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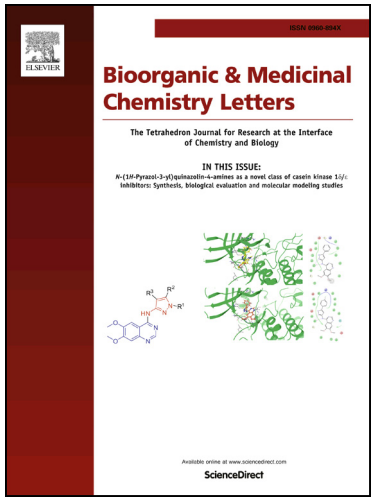

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Docetaxel prodrug self-assembled nanosystem: synthesis, formulation and cytotoxicity

Fanbo Jing [a], Qie Guo [a], Wen Xu [a], Haijun Qu [a], Zhongguo Sui [a,*]

[a]*Department of Pharmacy, Affiliated Hospital of Qingdao University, Qingdao, China*

*Corresponding author.

Email address: jingbf178@sina.com (Z. Sui)

Abstract

Conventional drug delivery systems of docetaxel (DTX) are challenged with low drug loading efficiency and potential carriers-induced toxicity. In this work, a docetaxel prodrug self-assembled nanosystem was designed and synthesized by conjugating docetaxel with oleic acid (OA) exploring a thioether as the linker, which is redox-sensitive to the redox environment within tumor cells. Notably, the carrier-free nanomedicine which does not need any carrier has obviously high drug loading that reaches 58%. Moreover, the cytotoxicity of DTX-S-OA maintains an equal level with DTX. The novel prodrug conjugate therefore has a promising perspective as carrier-free nanomedicine for cancer therapy due to its high drug loading property, redox-sensitive release and long circulation mechanism.

***Keywords*:** docetaxel, oleic acid, thioether bond, self-assembled nanosystem, redox-sensitive

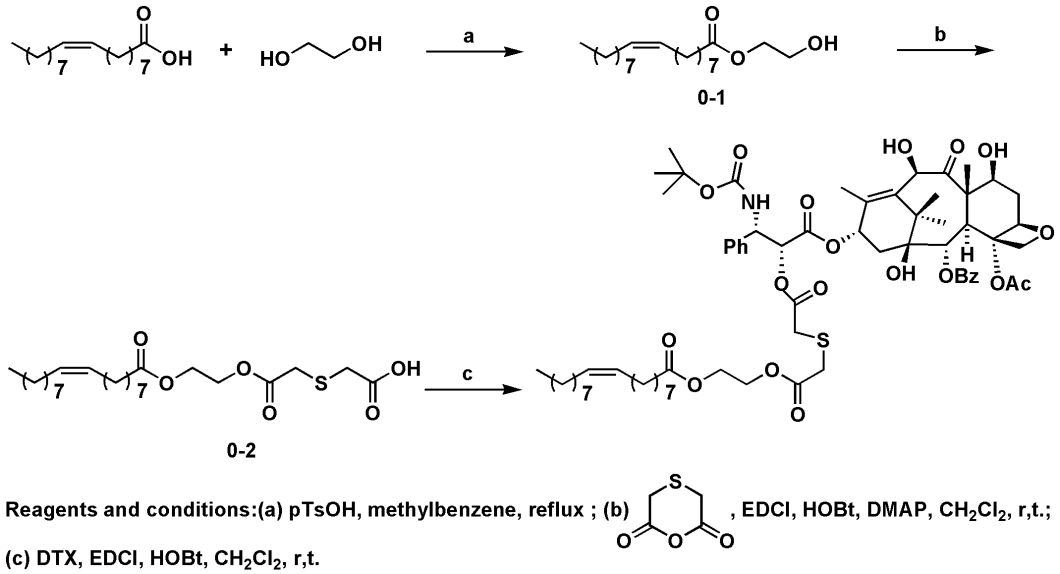
Nanosystem for drug delivery (nano-DD), especially for anticancer drug delivery, has been paid a lot of attention during the past few decades[1-5]. Although, carrier based nanosystem has brought great improvement for the usage of antitumor drug, there is still a long way for its clinical application. On one hand, it needs a carrier to load drug, which is usually much larger than the drug, leading to a low drug loading[6, 7]. On the other hand, the manufacturing process of it, in general, is very complicated and difficult to control, making it hard to get product of uniform quality[8]. Quite different from carrier based nanosystem, the self-assembled nanosystem is a kind of carrier-free nanomedicine, without the disadvantages of the traditional nano-DD mentioned above. The self-assembled nanosystem here is essentially made of prodrug, so its drug loading is much higher than the traditional nano-DD. Moreover, the manufacturing process of it is really simple, which will be described below.

