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Reduction-Sensitive Polymeric Micelles for Intracellular Doxorubicin Delivery

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Abstract: *The hazard of cancer is continuously increasing with age, Doxorubicin (DOX) encapsulated by liposomes as an effective formulation is widely used to treat malignancies. To avoid the cardiotoxicity of DOX and increase the half-life and targeting of liposomal drugs, redox-sensitive nanoparticle that improve the delivery of therapeutics to target tissue have become an extensive choice of medical treatment. In this work, we synthesised a kind of redox-sensitive product, PEG-redox-DOX micelles which were made of PEG-SS-COOH coupled with DOX by a reduction-sensitive disulfide bond. This micellar system has a mean diameter of 60nm with acceptable distribution (PDI=0.81) and remained unchanged in phate buffered saline (PBS, pH=7.4) for 24 hours. However, the disulfide bond between DOX and hydrophilic PEG broke readily in the presence of 10 mM DTT imitating intracellular condition. The micelle released more than 75% DOX within 48h at 10 mM DTT, whereas only 12% DOX were released from prodrug at 0 mM DTT. These results suggest that PEG-redox-DOX has expectable efficiency of drug-loading and release. Nowadays, redox-sensitive micelles are more and more taken into considered, demonstrating its developmental potential in cancer therapy categories by providing a new search direction.*

Keywords: *Reduction-Sensitive Polymeric,Doxorubicin,Micelles*

1. Introduction

Cancer is a disease that has threatened mankind for decades and is the primary cause of non-accidental death among adolescents and young adults.[1]To deal with such severe disease, scientist developed several cancer therapies including chemotherapy, radiation therapy, and surgical resection. However, these therapy all obsess some problems in common including adverse effects to normal tissues, incomplete resection for the nanoscale cancer cells, and drug resistant for chemotherapy. [2]

Doxorubicin (DOX), one of the chemotherapeutic DNA-damaging drugs, exhibits effective anticancer effects against several malignant tumors including ovarian cancer, liver cancer, and esophageal carcinoma. [3][4][5] Its pharmacological mechanism is as follows: previous studies suggest that Adriamycin is embedded between adjacent base pairs of DNA, cross-linking with DNA, inhibiting DNA polymerase and nucleic acid synthesis, blocking DNA replication and RNA transcription, so as to achieve the purpose of anti-tumor. [6]Nevertheless, like all cancer chemotherapy, low target to non-target ratio of therapeutic agents would also cause several adverse side effects. To be exact, after taking DOX may causing motor disability, blood circulation defects, and dose-dependent cardiotoxicity due to its low drug loading capacity (DLC), one of the most significant consideration for drug delivery system, which is the percentage of the drug that successfully load in the cancer cell.[7][8][9][10] Thus, developing new methods to improve the anticancer efficiency of DOX in a limited does is significant, and one efficient way to reduce most of the side effects and increase the tumor specificity is using the nanomedicines.[11]

Nanomedicines based on polymers including liposomes, micelles, albumin nanoparticles, and quantum dots have been extensively explored as one kind of cancer therapy. [12] These nanocarriers exhibited properties that could prolonged circulation time by evading the filtration of renal and reticuloendothelial systems, exhibiting the enhanced permeation and retention (EPR) effect(*Figure 1)*, and improving tumor-specific accumulation.[13][14][15] Stimuli-Sensitive polymeric micelles, one kind of nanocarriers, could release drugs efficiently and immediately in the cancer cells due to the change of the

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physical and chemical environment inside the cancer cells such as temperature, pH, and redox potential.[13]

The construction of the polymeric micelles has to be considered according to the difference intracellular environment of the cancer. One of the noticeable chemical differences that could take advantage of is that the intracellular compartments conclude relatively higher concentration of redox species than extracellular compartments.[16] To be specific, reducing glutathione (GSH) is the most abundant reducing molecule in most tumor cells (~ 10 mM in the cytosol) that decomposes the disulfide bond to stimulate the release of the drug when the nanocarrier is in the cancer cells.[2][16][17] Considering this specialty, the reduction-sensitive polymeric micelles could expect to be effective in drug delivery as it could keep stable in the extracellular environment and rapidly degrade and release the anticancer drug in the intracellular environment owing to the highly concentrated reducing species in the cell.[13]

