micelles are an attractive delivery system due to easy preparation, small size (10–100 nm), and the ability to solubilize hydrophobic drugs and accumulate preferentially within tumors.<sup>1–5</sup> We previously developed a nanocarrier based on polyethylene glycol (PEG)-derivatized farnesylthiosalicylate (FTS).<sup>6</sup> PEG<sub>5k</sub>-FTS<sub>2</sub> readily formed micelles (20–30 nm) that were highly efficient in loading hydrophobic anticancer drugs such as paclitaxel (PTX).<sup>6</sup> More importantly, PTX-loaded PEG<sub>5k</sub>-FTS<sub>2</sub> micelles achieved enhanced antitumor efficacy compared to Taxol formulation *in vivo*.<sup>6</sup> Different from most of the existing drug carriers that lack therapeutic effect, PEG<sub>5k</sub>-FTS<sub>2</sub> itself exhibits antitumor activity.<sup>6</sup> FTS is a potent and especially nontoxic Ras antagonist.<sup>7,8</sup> Ras gene mutations can be found in one-third of human cancers, with the highest incidence in adenocarcinomas of the pancreas (90%), colon (50%), and lung (30%) tumors.<sup>9</sup> FTS can effectively inhibit the growth of many different types of tumors via inhibition of Ras-dependent signaling involved in tumor maintenance and progression.<sup>10</sup> The mechanism involves the dislodgement of Ras from the cell membrane and subsequent degradation of the protein.<sup>11,12</sup>

As a dual function carrier, the cleavability of the linkage between PEG and FTS in PEG<sub>5k</sub>-FTS<sub>2</sub> conjugate is critical for its biological activity. In our previous study, we compared the

antitumor activity of a conjugate with a labile ester linkage, PEG<sub>5k</sub>-FTS<sub>2</sub>(L), with that of a similar conjugate with a relatively stable amide linkage, PEG<sub>5k</sub>-FTS<sub>2</sub>(S).<sup>6</sup> PEG<sub>5k</sub>-FTS<sub>2</sub>(L) alone showed a significantly higher level of cytotoxicity toward tumor cells compared to PEG<sub>5k</sub>-FTS<sub>2</sub>(S), presumably due to a more ready release of FTS from PEG<sub>5k</sub>-FTS<sub>2</sub>(L) inside tumor cells.<sup>6</sup> In addition, delivery of PTX via PEG<sub>5k</sub>-FTS<sub>2</sub>(L) micelles led to an improved antitumor activity *in vivo* over PTX formulated in PEG<sub>5k</sub>-FTS<sub>2</sub>(S) micelles.<sup>6</sup>

In this study, we propose to incorporate into PEG<sub>5k</sub>-FTS<sub>2</sub>(L) an additional cleavable linkage (disulfide bond) to further facilitate the release of FTS following intracellular delivery to tumor cells. We choose the disulfide linkage because tumor cells have significantly higher concentrations of glutathione (GSH) than those in the extracellular fluids and the disulfide linkage has been widely used to develop a reduction-sensitive delivery system to facilitate drug release at the tumor site.<sup>13–24</sup> Our data showed that incorporation into PEG<sub>5k</sub>-FTS<sub>2</sub>(L) of a disulfide linkage led to an enhanced release of FTS inside tumor cells, which was associated with an improved cytotoxicity against tumor cells. Interestingly, the conjugate with a disulfide

**Received:** July 3, 2014

**Revised:** August 6, 2014

**Published:** August 8, 2014

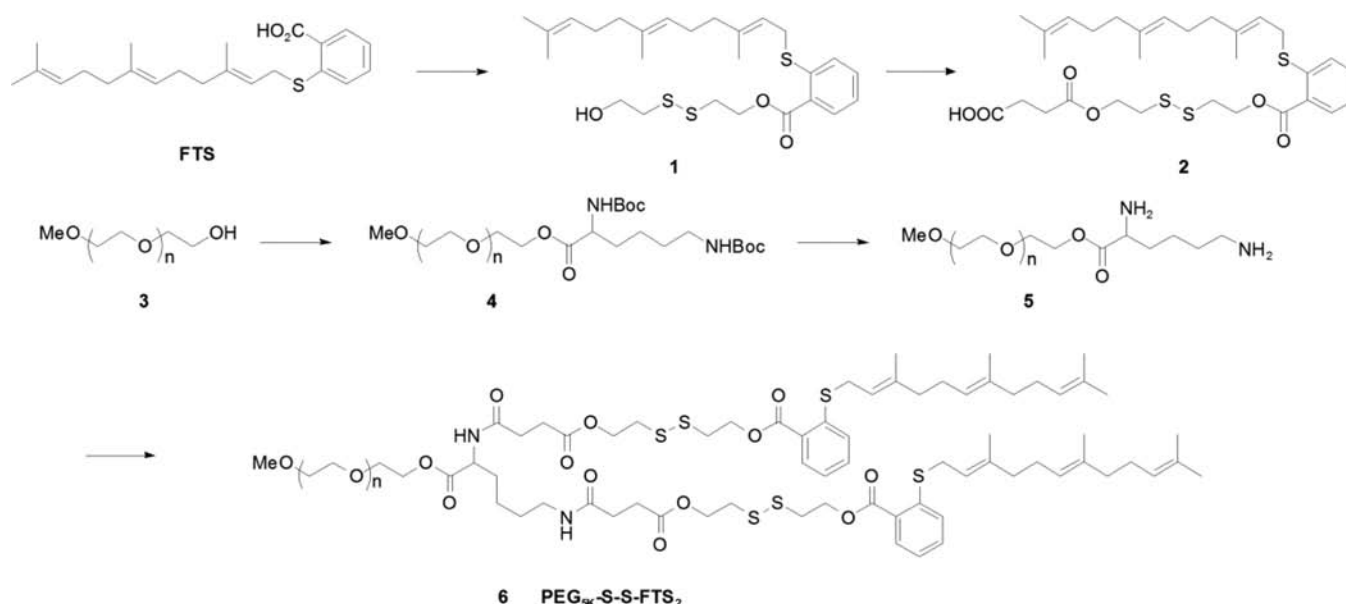


Figure 1. Synthesis scheme of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate.