The prodrug is usually designed to improve the physical, chemical or biopharmaceutical properties, such as solubility, stability, distribution, etc. by simply chemically modifying[9]. The prodrug is expected to be stable in blood until reaching the target site in order to get the intended efficacy and safety[10]. In fact, the cytoxicity of the prodrug is usually several hundreds of times lower than that of the parent drug, in which the selection of the linkage plays a really important role[11, 12]. The common linkers comprises disulfide, ester, thioether, etc[13]. The stability of disulfide linker is thought to be relevant to the concentration of GSH. Minimal disulfide linker breakage occurs in blood plasma, in which there are about 10 μM GSH. In comparison, plenty of disulfide linkers are cleaved at the tumor, the GSH concentration of which reaches 10 mM[14]. Therefore, disulfide linkers have caught a lot of attention of researchers. However, the results were not as intended as the researchers expected. The studies performed up to now indicate that disulfide bonds can be cleaved during blood circulation, indicating more stabilization needed[15, 16].

A thioether linked conjugate of trastuzumab and antimitotic agent DM1 was synthesized and studied by Phillips et al., abbreviated as T-DM1. The outcomes of which demonstrated that DM1 concentrations of T-DM1 decreased slowly in the plasma, however, T-DM1 showed fast tumor catabolism[10, 17]. Moreover, PTX-S-OA,

short for the conjugate of paclitaxel and oleic acid with a single thioether inserted was investigated by Cong Luo, etc. and the results indicated that PTX-S-OA showed intended drug release and antitumor efficacy[18]. Accordingly, we hypothesize that thioether may be an appropriate option for prodrug as a linker.

To test the hypothesis, we synthesized a conjugate of docetaxel (DTX) and oleic acid with a thioether linker inserted into spacer in order to investigate whether the prodrug could release DTX responding to high concentration of reducing agent or oxidant and achieve similar antitumor efficacy as DTX. Afterwards, the synthetic prodrug is prepared into nanoparticles by nano-coprecipitation method and a series of studies were carried out.



Scheme 1. The synthetic route of DTX-S-OA.

First of all, one esterification reaction occurred between oleic acid and ethylene glycol, obtaining intermediate O-1. Then intermediate O-1 reacted with 2,2'-thiobisacetic anhydride, obtaining intermediate O-2. And finally intermediate O-2 reacted with docetaxel to gain the target compounds (Scheme 1).

The chemical structure of docetaxel prodrug was characterized by ^{1}H NMR (Figure S1). The ^{1}H NMR spectra of the produg was analyzed as shown below： δ 8.11(d, 2H, *o*-Bz-2-H), 7.62(t, 1H, *p*-Bz-2-H), 7.51(t, 2H, *m*-Bz-2-H), 7.40(t, 2H, *m*-Ph-3'-H), 7.34(t, 3H, *o*-Ph-3'-H, *p*-Ph-3'-H), 6.23(s, 1H, 13-H), 5.69(d, 1H, 3'-H), 5.48(s, 1H,

-NH-), 5.33-5.36(m, 2H, -CH=CH-), 5.22(s, 1H, 10-H), 4.98(d, 1H, J=8.4Hz, 5-H), 4.29-4.36(m, 5H, 20α-H, -O-CH_2CH_2-O-), 4.27(t, 1H, 7-H), 4.21(d, 1H, J=8.5Hz, 20β-H), 3.94(d, 1H, J=7.0Hz, 3-H), 3.49-3.62(m, 4H, -CO-CH_2-S-CH_2-CO-), 2.59(m, 1H, 6α-H), 2.43(s, 3H, 4-$COCH_3$), 2.10-2.34(m, 4H, 14α-H, 14β-H, -CH_2CO-), 2.02(d, 4H, -CH_2-CH=CH-CH_2-), 1.95(s, 3H, 18-H), 1.87(t, 1H, 6β-H), 1.76(s, 3H, 19-H), 1.63(t, 6H, -CH_2CH_2CO-, H_2O), 1.23-1.33(m, 32H, 17-H, *t*-BuO-H), 1.13(s, 3H, 16-H), 0.88(t, 3H, -CH_3).