Recently, several studies have developed the nanocarriers that utilized the redox-sensitive materials for intracellular drug delivery. Yin and coworkers developed octreotide (Phe)-polyethene glycol-disulfide bond-paclitaxel [OCT(Phe)-PEG-ss-PTX] for PTX delivery, which exhibit superior tumor-targeting ability and antitumor activity and minimized the side effect of PTX.[18] Xia H et al. have designed polymeric micelle mPEG-S-S-PCL-Por (MSLP) to amplify the effect of oxidative stress in order to strengthen the effectiveness of the drug.[19]Yi X et al. developed a one-pot synthesized amphiphilic polycarbonates with disulfide crosslinked structure for DOX delivery, showing great efficiency for the delivery of DOX to the cell nuclei.[20] The results of other methods like only using pH-responsive micelles or nanoscale carriers reveals not desirable.[21][22]Thus, using reduction-sensitive polymeric micelles might be a potential approach for DOX delivery.

In this study, we constructed reduction-sensitive prodrugs based on an amphiphilic polymer, PEG-redox-DOX for intracellular delivery of anticancer drug. Polymer PEG-redox-DOX was prepared by an easy fabrication method including two reactions. 3,3'-Dithiodipropionic acid and PEG-OH were used to compound PEG-SS-COOH and then a chemical reaction between DOX and carboxyl group occurred to assemble PEG-redox-DOX. During the delivery of this DOX-loaded nanoparticle, they accumulated and penetrated through blood vessel from blood into cancer tissue based on EPR effect. Once the particles entered the target cell, they would fuse with endosome or liposome leading to the release of DOX and made an effect to nucleus eventually (S*cheme 1*). The physicochemical characteristics of micelles were assessed by ^{1}H NMR, dynamic light scattering (DLS) and transmission electron microscopy (TEM). In addition, to evaluate the feasibility of PEG-redox-DOX, we investigated the micelles stability in PBS (0.5 mg/ml, pH=7.4, 37 centigrade) for 24 hours. Moreover, the behaviour of the intracellular release of DOX was formulated by the analysis and size change and *in vitro* release study with and without the presence of 10 mM DTT in PBS (0.5 mg/ml, pH=7.4, 37 centigrade) for 24 hours.

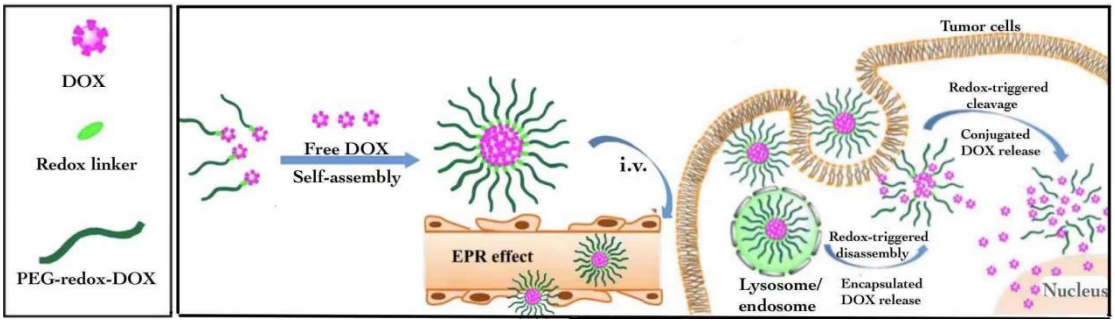

Figure 1. Schematic illustration of formation and delivery of the PEG-redox-DOX *micelles.*