linkage (PEG<sub>5K</sub>-S-S-FTS<sub>2</sub>) exhibited a reduced critical micelle concentration (CMC) in addition to improved drug loading capacity and formulation stability. Finally, paclitaxel (PTX) formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles was significantly more effective than the PEG<sub>5K</sub>-FTS<sub>2</sub>(L) formulation in inhibiting the tumor growth in a murine breast cancer model (4T1.2).

## RESULTS

**Synthesis of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> Conjugate.** To facilitate the retention of the antitumor activity of FTS, a cleavable disulfide linkage was used to couple FTS to a hydrophilic PEG (PEG<sub>5K</sub>-S-S-FTS<sub>2</sub>). The chemical structure of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> is shown in Figure 1. As shown in <sup>1</sup>H NMR spectra (Supporting Information Figure S1), the signals at 3.63 ppm and 7–8 ppm were attributed to the methylene protons located at the terminus of PEG and the benzene ring protons of FTS, respectively. Additionally, the chemical shift of -CH<sub>2</sub>-S-S-CH<sub>2</sub>- (2.9–3.1 ppm) could be observed, which confirmed the presence of a disulfide linkage (Supporting Information Figure S1). The molecular weight of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> was determined by MALDI-TOF mass spectrometry (Supporting Information Figure S2), which is close to the theoretical value of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub>. Both <sup>1</sup>H NMR and MALDI-TOF mass spectra indicated the successful synthesis of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate.

**Characterization of PTX-Free and PTX-Loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> Micelles.** PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> was soluble in aqueous solution and readily self-assembled to form micelles with size around 30 nm (Figure 2A). Figure 2C shows the TEM images of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles. Spherical particles of uniform size were observed and the sizes of the particles observed under TEM were consistent with those measured by DLS. Figure 3 shows the CMCs of PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles using pyrene as a fluorescence probe. It is interesting to note that incorporation into PEG<sub>5K</sub>-FTS<sub>2</sub> of a disulfide linkage led to a ~4-fold decrease in CMC. PTX can be readily loaded into PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles. The spherical shape and size of the micelles were well retained following incorporation of PTX (Figure 2B,D). We then evaluated the loading capacity and stability of PTX-loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles and compared to those of PEG<sub>5K</sub>-FTS<sub>2</sub> formulation. As shown in Table

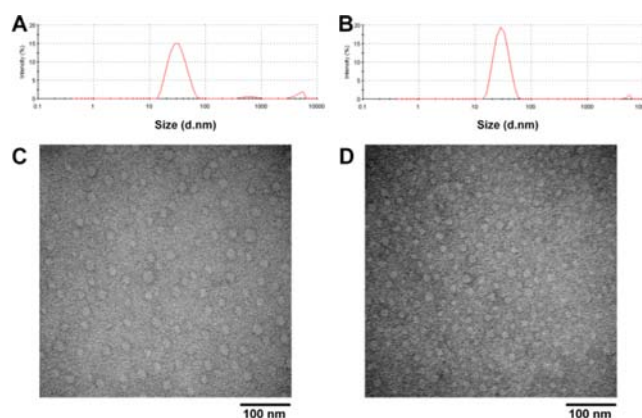
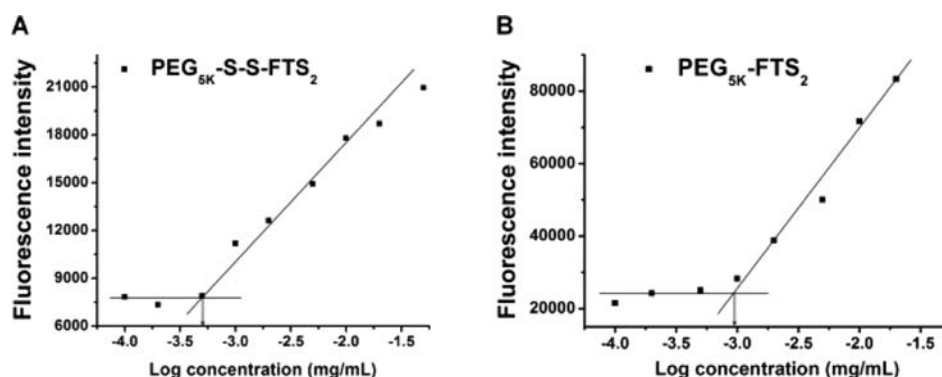


Figure 2. Particle size distribution of PTX-free PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> (A), and PTX-loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles (B). TEM images of PTX-free PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> (C), and PTX-loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles (D).