The docetaxel prodrug was also determined by Bruker micro TOF-Q time of flight mass spectrometer. The accurate m/z 1270.5955 ions were determined as sodium adduct for the final product (Figure S2). The actual molecular formula is matched with the predicted one.

We can also clearly observe the characteristic peaks of docetaxel prodrug from the FT-IR spectra (figure S3): The stretching vibration of the hydroxyl group arose at 3450 cm^{-1} which showed strong peak intensity and a wide peak. The methylene stretching vibration fused at 2927 cm^{-1} and 2854 cm^{-1} and the carbonyl stretching vibration fused at 1739 cm^{-1}. For the double carbon-carbon bond in the aliphatic chain, the stretching vibration appeared at 1602 cm^{-1} and 1497 cm^{-1}. The 1272 $cm^{-1}$and 1243 cm^{-1} were the stretching vibration of ether bond. And the plane bending vibration of the C-H appeared at 709 cm^{-1}. These data confirmed the structure of the new compound for docetaxel.

The above results proved the oleic acid was successfully conjugated to docetaxel via thioether bond.

The nano-precipitation method has been widely used in the preparation of nanoparticles[19]. The carrier-free docetaxel prodrug self-assembled nanosystem was simply prepared by the same way. DLS (Dynamic Light Scattering) showed that the size of nanoparticles was 153.1 ± 0.25 nm, PDI 0.078 ± 0.011 and Zeta potential -21.1 ± 2.37 mV. The appropriate size of the docetaxel prodrug self-assembled nanosystem was just between the fenestrations of vasculatures of tumor and the endothelial junctions of normal vessels[20], giving it an advantage that it could accumulate in tumor site by EPR effect (Enhanced permeability and retention effect).

And TEM (Transmission electron microscope) micrograph (Figure 1A) revealed that the docetaxel prodrug self-assembled nanosystem was spherical and the distribution of particle size was narrow, which was same with the results recorded by DLS (Figure 1B). Additionally loading content of the carrier-free docetaxel prodrug self-assembled nanosystem was 57.80 wt%, which was much higher than typical nanomedicine such as polymeric micelles, nanoemulsions,[21] liposomes, and polymer nanoparticles for delivering taxane drug[12-24]. And we also used DSPE-PEG2000 as the coating materials in the nanoparticles, which can make the nanosystem stealth from the immune system of host by preventing non-specific interaction with plasma protein and prolong its circulatory time *in vivo* by reducing RES clearance[25,26].

