Preparation of pH-Sensitive Polymeric Nanoparticles for the Targeted Delivery of Doxorubicin with High Drug Capacity

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Abstract. In this study, pH-responsive prodrug nanoparticles PEG-Schiff-doxorubicin (PEG-Schiff-DOX) were designed and synthesised using chemical conjugation method and can self-assembly into spherical micelles in aqueous solution. These nanoparticles show good storage stability which can be stored over one week under normal condition. The acid-liable Schiff linker is stable under neutral pH and cleave under acidic environment, allowing the prodrug micelles to withhold DOX anticancer drug when being delivered in human circulation and disassemble to release drug once enter tumor cell tissue or taken into intracellular endosomal and lysosomal compartments. In addition to the enhanced permeation and retention (EPR) effect possessed by nanoparticle [1], the nanoparticle prodrug PEG-Schiff-DOX possess an advanced drug release behaviour, resulting in higher intracellular drug concentration in cancer cells and prolonged time of action. The superior anticancer effect of these nanoparticles against Hela cells is also investigated with CCK-8 assays, demonstrating the great potential for clinical application of PEG-Schiff-DOX in cancer treatment.

Keywords: Doxorubicin, Nanomedicine, Prodrug, pH-responsive, Schiff bond, PEG polymer.

1. Introduction

Nowadays, with rapid development of medical system and technique word-wise, cancer treatment has taken significant improvements. However, despite those promising improvements, most anti-cancer drug therapy on market still suffers from dangerous adverse-effects and suboptimal treatment response. During decades, polymer nanomedicines have become an emerging area of focus in cancer treatment. With extensive studies, nanomedicines with extended circulation time within human body and more targeted therapeutic effects profile have shown outstanding properties over those typical anticancer drugs [2-6]. Among various types of nanomedicines (prodrugs, vesicles, micelles, nanogels, liposomes), prodrug nanomedicines have exabit attractive benefits including high drug loading capacity, simple structure, efficient targeting, and less side effects [7].

In this study, we designed and synthesized an amphiphilic polymer-drug conjugated poly (PEG)-Schiff-Doxorubicin (Fig.1), which could self-roundup into micellar structures which can disassemble under acidic condition. Doxorubicin is a potent drug commonly used in chemotherapy of cancer treatment. With the ability of rapid cell penetration and perinucleolar chromatin binding, it can bind to tumor cell DNA with effective inhibition effect of nucleic acid synthesis and mitotic activity. DOX is used in various cancer types including breast cancer, lymphomas, sarcomas, bladder cancer, Wilms' tumor, neuroblastoma, and multiple myeloma [8]. Unfortunately, despite having broad clinical application and effective mechanism of action, conventional DOX drug is associated with low intracellular absorption by tumor cells and rapid clearance rate from kidney due to its hydrophilic property [9]. High aqueous solubility cause DOX drug molecule to interact with blood cells, cellular membrane, serum proteins and biomacromolecules. It is also associated with a risk of extravasation during intravenous administration, causing DOX to be absorbed by healthy human tissue which led to severe tissue necrosis. Therefore, developing an alternative delivering method of DOX with more targeted effect is essential to improve its efficacy and toxicity profile.

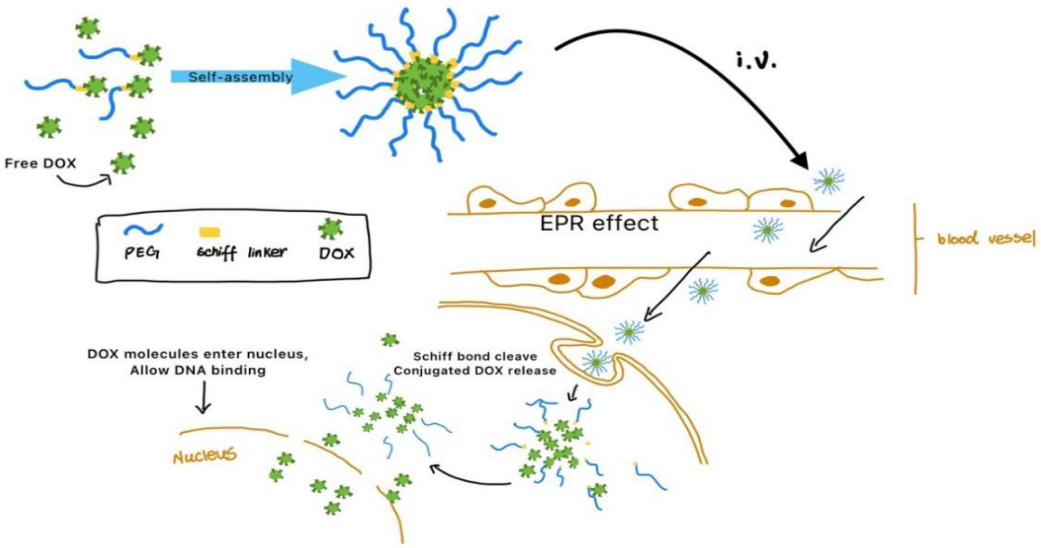

Figure 1. Schematic illustration of formation and delivery of DOX within the PEG-Schiff-DOX nanoparticles.