1, PTX could be loaded in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles at a carrier/drug molar ratio as low as 1/1. In contrast, a minimal carrier/drug molar ratio of 2.5/1 was needed to formulate PTX in PEG<sub>5K</sub>-FTS<sub>2</sub> micelles. Increasing the carrier/drug molar ratio was associated with an improvement in both drug loading efficiency (DLE) and the colloidal stability of PTX-loaded micelles (Table 1). In addition, PTX-loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles showed better colloidal stability than PEG<sub>5K</sub>-FTS<sub>2</sub> formulation at all carrier/drug ratios examined (Table 1).

**In Vitro Cytotoxicity of Drug-Free Micelles.** The antitumor activities of two PTX-free micelles, PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> and PEG<sub>5K</sub>-FTS<sub>2</sub>, were tested in HCT-116 and DU-145 cancer cell lines and compared to free FTS (Figure 4). PEG<sub>5K</sub>-FTS<sub>2</sub> conjugate with an ester linkage was used as a reduction-insensitive control. As shown in Figure 4A, free FTS inhibited the HCT-116 cell growth in a concentration-dependent manner. PEG<sub>5K</sub>-FTS<sub>2</sub> with a reduction-insensitive ester linkage was less active than free FTS in cytotoxicity (Figure 4A). Interestingly, incorporation into PEG<sub>5K</sub>-FTS<sub>2</sub> of an additional disulfide linkage led to a significant improvement in cytotoxicity

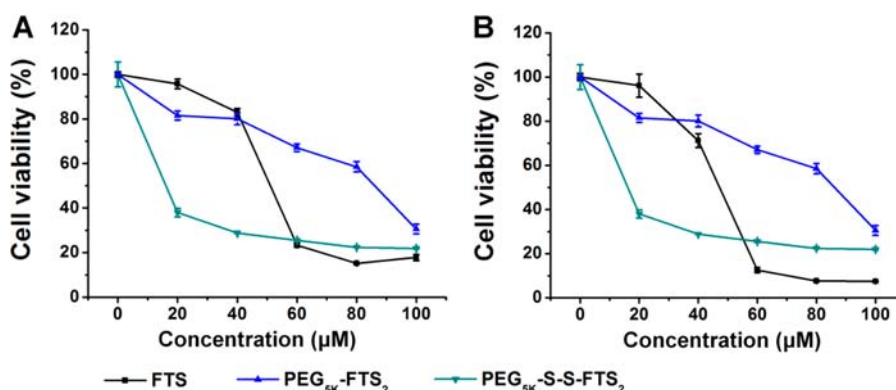


**Figure 3.** Critical micelle concentration (CMC) of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> (A) and PEG<sub>5K</sub>-FTS<sub>2</sub> (B) micelles.

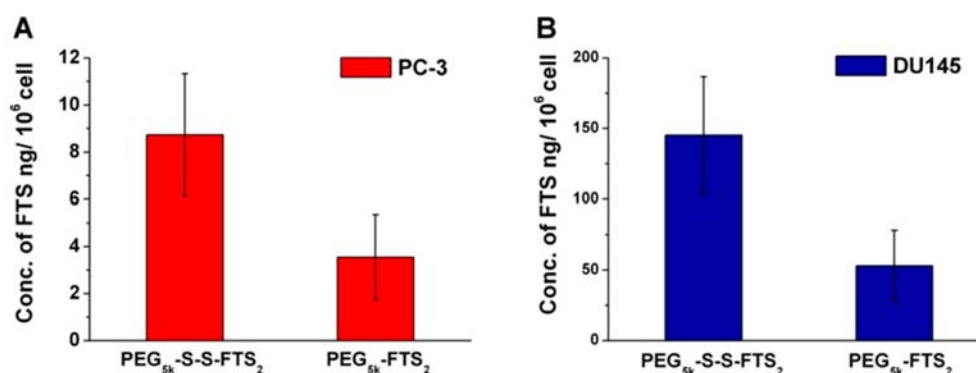
**Table 1.** Physicochemical Characterization of Free Drug and PTX-Loaded PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> Micelles

micelles	molar ratio	conc. of PTX <sup>a</sup> (mg/mL)	size <sup>b</sup> (nm)	PDI <sup>c</sup>	DLC <sup>d</sup> (%)	DLE <sup>e</sup> (%)	stability <sup>f</sup> (h)
PEG <sub>5K</sub> -FTS <sub>2</sub>	-	-	17.6	0.20	-	-	-
PEG <sub>5K</sub> -FTS <sub>2</sub> :PTX	2.5:1	1	24.9	0.35	5.5	81.2	2
PEG <sub>5K</sub> -FTS <sub>2</sub> :PTX	5:1	1	25.6	0.23	2.8	97.6	20
PEG <sub>5K</sub> -S-S-FTS <sub>2</sub>	-	-	32.4	0.21	-	-	-
PEG <sub>5K</sub> -S-S-FTS <sub>2</sub> :PTX	1:1	1	28.5	0.22	12	85.2	1
PEG <sub>5K</sub> -S-S-FTS <sub>2</sub> :PTX	2.5:1	1	32.0	0.30	5.2	89.7	3.5
PEG <sub>5K</sub> -S-S-FTS <sub>2</sub> :PTX	5:1	1	30.2	0.35	2.6	94.8	30

<sup>a</sup>PTX concentration in micelle was kept at 1 mg/mL. Blank micelle concentration was 20 mg/mL. Values reported are the mean  $\pm$  SD for triplicate samples. <sup>b</sup>Measured by dynamic light scattering particle size. <sup>c</sup>PDI = polydispersity index. <sup>d</sup>DLC = drug loading capacity. <sup>e</sup>DLE = drug loading efficiency. <sup>f</sup>Data means there was no noticeable size change during the follow-up period.