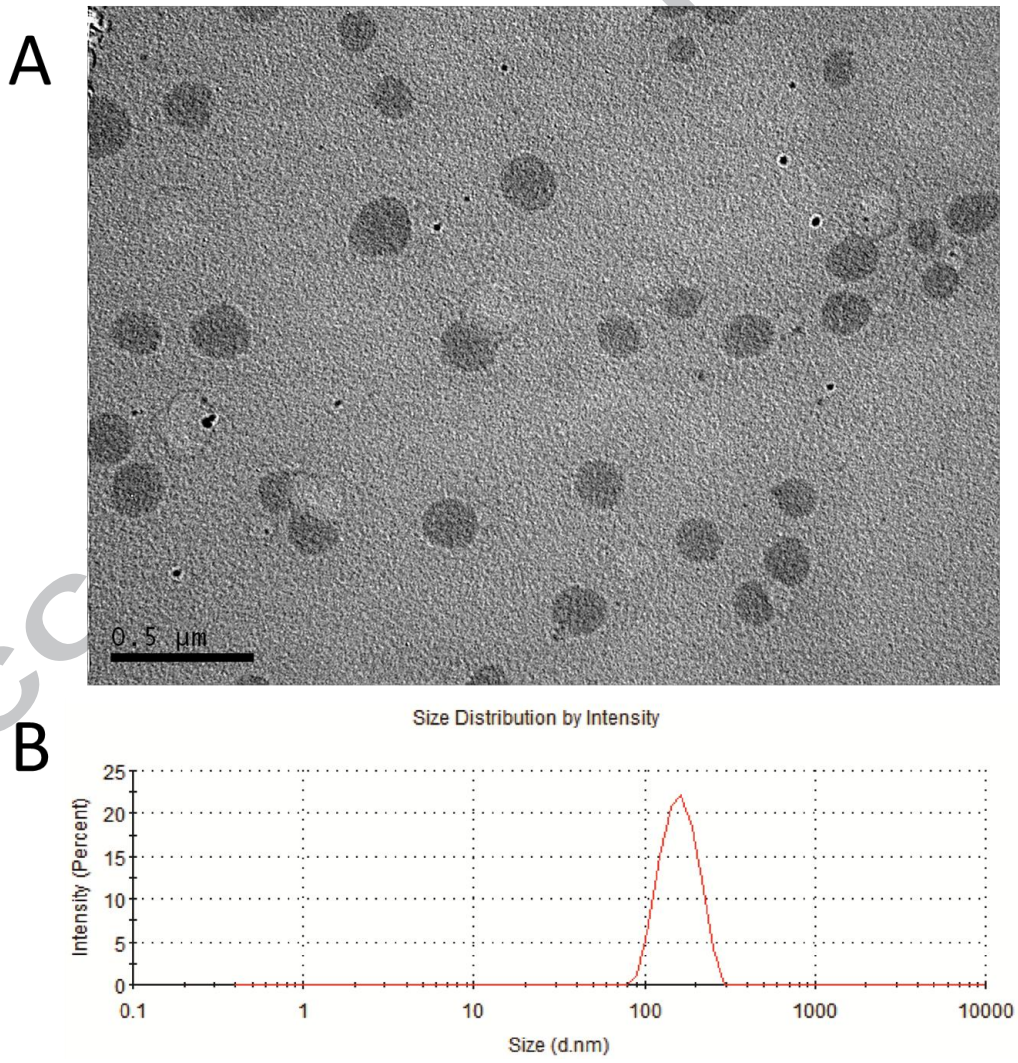


Fig. 1. TEM image of docetaxel prodrug nanoparticles (A) and size distribution of docetaxel prodrug nanoparticles determined by DLS (B).

Tab. 1. Characteristics of docetaxel prodrug self-assembled nanosystem (mean ± SD, n = 3).

Formulations	Mean size (nm)	PDI	Zeta potential (mV)	DLC (%)[a]
Docetaxel prodrug nanoparticles	153.1 ± 0.25	0.078 ± 0.011	-21.1 ± 2.37	57.80

[a] Drug loading content (%) = (amount of the drug encapsulated in NPs/weight of the NPs).

Notably, the storage stability test results of docetaxel prodrug self-assembled nanosystem was evaluated by DLS for one month (Figure S4), which indicated that the size and PDI of nanoparticles had no significant changes during test period. The particle size was nearly 150 nm in the whole period of storage and the PDI was always nearly 0.1, suggesting that the nanoparticles stored at 4 °C were stable enough. All these results indicated that the spherical docetaxel prodrug self-assembled nanosystem with good stability and monomodal particle size distribution was successful formed.

The *in vitro* release of docetaxel from docetaxel prodrug self-assembled nanosystem was studied under two reductive conditions containing 10 mM and 10 μM dithiothreitol (DTT), respectively. The experimental temperature was carried out at 37 °C simulating the body temperature. DTT is a kind of strong reducing agent. The reduction of disulfide bond by DTT is made up of two continuous mercapto - disulfide bond exchange reactions[15]. The results showed that the high concentration of DTT can apparently accelerate the release rate of docetaxel (Figure 2A). For instance, 51.3% of docetaxel were released from the prodrug nanoparticles in the condition of PBS (pH 7.4) with 10 mM DTT, which was much higher than that released (9.7%) in the condition of PBS (pH 7.4) with 10 μM DTT at the same time point (24h). It is known that the break of the thioether bond in the docetaxel prodrug caused by the high concentration of DTT would not directly generate docetaxel, since the degradation product had an easy attacked ester bond that can be cleaved for the release of docetaxel. To our best knowledge, the high concentration of DTT (10 mM - corresponding to the high redox condition within tumor cells) would promote more