To overcome this problem, researchers have developed several drug-carriers via either physical entrapment or chemical conjugation to deliver cancer drugs, including liposomal DOX [10] and PEG-DOX prodrugs [11-12]. However, those drug-carriers are often related to two essential drawbacks, that is uncontrolled drug release and low drug loading capacity [13]. On the one hand, uncontrolled drug release before reaching tumor cells can lead to serious side-effects such as tissue necrosis and organ damage [14]. To avoid this, it is important for drug-carrier to only release drug under certain conditions that are only present in tumor cells. Luckily, research found tumor cells has an extra acidic microenvironment and intracellular endosomal and lysosomal compartments compared to normal human cells due to glycolysis, hypoxia, and lack of blood perfusion. Therefore, various polymer structures such as pH and Redox responsive micelles have been studied to chemically conjugate small drug molecules to drug carriers to avoid undesired drug release [15]. On the other hand, high drug loading capacity is assonated with lower cost of production as less carrier material are needed, and higher drug delivery efficacy which enhance treatment therapeutic response [26]. Because of the low critical aggregation concertation (CAC) DOX possess [17-18], high drug loading capacity (DLC) can be achieved with this nanoparticles. Additionally, the protective effect of the outer PEG corona and acid-liable Schiff linker allows more targeted drug release into cancer tissue [19], decrease drug toxicity caused to health human tissue cells.

In this study, Schiff-bond would be used to link PEG polymer and DOX anticancer drug. Within the acidic environment, pH triggered cleavage of Schiff bond will lead to disassociation of DOX from PEG polymer, causing disintegration of PEG-Schiff-DOX prodrug and rapid release of DOX free drug molecule to kill cancer cells. When being delivered in blood circulation under neutral pH, the Schiff bond will stay stable, enabling DOX drug molecules to be tightly bonded to PEG polymer, decreasing undesired drug leakage and side effects [20-29]. The acid responsiveness of Schiff-bond will be investigated in this study.

2. Materials and experimental methods

2.1. Materials

4-dimethylaminopyridine, p-carboxybenzaldehyde, doxorubicin, anhydrous N, dimethyl sulfoxide and N-dimethylformamide were directly obtained from Energy Chemical Company. Ultra-pure water was used during the experiment. Other used chemical reagents were directly purchased and used from the Beijing Chemical Company without any other purification steps.

2.2. Characterization

^{1}H NMR: Avance 400 (400 MHz) spectrometers was applied using the deuterated reagents of deuterated acetone (Actone-D_6), deuterated dimethyl sulfoxide (DMSO-D_6) ans deuterated chloroform ($CDCl_3$) within the TMS was the internal standard, and the measured condition was at 25 ^{o}C.

Transmission electron microscope (TEM) was employed on JEM-2200FS (JEOL, Japan) electron microscope with the acceleration voltage of 100 kV. Especially, 3 μL of sample was dripped onto the copper mesh (300 mesh) covered with carbon film, and the excess liquid was directly removed with a filter paper to dry for the observation. The electron microscope pictures were recorded with Gatan multiscan CCD and processed with Digital Micrograph.

The result of dynamic light scattering (DLS) was obtained by Malvern Zetasizer Nano ZS dynamic light scattering particle size analyser, which was equipped with a 633 nm of He-Ne laser with a detection angle of 173 °. The samples were contained in quartz cuvette for measurements.

UV-Vis curve was performed on the Shimadzu TU1901 UV-Vis spectrophotometer.

2.3. pH-responsive prodrug synthesis

The synthesis of pH sensitive PEG-Schiff-DOX drug molecule was performed within two steps in Fig. 2. Schiff bonds allow DOX drug molecules to be chemically conjugated to PEG polymer, lower the risk of undesirable drug release can be achieved compared to physical entrapment.

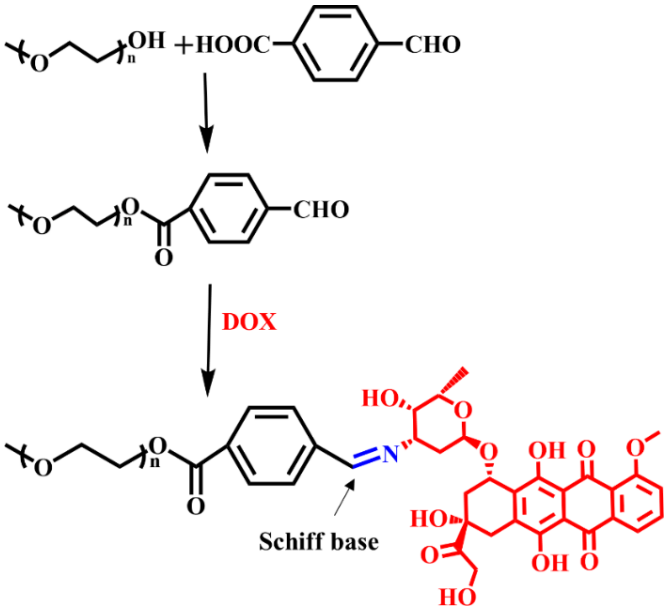

Figure 2. Synthetic route of the PEG-Schiff-DOX prodrug.