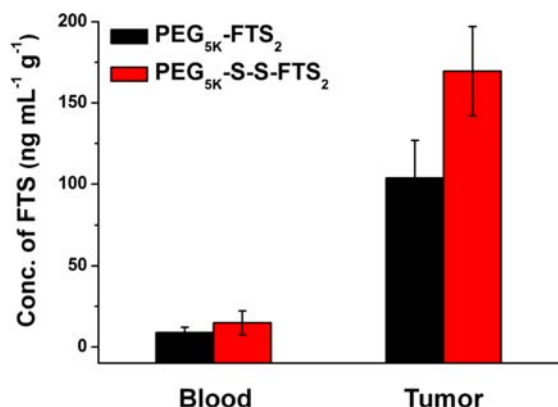
**Figure 4.** Cytotoxicity of drug free PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles in comparison to free FTS in HCT-116 human colon carcinoma cell line (A) and DU-145 human prostate cancer cell line (B). Cells were treated for 72 h and cytotoxicity was determined by MTT assay.



**Figure 5.** HPLC analysis of the amounts of released free FTS in PC-3 or DU-145 prostate cancer cells 72 h following treatment with PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> or PEG<sub>5K</sub>-FTS<sub>2</sub> at a FTS concentration of 20  $\mu$ M.

compared to both free FTS and PEG<sub>5K</sub>-FTS<sub>2</sub> (Figure 4A). A similar result was observed in DU-145 cell line (Figure 4B).

**Release of FTS from the PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> and PEG<sub>5K</sub>-FTS<sub>2</sub> Conjugates.** To investigate whether the improved cytotoxicity of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> over PEG<sub>5K</sub>-FTS<sub>2</sub> is attributed to a more effective release of FTS, HPLC-MS was employed to analyze FTS release inside PC-3 or DU-145 human prostate cancer cells 72 h following treatment with PEG<sub>5K</sub>-FTS<sub>2</sub> or PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles. We focused on the detection of the signal of parent FTS. The FTS extraction protocol had minimal impact on the integrity of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> as demonstrated in a preliminary study (data not shown). Figure 5A shows that incorporation into PEG<sub>5K</sub>-FTS<sub>2</sub> a disulfide linkage led to a 2- to 3-fold increase in the amounts of free FTS detected in PC-3 cells (Figure 5A). A similar result was observed in DU-145 cell line (Figure 5B). We also conducted a preliminary study on the release of FTS in tumor tissues *in vivo*. Female BALB/c mice bearing 4T1.2 tumor (~1 cm) received i.v. injection of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> and PEG<sub>5K</sub>-FTS<sub>2</sub> micelles at the same dose and the amounts of free FTS in the tumor tissues were examined 24 h later. As shown in Figure 6, a strong signal of FTS was detected



**Figure 6.** HPLC-MS analysis of FTS in blood and tumors 24 h following i.v. administration of PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub>.

in the tumor tissues while very little FTS signal was found in the blood. It is also apparent that significantly greater amounts of free FTS were found in the tumors treated with PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> compared to the PEG<sub>5K</sub>-FTS<sub>2</sub>-treated tumors (Figure 6),

indicating that FTS is more readily cleaved from PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles at the tumor sites.

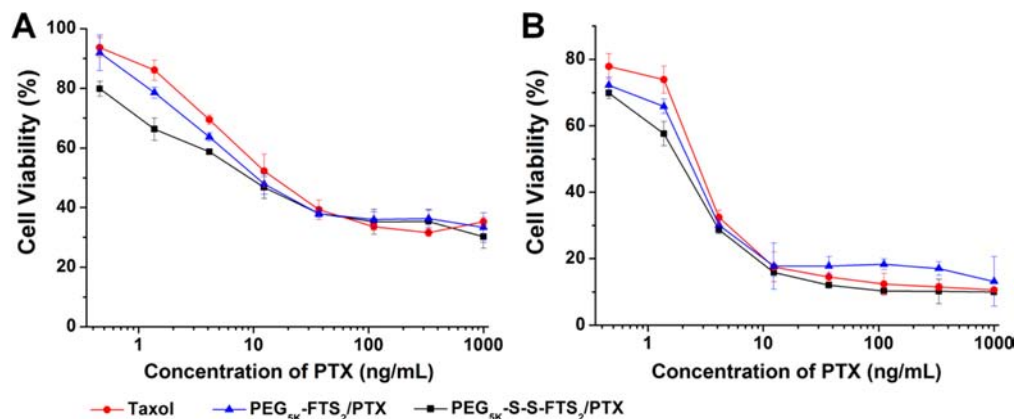
**In Vitro Cytotoxicity of Drug-Loaded Micelles.** Figure 7 shows the *in vitro* cytotoxicity of PTX formulated in PEG<sub>5K</sub>-FTS<sub>2</sub> or PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles, in comparison with Taxol formulation in MCF-7 and HCT-116 cells. Taxol inhibited the proliferation of MCF-7 breast cancer cells in a concentration dependent manner (Figure 7A). Delivery of PTX via PEG<sub>5K</sub>-FTS<sub>2</sub> micelles led to a slight increase in cytotoxicity against MCF-7 tumor cells. More importantly, PTX formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles were more active than both Taxol formulation and PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub> micelles in inhibiting the tumor cell growth, particularly at low PTX concentrations (Figure 7A). A similar result was observed in a colon carcinoma cell line, HCT-116 (Figure 7B).