cleavage of thioether bonds of docetaxel prodrug to release docetaxel compared with low concentration of DTT (10 μM) which simulated the slightly-redox environment in blood plasma[16, 26]. The results indicated that the docetaxel prodrug self-assembled nanosystem could selectively release docetaxel within tumor site and decrease the unwanted drug release at blood circulation and thus reduce systemic toxicity.

As reactive oxygen species (ROS) is also simultaneously overproduced in some tumor cells, which leads to increased oxidative stress.[26] The *in vitro* release study was also conducted under three oxidative conditions containing 10 mM, 1 mM and 0 mM H_2O_2, respectively. The results showed that the high concentration of H_2O_2 can apparently accelerate the release rate of docetaxel (Figure 2B). For instance, 91.2%, 24.3% and 4.7% of docetaxel were released from the prodrug nanoparticles at 37 °C in 12h in PBS (pH 7.4) with 10 mM H_2O_2, 1 mM H_2O_2 and 0 mM H_2O_2, respectively. The result indicates that the thioether linked prodrug has the potential of releasing parent drug rapidly in the high oxidative environment of tumor cells.

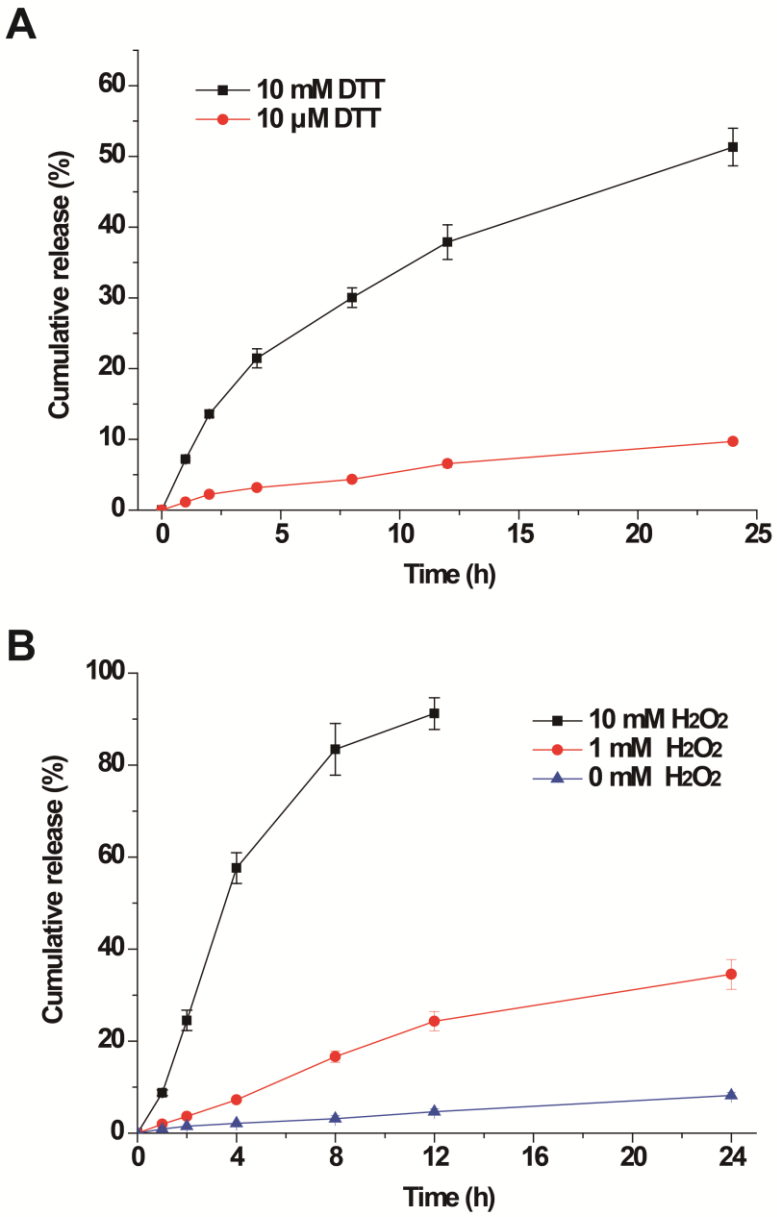


Fig. 2. *In vitro* release test. The redox-sensitive release of docetaxel from docetaxel prodrug self-assembled nanosystem was studied at 37 °C with 10 mM and 10 μM DTT, respectively(A), and it was studied at 37 °C with 10 mM, 1 mM and 0 mM H_2O_2, respectively (B). (n = 3).

The result of the in vitro cytotoxicity test was shown in Figure 3. And the IC_{50} values, derived from the MTT assay above, were calculated and summarized in Table 2. The data indicated that the DTX prodrug nanoparticles generated slightly little anti-tumor effect than the DTX solutions on both PC3 and MCF-7 cells and all the four IC_{50} values were within the range of 1~10 nM.

As we know, the toxicity usually dropped at least two logs when synthesizing the

prodrug out of the parent drug[11, 12]. On the one hand, it may be because the efficacy of linkers' cleavage was not enough to release the drug in an ample way, and on the other hand, the nano-DD usually include not only drug but much polymer as the carrier, hence, the capacity for the drug coming out of the carrier may influence the quantity of drugs reaching the target site. Opposite to this, the DTX prodrug nanoparticles with thioether inserted and without any polymer as a carrier could release drug efficiently. The result provided supports for our hypothesis that thioether may be an appropriate option for prodrug as a linker.

Tab. 2. IC_{50} of PC-3 and MCF-7 cells incubated with DTX prodrug nanoparticles and DTX solutions at 24 h (n = 3).