2. Experimental section

2.1 Synthesis of PEG-SS-COOH

Dissolve 3,3'-Dithiodipropionic Acid (630 mg,3 mmol), EDCI (191.7 mg, 1 mmol) and DMAP (61 mg, 0.5 mmol) in ultra-dry DMF. Peg-oh (375 mg, 0.5 mmol) was then added under the protection of nitrogen. The whole system was stirred at 37℃ for 24 hours, and then washed with 1 M HCl, saturated NaHCO3 and saturated salt water for three times. The organic phase was collected, dried with anhydrous magnesium sulfate, the reaction solvent was removed by rotary evaporation, and the product was obtained after three times of ether precipitation.

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2.2 Synthesis of PEG-redox-DOX

Peg-SS-COOH (100 mg, 0.1 mmol), deionized doxorubicin (DOX, 50 mg, 90 μmol) and TEA (70 μL, 500 μmol) were dissolved overnight in 3 mL anhydrous DMF. After the solvent was removed by rotary evaporation, the product was dissolved with a large amount of DCM, extracted with saturated salt water for three times, and precipitated in cold ether for three times.

2.3 Preparation of the nanomedicine

Dissolved PEG-redox-DOX in water and stirred to obtain the solution with nanomedicine.

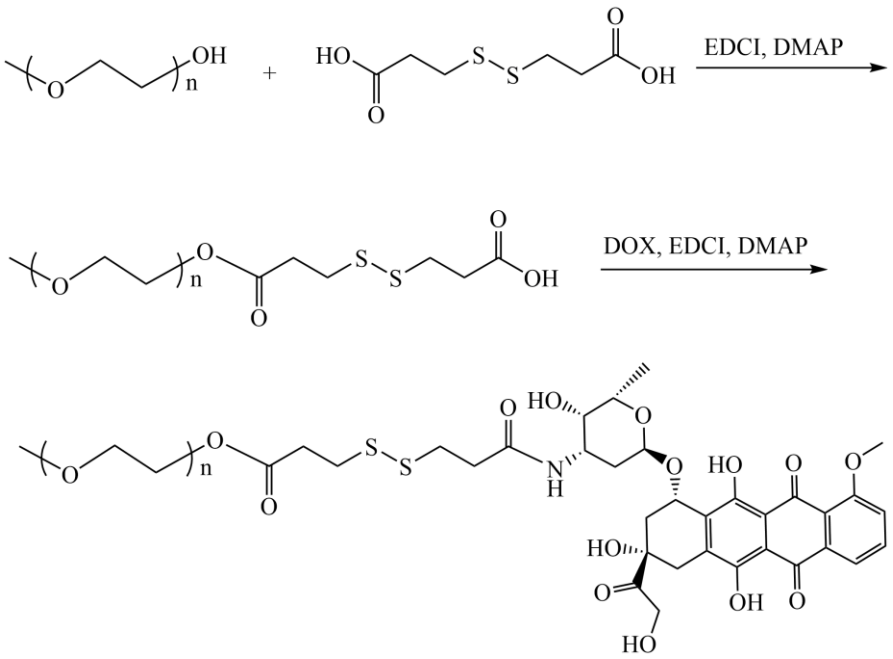

Figure 1. Synthesis roadmap of PEG-REDOX-DOX

3. Results and discussion

3.1 Preparation of nanodrug PEG-redox-DOX

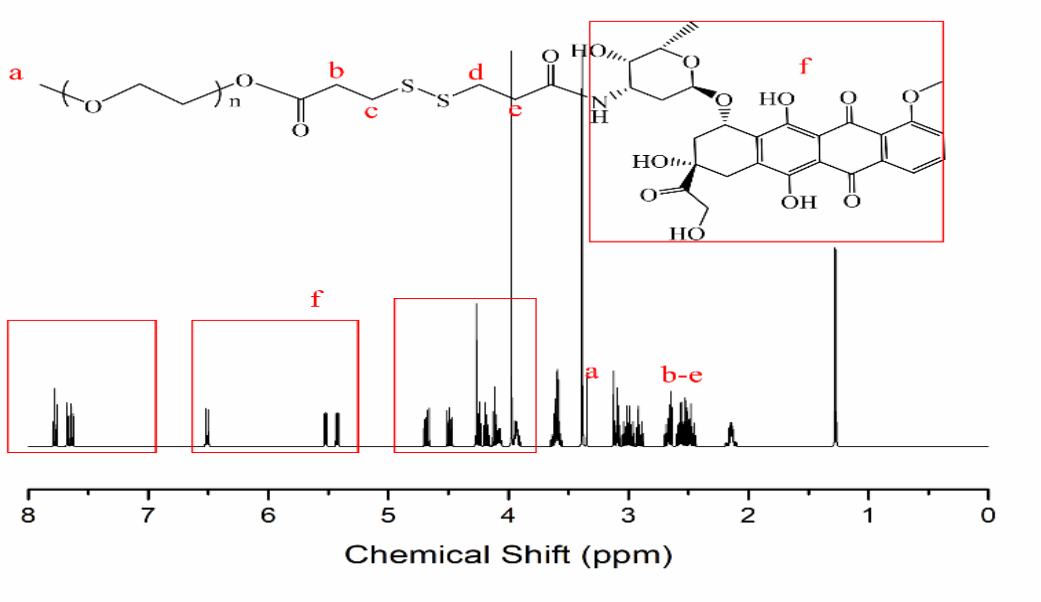