**In Vivo Therapeutic Study.** Figure 8 shows the *in vivo* therapeutic activity of PTX formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> in an aggressive murine breast cancer model (4T1.2). PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles alone showed no effects in inhibiting the tumor growth at the concentration used. This is due to a relatively low concentration of FTS in this group. Taxol formulation showed a modest tumor growth inhibition at a dose of 10 mg PTX/kg (Figure 8A). In contrast, both PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles were more effective than Taxol formulation at the same dose (Figure 8A). More importantly, PTX formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles exhibited even more potent tumor growth inhibition than PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub> micelles ( $P < 0.05$ ) (Figure 8A). No significant changes in body weight were detected in all treatment groups compared to PBS control group (Figure 8B).

## DISCUSSION

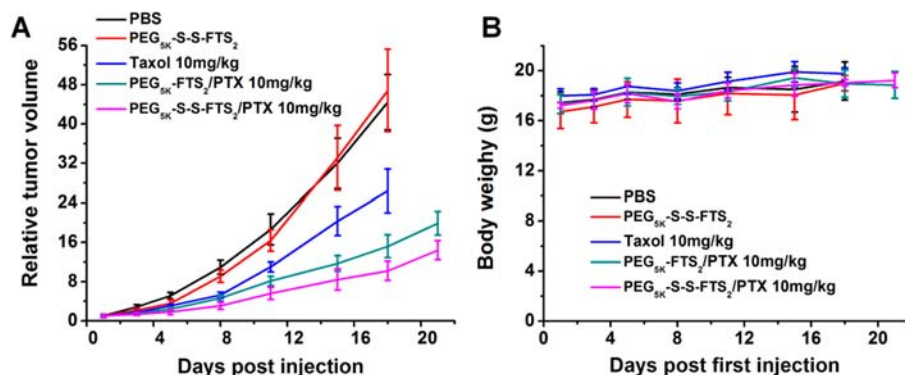
We have previously reported that PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub>(L) micelles showed better antitumor activity than Taxol formulation, which could be ascribed to their preferential tumor accumulation and a possible synergistic effect between PEG<sub>5K</sub>-FTS<sub>2</sub>(L) carrier and loaded PTX.<sup>6</sup> In this study, we have shown that inclusion of a disulfide linkage led to a further improvement in the therapeutic activity compared to PTX formulated in PEG<sub>5K</sub>-FTS<sub>2</sub>(L) micelles and Taxol *in vivo* (Figure 8).

Similar to PEG<sub>5K</sub>-FTS<sub>2</sub>(L), PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> readily self-assembled to form micelles in aqueous solution with relatively

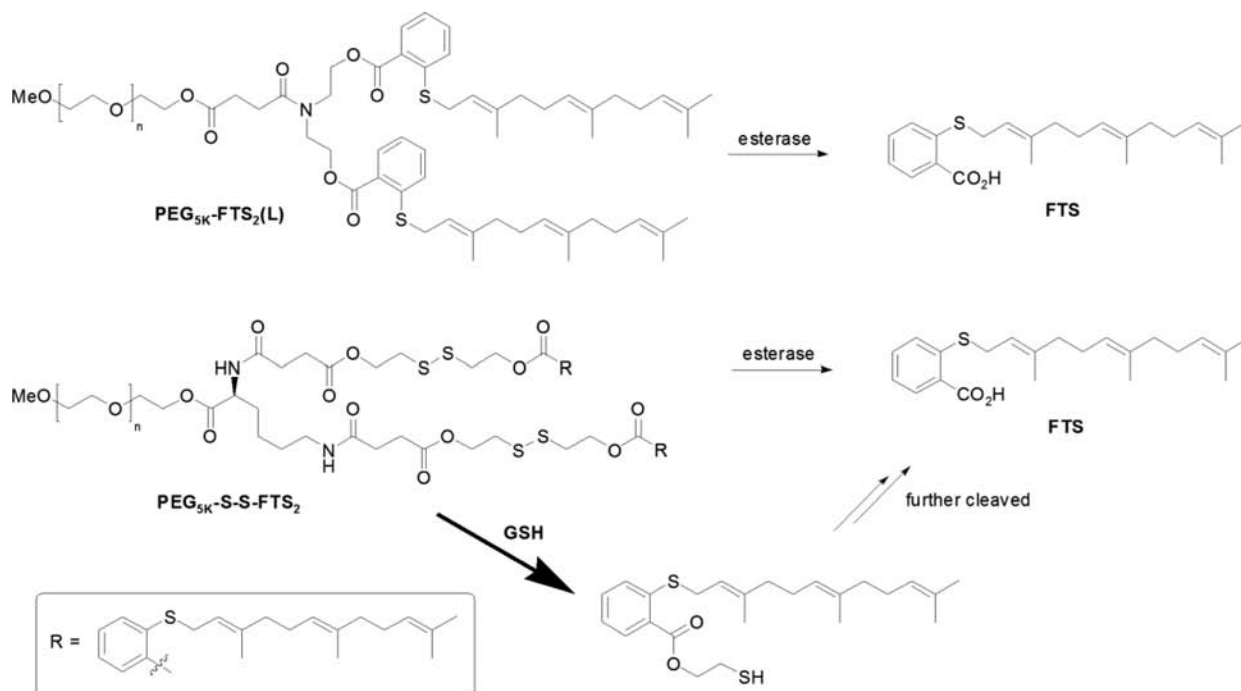


**Figure 7.** Cytotoxicity of PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles in comparison to Taxol formulation in MCF-7 human breast carcinoma cell line (A) and HCT-116 human colon carcinoma cell line (B). Cells were treated for 72 h and cytotoxicity was determined by MTT assay.