Compd.	IC_{50} (nM) PC-3	IC_{50} (nM) MCF-7
DTX-S-OA	9.17	3.99
DTX	5.49	3.33

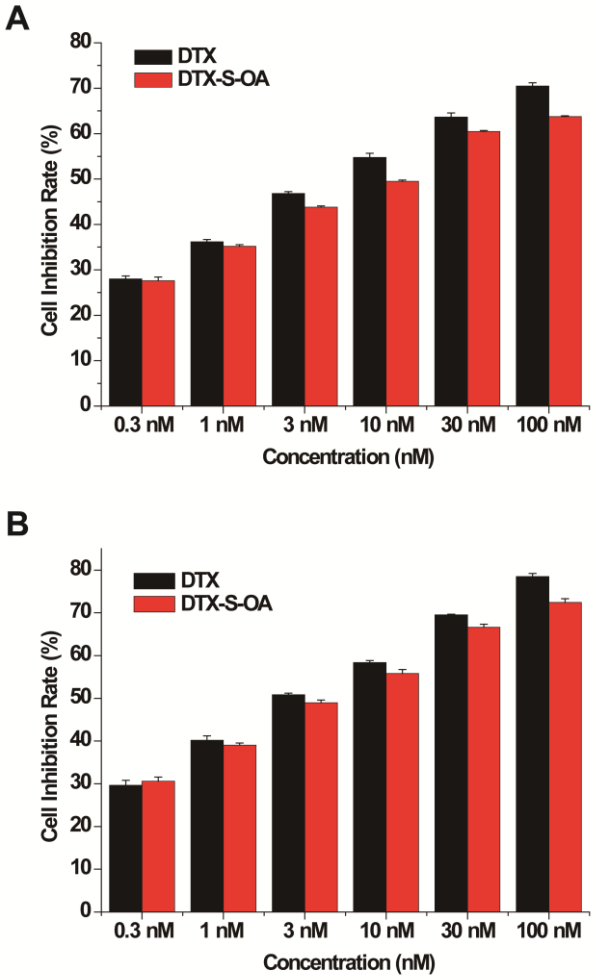
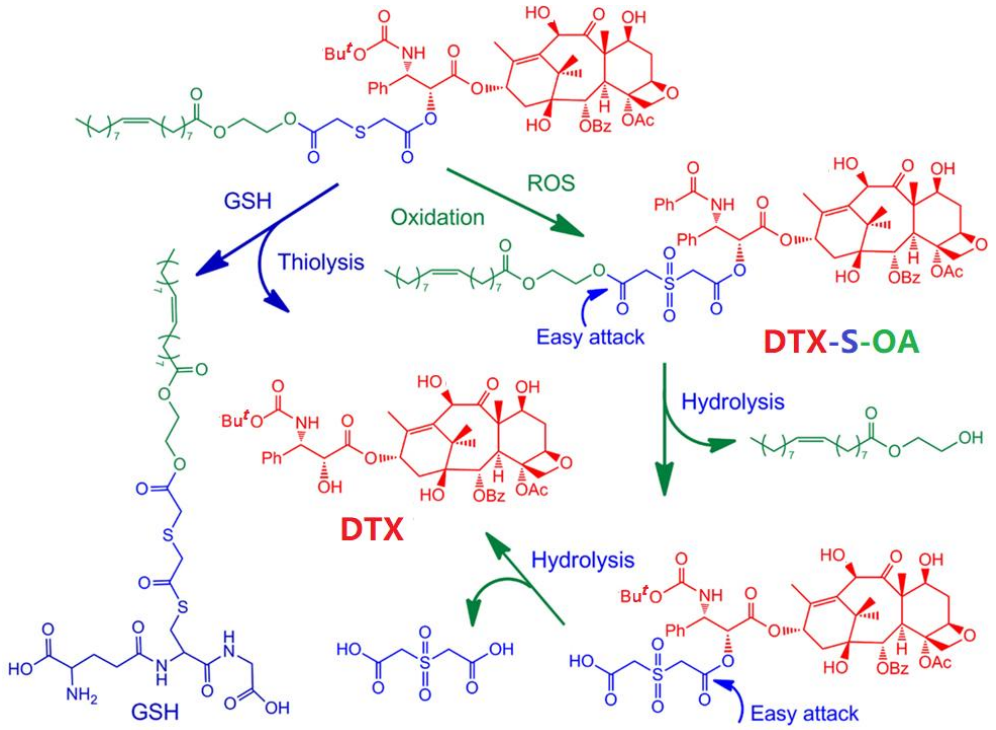


Fig. 3. MTT assays of docetaxel solutions and DTX-S-OA nanoparticles on PC-3 cell line (A) and MCF-7 cell line (B). Both of the two kinds of cells were incubated with preparations for 48h at concentrations varying from 0.3 nM to 100 nM. Data are presented as mean ± SD (n = 3), $p > 0.05$, when DTX-S-OA compared with free DTX at different concentrations.

In general, we have successfully developed a carrier-free docetaxel prodrug self-assembled nanosystem. The docetaxel prodrug was synthesized by conjugating docetaxel with oleic acid with thioether bond inserted into the spacer. Indubitably, the well-defined docetaxel prodrug nanoparticles have a clear superiority: (i) The preparation method is very simple and the formulations are significantly stable, obtaining spherical nanoparticles of uniform size. (ii) The drug loading capacity is significantly higher than other typical nanosystem such as liposomes, polymeric micelles and polymeric nanoparticles. (iii) The embeded PEG chain could protect the nanoparticles from the recognition of RES. (iv) The thioether bond is redox-sensitive

so that the docetaxel prodrug nanoparticles can reduce the general toxicity and rapidly release docetaxel in tumor cells (Scheme 2). (v) The cytotoxicity was comparable.



Scheme 2. Redox-sensitive drug release mechanism of DTX-S-OA triggered by GSH/ROS.

Based on the results above, we can come to the conclusion that the DTX prodrug with a thioether linker inserted into spacer can release DTX responding to high concentration of reducing agent or oxidant and achieve similar antitumor efficacy as DTX. And the thioether bond is an appropriate choice for DTX prodrug as a linker, since really plays an important part in the process of the development of new prodrug compound. The DTX prodrug conjugate synthesized in this study therefore has a hopeful perspective as carrier-free nanomedicine due to its high drug loading capacity, redox-sensitive release and long circulation mechanism.

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Graphical Abstract