Figure 2. 1H NMR spectrum of PEG-Schiff-DOX

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The chemical structure of the obtained polymer was first characterized by 1H NMR spectroscopy. As shown in Figure 2: In the 1H-NMR spectrum, the peak f-shape of the drug molecule is more heterogeneous and dwarfed with scattered distribution of absorption peaks. Two strong peaks were observed at 1.30, 3.92 ppm for the 8-hydroxyacetyl (-COCH2OH) terminal hydroxyl group of adriamycin, and H on 1-methoxy (-OCH3) of adriamycin. using the PEG terminal on the methyl group as the starting point for the analysis. At 3.40 ppm is the methyl (-CH3) peak at the end of the PEG chain a, which corresponds essentially to the area of the main chain peak, and the area was found to correspond essentially after integration. Multiple peaks at 3.00 ppm are α-H on the methyl group attached at both ends of the disulfide bond (at c, d). Multiple peaks at 2.50 ppm are H at b, e. In particular, the appearance of e peak at 2.48 ppm indicates the formation of amide bond and a significant shift in absorption coefficient due to α-hydrogen in carboxylic acid of the original drug carrier, which becomes α-hydrogen in amide group after drug loading, indicating that DOX was successfully attached to PEG-Schiff drug carrier and it can be proved that PEG- redox-DOX was successfully prepared.