**Figure 8.** Antitumor activity of PTX formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles in a syngeneic murine breast cancer model (4T1.2) (A).  $P < 0.05$  (PTX/PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> vs PTX/PEG<sub>5K</sub>-FTS<sub>2</sub>).  $N = 5$ . Changes of body weight in mice receiving different treatments (B).



**Figure 9.** Proposed mechanism for the release of FTS from PEG<sub>5K</sub>-FTS<sub>2</sub> and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugates following intracellular delivery to tumor cells.

small size ( $\sim 30$  nm) (Table 1). Such small size shall enable the carrier to be highly effective in passive accumulation at and deep penetration into solid tumors, including the poorly vascularized tumors.<sup>1,25</sup> Interestingly, inclusion of a disulfide linkage led to an improvement in both PTX loading capacity and the colloidal stability of PTX-loaded micelles (Table 1). We further noticed that incorporation into PEG<sub>5K</sub>-FTS<sub>2</sub>(L) a disulfide linkage resulted in a 4-fold decrease in CMC (Figure 3). A lower CMC will enable the micelles to be more stable in the blood circulation following systemic administration.<sup>26</sup> This is likely due to an improved cooperation in both carrier/carrier and carrier/drug interactions following incorporation of a flexible disulfide linkage into the lipid motif of the carrier. The increase in the chain length of lipid motif following inclusion of a disulfide linkage may also contribute to the improved PTX loading capacity and formulation stability.

HPLC-MS analysis showed that parent FTS can be detected from cultured cancer cells following treatment with PEG<sub>5K</sub>-FTS<sub>2</sub>(L) micelles (Figure 5), suggesting that FTS can be released from the conjugate via intracellular esterases. It is also

apparent that significantly higher levels of parent FTS were found in tumor cells treated with PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate (Figure 5). A similar result was found in tumor tissues (Figure 6) in tumor-bearing mice receiving i.v. administration of PEG<sub>5K</sub>-FTS<sub>2</sub>(L) or PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate. FTS can be released from the PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate via two different mechanisms: one is direct cleavage of the ester linkage adjacent to FTS to generate the parent FTS, and the other involves reduction of the disulfide linkage first, followed by cleavage of the ester linkage to generate the parent FTS (Figure 9). The fact that significantly greater amounts of parent FTS were released from cells treated with PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> would suggest that the latter is the major mechanism for FTS release (Figure 9). This is probably because direct release of FTS from PEG<sub>5K</sub>-FTS<sub>2</sub>(L) by intracellular esterases is relatively ineffective due to the steric hindrance imposed by PEG. On the other hand, cleavage of disulfide linkage is a more effective process inside tumor cells due to the significantly increased GSH levels in tumor cells.<sup>15,17</sup> Once released from the conjugate, the small

molecule intermediate can be effectively hydrolyzed by the intracellular esterases to generate the parent FTS (Figure 9).

*In vitro* cytotoxicity showed that PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate was significantly more effective in inhibiting the proliferation of cultured tumor cells compared to PEG<sub>5K</sub>-FTS<sub>2</sub>(L) conjugate (Figure 4). The increased cytotoxicity of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> is most likely due to a facilitated release of FTS following inclusion of an additional cleavable disulfide linkage. It is interesting to note that PEG<sub>5K</sub>-SS-FTS<sub>2</sub> also exhibited higher levels of cytotoxicity than free FTS at low concentrations (Figure 4). This is unlikely due to the surfactant activity of the conjugate as PEG<sub>5K</sub>-SS-FTS<sub>2</sub> showed minimal hemolytic activity at even much higher concentrations (data not shown). It is possible that PEG<sub>5K</sub>-SS-FTS<sub>2</sub> is more effectively taken by tumor cells than free FTS, which will be further examined in the future. PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> not only retained the biological activity well, but also served as an efficient carrier to deliver hydrophobic drug PTX. PTX formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles showed a level of cytotoxicity that was higher than that of either Taxol or PTX formulated in PEG<sub>5K</sub>-FTS<sub>2</sub>(L) micelles (Figure 7), particularly at low PTX concentrations.

*In vivo* antitumor study showed that PTX formulated in PEG<sub>5K</sub>-FTS<sub>2</sub>(L) micelles were significantly more effective than Taxol formulation in a syngeneic murine breast cancer model (Figure 8), which is consistent with our previous report.<sup>6</sup> It is also apparent that inclusion of a disulfide linkage in PEG<sub>5K</sub>-FTS<sub>2</sub>(L) micellar system led to a further improvement in antitumor activity (Figure 8). The improved performance is likely attributed to a more effective cleavage of FTS from the conjugate in the tumor tissue, which shall lead to not only enhanced intracellular delivery of FTS but also facilitated release of loaded drug following disassembly of micelles. It remains to be investigated whether the improved carrier/drug interaction and the reduced CMC of the PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micellar system also contribute to the overall improvement in antitumor activity.

## CONCLUSION

An improved dual-functional micellar carrier composed of a PEG shell and FTS core via a disulfide linkage (PEG<sub>5K</sub>-S-S-FTS<sub>2</sub>) was developed. PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> retained the FTS biological activity well, which was attributed to an effective release of FTS from the conjugate following intracellular delivery. In addition, PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> readily self-assembled into small-sized micelles and formed stable mixed micelles with PTX. More importantly, PTX-loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles demonstrated more effective therapeutic effects *in vivo* over Taxol formulation and PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub> micelles.