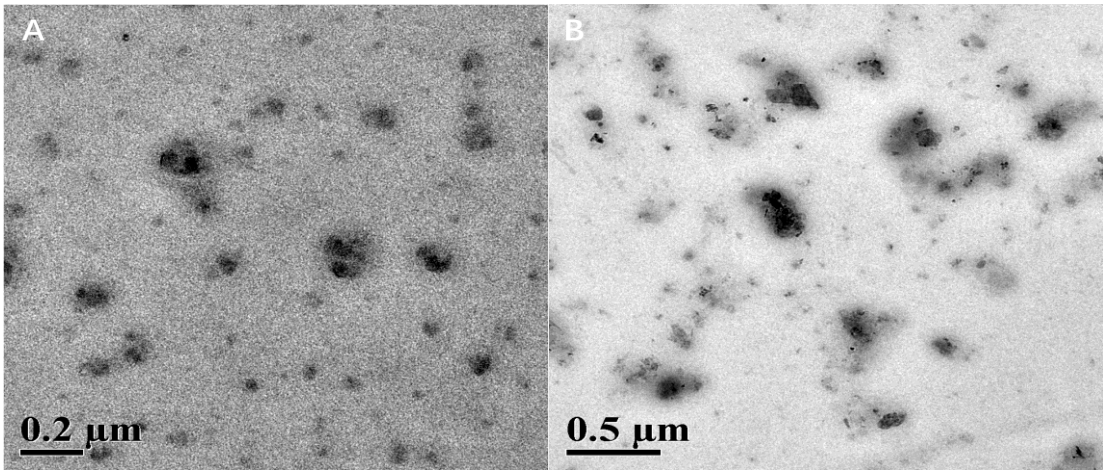

Figure 3. Morphology of PEG-redox-DOX-loaded nanoparticles before and after 10 mM DTT immersion (electron microscopy)

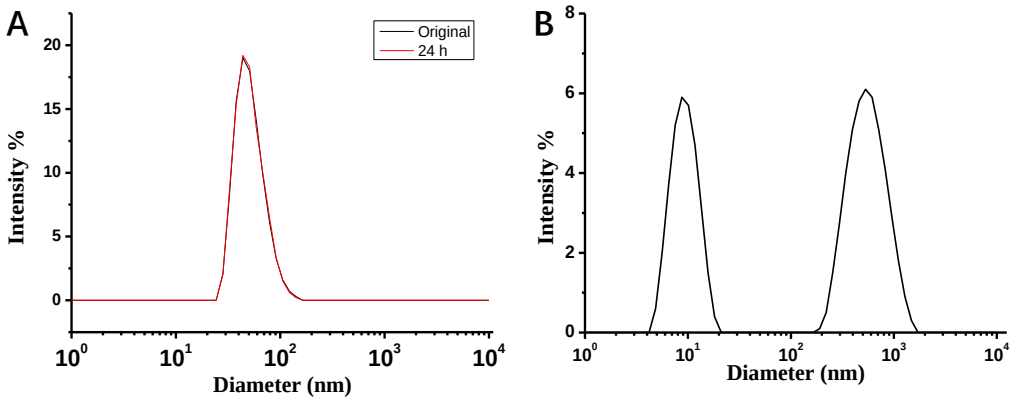

Figure 4.(A) Stability of PEG-redox-DOX-loaded nanoparticles. (B) Particle size of PEG-redox-DOX-loaded nanoparticles after 10 mM DTT immersion (DLS)

3.2 Stability studies of nanodrugs

The size of micelles is a very important parameter for intracellular drug delivery, as the small size of micelles (<200 nm) helps to maintain reduced reticuloendothelial system (RES) uptake levels, meet minimal renal excretion, and effectively passively target tumors for EPR effects.[23]

We measured and observed the size and morphology of PEG-redox-DOX micelles using transmission electron microscopy. Transmission electron microscopy microscopy images (Figure 3A) showed that PEG-redox-DOX could aggregate in water into nearly spherical micelles with a micelle diameter of less than 100 nm, the size of which depended on the length of the grafted PEG. Longer PEG side chains may

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lead to the formation of larger size micelles due to the formation of a thicker hydration layer. The size of PEG-redox-DOX micelles was determined by dynamic light scattering (DLS) measurements. As shown in Figure 4A, the histogram of the size distribution of micelles in aqueous solution shows a single peak with an average hydrodynamic diameter of 60 nm and a polymer dispersibility index (PDI) of 0.18. On the other hand, the average diameter is close to the TEM measurements. Typical transmission electron micrographs and DLS results show a spherical morphology and a uniform, medium-sized distribution of micelles. These results suggest that the size of PEG-redox-DOX micelles is suitable for intracellular drug delivery.

Storage stability is very important for drug formulations. The nanodrug was dissolved in a PBS buffered solution (0.5 mg/mL) simulating the human environment pH=7.4, and the particle size distribution was tested by dynamic light scattering and again after 24 h in a constant temperature water bath at 37 ℃. The comparison revealed that the particle size was almost unchanged. It can be inferred that in vivo, PEG-redox-DOX micelles have good stability and do not release irregularly in vivo, which ensures targeted release of the drug after entering the body, avoiding drug action at other sites outside the target tissue, improving bioavailability in the affected area, and reducing toxic side effects.

3.3 Nanodrug redox responsiveness studies

Disulfide bonding between the hydrophobic DOX and the hydrophilic PEG shell causes the PEG-redox-DOX micelles to fragment reductively in response to the reducing agent. This is due to the fact that reduced glutathione (GSH) is the most abundant reducing molecule in the intracellular compartment (~111 mM in the cytoplasm) and in micromolar concentrations in the extracellular environment (~10 μM in plasma.) GSH levels are approximately 10-100 μM in the circulation and elevated to 110 mM in tumor cells.This large difference may trigger disulfide bond cleavage and micelle breakdown, resulting in the incorporation of abrupt release of drug from micelles into tumor cells. Therefore a DTT level of 10 mM was chosen to mimic the level of reducing agent in the cytoplasmic compartment. To investigate the reductive responsiveness, nanodrugs were dissolved (0.5 mg/mL) in 10 mM DTT in pH=7.4 PBS buffer and placed in a constant temperature water bath at 37 ˚C for 24 h (after shaking at 37 ˚C for 24 h in 10 mM DTT, pH=7.4 buffer).[24]

As shown in Figure 3B, compared with the control group of micellar solution without DTT treatment, there were significant changes in the morphology and particle size of nanoparticles after 24 h treatment with DTT, and some nanoparticles had completely disintegrated into nanoscale fragments and some aggregated into micrometer scale. This phenomenon confirmed the redox-responsive degradation of the nanoparticles. The corresponding DLS plot (Fig. 4B) also shows that the average diameter of micelles increased from 60 nm to several hundred nm or micrometers with a narrowed particle size distribution, indicating that the inhomogeneous nanoparticles disintegrated in the presence of high concentrations of the reducing agent DTT, releasing the drug. It can be inferred that in vivo, PEG-redox-DOX micelles have good redox responsiveness and can be degraded at tumor tissues or cells in the presence of reducing agents by disulfide bond breakage of drug carriers, releasing the drug and providing a targeted release effect to improve bioavailability at the affected site.

3.4 Nanodrug and gel-loaded drug testing

The test results are more intuitive for gel-loaded drugs. Characterization was performed by UV spectroscopy at 480 nm, which is the maximum absorbance of DOX in solution. In vitro drug release was performed at 37°C in a solution containing 0.1 mM of DTT as shown in Figure 5. As a control, drug release was also performed in a normal solution without DTT. However, as hypothesized, the DOX not immersed in DTT released relatively slowly and the drug-laden nanoparticles hardly released the drug. In the normal PBS solution, there was a slow release of about 12% of DOX over 48 hours. In contrast, in the DTT solution containing 0.1 mM at 37°C, the release of the co-micelles was much faster, with an exponential increase in release of 20% in the first hour, up to 68% in the first 12 hours, and over 75% at 48 hours, with a tendency to continue releasing. The release behavior was significantly enhanced, with increased release and prolonged release at a significantly faster rate than in normal solution, especially during the first 12 hours. This suggests that in the presence of reducing stimuli, disulfide bonds in PEG-Schiff-DOX micelles can be reduced and broken, which destabilizes the micelle structure and increases the rate and duration of DOX release, achieving a secondary programmed drug release, i.e. encapsulated DOX is released rapidly at an early stage to reach high drug concentrations to invade tumor cells, and coupled DOX provides a longer release period and extended treatment time, thereby enhancing

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therapeutic efficacy.