## EXPERIMENTAL PROCEDURES

**Materials.** Paclitaxel (98%) was purchased from AK Scientific Inc. (CA, USA). FTS and PEG<sub>5K</sub>-FTS<sub>2</sub> conjugate were synthesized according to published literature.<sup>6,7</sup> Poly(ethylene glycol) methyl ether (MeO-PEG-OH, MW = 5000 kDa), dimethyl sulfoxide (DMSO), succinate anhydride, diethanolamine, trypsin-EDTA solution, Dulbecco's Modified Eagle's Medium (DMEM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma-Aldrich (MO, USA). Di-Boc-lysine, triethylamine (TEA), and trifluoroacetic acid (TFA) were obtained from Acros Organic (NJ, USA). Bis(2-hydroxyethyl) disulfide, dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide

(NHS) were purchased from Alfa Aesar (MA, USA). 4-(Dimethylamino)pyridine (DMAP) was purchased from Calbiochem-Novabiochem Corporation (CA, USA). All solvents used in this study were HPLC grade.

**Cell Culture.** MCF-7 is a human breast carcinoma cell line. 4T1.2 is a mouse metastatic breast cancer cell line. HCT-116 is a human colon carcinoma cell line. PC-3 and DU-145 are human prostate cancer cell lines. All cell lines were cultured in DMEM containing 5% FBS and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

**Animals.** Female BALB/c mice, 4–6 weeks in age, were purchased from Charles River (Davis, CA). All animals were housed under pathogen-free conditions according to AAALAC guidelines. All animal-related experiments were performed in full compliance with institutional guidelines and approved by the Animal Use and Care Administrative Advisory Committee at the University of Pittsburgh.

**Synthesis of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> Conjugate.** Figure 1 shows the synthesis route of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate. Synthesis and structural characterizations are detailed below.

**Compound 1.** Bis(2-hydroxyethyl) disulfide (1.54 g, 10 mmol) was added to a solution of FTS (3.58 g, 10 mmol), DCC (3.09 g, 15 mmol), and DMAP (122 mg, 1 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL). The mixture was stirred at room temperature until TLC showed completion of reaction. The mixture was filtered through cotton and the filtrate was concentrated on a rotary evaporator. The residue was chromatographed (1:4 EtOAc/PE) on silica gel to afford the compound 1 (3.2 g, 6.5 mmol, 65%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.99–7.97 (m, 1H), 7.45–7.41 (m, 1H), 7.32–7.28 (m, 1H), 7.17–7.13 (m, 1H), 5.36–5.32 (m, 1H), 5.10–5.07 (m, 2H), 4.59 (t, *J* = 6.8 Hz, 2H), 3.88–3.87 (m, 2H), 3.58–3.56 (m, 2H), 3.06 (t, *J* = 6.8 Hz, 2H), 2.89 (t, *J* = 6 Hz, 2H), 2.09–1.97 (m, 8H), 1.73 (s, 3H), 1.68 (s, 3H), 1.60 (s, 6H).

**Compound 2.** Succinic anhydride (2 g, 20 mmol) was added to a solution of compound 1 (4.94 g, 10 mmol) and DMAP (2.44 g, 20 mmol) in CHCl<sub>3</sub> (50 mL), and the mixture was refluxed until TLC showed completion of reaction. The mixture was concentrated on a rotary evaporator and the residue was chromatographed (1:1 EtOAc/PE) on silica gel to afford the compound 2 (5.8 g, 6.5 mmol, 97%). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.99–7.97 (m, 1H), 7.41–7.38 (m, 1H), 7.29–7.27 (m, 1H), 7.14–7.10 (m, 1H), 5.30–5.27 (m, 1H), 5.06–5.03 (m, 2H), 4.54 (t, *J* = 6.8 Hz, 2H), 4.33 (t, *J* = 6.8 Hz, 2H), 3.55–3.53 (m, 2H), 3.03 (t, *J* = 6.4 Hz, 2H), 2.89 (t, *J* = 6.4 Hz, 2H), 2.62–2.61 (m, 4H), 2.04–1.92 (m, 8H), 1.69 (s, 3H), 1.64 (s, 3H), 1.55 (s, 6H).

**Compound 5.** Compound 5 was synthesized from compound 3 following a published method.<sup>6</sup>

**Compound 6.** DCC, DMAP, compound 2, and compound 5 were dissolved in CH<sub>2</sub>Cl<sub>2</sub> with a molar ratio of 1:6:3:0.3 and allowed to react overnight at room temperature. The solution was filtered and precipitated in diethyl ether and ethanol twice, respectively. Compound 6 was obtained by further drying under vacuum.

**Preparation and Characterization of PTX-Loaded PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> Micelles.** PTX-solubilized PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles were prepared via a solvent evaporation method following our published protocol.<sup>6,18</sup> Briefly, PTX (10 mM in chloroform) and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate (10 mM in chloroform) were mixed with various carrier/drug ratios. A film of drug/carrier mixture was formed by removed the organic solvent and the film was further dried under vacuum.