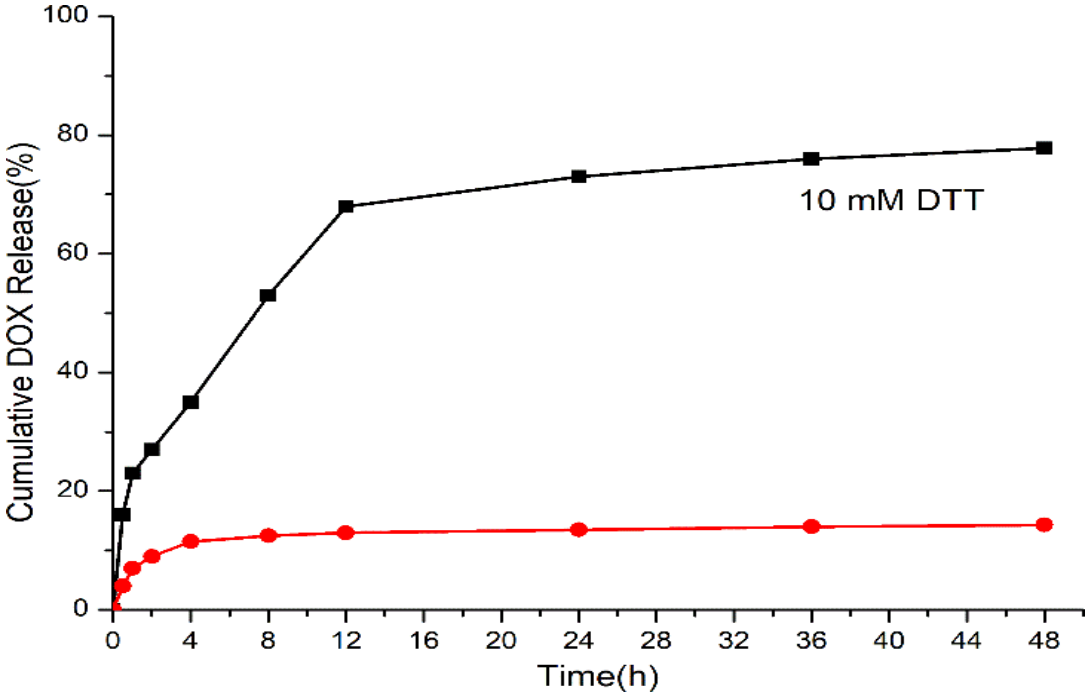

Figure 5. In vitro release profile of PEG-Schiff-DOX drug-loaded nanoparticles

In vitro drug release showed that PEG-Schiff micelles loaded with dox would rapidly release a large amount of loaded drug when stimulated by high concentration of reducing agent DTT in the endosomal environment, especially in the tumor microenvironment, ensuring targeted drug release in the target tissue (tumor site), resulting in high local drug concentration and good bioavailability (EBA). At the same time, the reduction responsiveness of its disulfide bond can also prolong the drug release time, with the continuous reduction reaction of the drug carrier, continuous release of the drug, with longer drug action time. And little or no release of drug under normal conditions also ensures blood concentration of drug at normal sites and reduces toxic side effects.

4. Conclusion

In this study, we synthesised PEG-redox-DOX micelles, a kind of Adriamycin polymer prodrug with redox-sensitivity, and the drug release was investigated under the condition of 10mM DTT. These redox-sensitive nanoparticles possess several specific features: (i) the chemical structure is organised regularly and they can be prepared easily; (ii) the drug loading efficiency is high; (iii) they have excellent storage stability; (iv) the release of DOX from PEG-redox-DOX under physiological condition is limited, while it is markedly enhanced with the high concentration of reductant in tumour micro-environment, contributing to remaining drug curative efficacy and decreasing drug toxic side-effects; (v) the secondary programmed release method helps to make DOX accumulate at the target site. Multi-stage release system not only prolongs the period during which DOX activates successfully, but responses immediately to the stimulus of reductant, demonstrating some bright medical prospect. Consequently, these nano-drugs based on polymer prodrugs provide options for the development of new drug formulations and are expected to achieve good applications in cancer treatment.

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