PTX-loaded micelles were formed by adding DPBS to hydrate the thin film followed by gentle vortexing. The PTX loading efficiency was measured by high performance liquid chromatography (HPLC) (Alliance 2695–2998 system) as described previously.<sup>6</sup> Drug loading capacity (DLC) and drug loading efficiency (DLE) were calculated according to the following equation:

$$\text{DLC}(\%) = [\text{weight of drug used} / (\text{weight of polymer} + \text{drug used})] \times 100\%$$

$$\text{DLE}(\%) = (\text{weight of loaded drug} / \text{weight of input drug}) \times 100\%$$

Morphology, micelle size, and size distribution were assessed by transmission electron microscopy (TEM) and dynamic light scattering (DLS) following our published protocol.<sup>4</sup> The critical micelle concentration (CMC) of PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles was determined using pyrene as a fluorescence probe.<sup>27</sup>

**Release of FTS from the PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> and PEG<sub>5K</sub>-FTS<sub>2</sub> Conjugates.** DU-145 and PC-3 cells were seeded in 6-well plates. After 24 h of incubation in DMEM with 5% FBS, the old medium was removed and the cells were incubated for 72 h in the presence of drug-free PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> or PEG<sub>5K</sub>-FTS<sub>2</sub> micelles. The cells were washed with ice-cold PBS three times and solubilized via a mixture of MeOH and H<sub>2</sub>O. The lysates were vortexed and then centrifuged at 14 000 rpm for 10 min at 4 °C. Supernatants were transferred to another set of 1.5 mL microtubes and stored at –80 °C for MS analysis. The amount of FTS in each sample was quantified by Waters' SYNAPT G2-S mass spectrometer according to the literature.<sup>28,29</sup> Chromatographic separation of FTS was performed on an Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters). The mobile phase A (MPA) was 0.1% formic acid in acetonitrile, and mobile phase B was 0.1% formic acid in water. The flow rate of mobile phase was 0.40 mL/min and the column temperature was maintained at 50 °C. Data were processed using QuanLynx (v 4.1, Waters). Extracted ion chromatograms (EICs) were extracted using a 20 mDa window centered on the expected *m/z* 357.189 for FTS.

Similarly, FTS release was examined with tumor tissues from female BALB/c mice bearing 4T1.2 tumor (~1 cm). Groups of 4 mice received i.v. injection of PEG<sub>5K</sub>-FTS<sub>2</sub> or PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> conjugate at a dose of 58.5 μmol/kg. One day post injection, blood and tumors were collected. Samples were homogenized with PBS, and then mixed with 2 volumes of acetonitrile. The mixture was vortexed for 1 min, incubated for 5 min, and centrifuged at 14 000 rpm for 10 min at 4 °C. The supernatants were decanted from each sample into a clean centrifugation tube for MS analysis and FTS was then analyzed as described above.

**In Vitro Cytotoxicity Study.** The cytotoxicity of PTX formulated in PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles was assessed with several cancer cell lines and compared to Taxol and PTX loaded in PEG<sub>5K</sub>-FTS<sub>2</sub> micelles, respectively. Briefly, MCF-7 cells (5000 cells/well) and HCT-116 cells (1000 cells/well) were seeded in 96-well plates. After incubation in DMEM with 5% FBS and 1% streptomycin–penicillin for 24 h, the old medium was removed and the cells were further incubated for 3 days in the presence of indicated concentrations of PTX formulated in Cremophor/EL, PEG<sub>5K</sub>-S-S-FTS<sub>2</sub>, or PEG<sub>5K</sub>-FTS<sub>2</sub> micelles. Cell viability was then assessed by MTT assay

following our published literature.<sup>6</sup> Similarly, the cytotoxicity of drug-free PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles was examined and compared to free FTS and PEG<sub>5K</sub>-FTS<sub>2</sub> micelles as described above.

**In Vivo Therapeutic Study.** An aggressive murine breast cancer model (4T1.2) was used to examine the *in vivo* therapeutic effect of different formulations of PTX. Tumors were induced by inoculation of 4T1.2 cells (1 × 10<sup>5</sup>) in 100 μL PBS at the right flank of female BALB/c mice. After tumors in the mice reached a tumor volume of ~40 mm<sup>3</sup>, treatments were started and this day was designated as day 1. On the first day, tumor (4T1.2)-bearing mice were randomly divided into five groups (*n* = 5) and administered i.v. with PBS (control), drug-free PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles, Taxol (10 mg PTX/kg), PTX-loaded PEG<sub>5K</sub>-FTS<sub>2</sub>, and PEG<sub>5K</sub>-S-S-FTS<sub>2</sub> micelles (10 mg PTX/kg), respectively, on days 1, 3, 6, 8, 10, and 12. Tumor sizes were measured twice a week and tumor volumes were calculated by the formula:  $(L \times W^2)/2$ , where *L* and *W* represent the longest and shortest in tumor diameters (mm). Each group was compared by relative tumor volume (RTV) (where RTV equals to the tumor volume divided by the initial tumor volume before treatment). The body weights of all mice from each group were measured twice a week.

**Statistical Analysis.** Data are presented as mean ± standard deviation (SD). Statistical analysis was performed by Student's *t* test for comparison of two groups, and comparisons for multiple groups were made with one-way analysis of variance (ANOVA), followed by Newman-Keuls test if the overall *P* < 0.05. In all statistical analysis, the threshold of significance was defined as *P* < 0.05.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Additional figures as discussed in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This work was supported by NIH grants R01CA174305, R01GM102989, and R21CA173887. We would like to thank Drs. Donna Stolz and Ming Sun for their help with negative EM study. We would like to thank Dr. Raman Venkataramanan and Wenchen Zhao for their help with drug loading efficiency test.

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