2.4. Synthesis of PEG-CHO

P-carboxybenzaldehyde (300 mg, 2 mmol), EDCI (383 mg, 2 mmol), DMAP (122 mg, 1 mmol) were dissolved in ultra-dry DCM, followed by adding the PEG-OH (750 mg, 1 mmol) polymer under nitrogen protection. System was stirred for 24h under 37 ℃, then washed for three times with 1 M HCl, saturated salt water and saturated $NaHCO_3$ solution. The organic materials were collected, dried and filtered by anhydrous magnesium sulfate. The final yield was 86%.

2.5. PEG-Schiff-DOX synthesis

TEA (70 μL, 500 μmol), PEG-CHO (100 mg, 110 μmol), and doxorubicin (50 mg, 90 μmol) were dissolved with anhydrous DMF (3mL) after overnight stirring. The resultant reaction solvent was obtained by rotary evaporation, which is then redissolved in a large amount of DCM and extracted for three times with saturated salt water and finally precipitated in the cold ether. The final yield was about 78%.

2.6. Study on stability and pH-response of prodrug

The prodrug particles was divided into two groups which are dissolved in 0.5 mg/mL of PBS solution with pH = 7.4 and pH – 5.0 respectively. The two groups are then placed in water bath under constant temperature at 37 ℃ for 2 h, followed by the measurement using dynamic light scattering to determine the size and size distributions of prodrug particles in those two groups individually.

3. Results

3.1. DOX prodrugs synthesis

The production of DOX prodrug is confirmed by its ^{1}H NMR spectrum (Fig. 3). In Fig. 3A we can see that the methyl peak at the end of the ^{1}H NMR spectrum is consistent with the benzene ring on PEG-CHO structure, indicating PEG-CHO has been successful. In regarding to PEG-Schiff-DOX synthesis, as seen in Fig. 3C, the new peak at around δ = 8.4 ppm has been formed, which indicates the formation of Schiff linkers have been identified. In addition, the peaks of drug molecules are relatively short and cluttered in spectrum of Fig. 3C, from the methyl group attached to the end of PEG molecule, we can see methyl peak was basically corresponded to the area of the main chain peak. Meanwhile, the peak in the low field region was believed to come from the Schiff bond and benzene ring for a total of H, and the area after integration was basically consistent. Therefore, the results shows PEG-Schiff-DOX polymers were successfully synthesized.

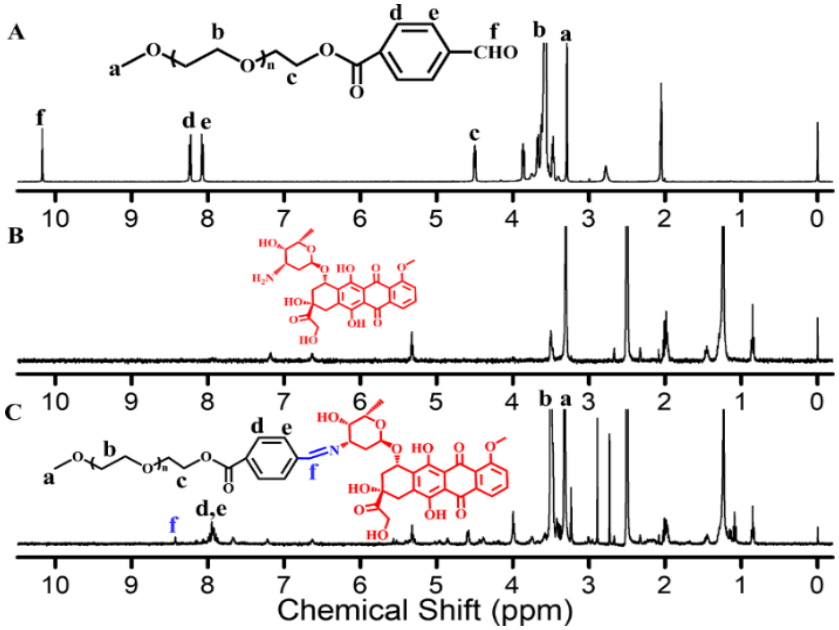

Figure 3. The ^{1}H NMR spectra of (A) PEG-CHO, (B) Doxorubicin and (C) PEG-Schiff-DOX.

3.2. The self-assembly and degradation behaviour of PEG-Schiff-DOX

Acid responsiveness is a very important feature of PEG-Schiff-DOX nanomedicine which determines its targeting effect to cancer cells and effectiveness of drug release. PEG-Schiff-DOX is able to self-assemble into a spherical micelle with a size less than 200 nm in aqueous solution under pH = 7.4 in Fig. 4A. When these nanoparticles are placed in aqueous solution with pH = 5.0, the acid-liable Schiff linker cleave, resulting in evident damage of those nanoparticles and present of amorphous structures. The degradation of nanoparticles allowed DOX to be disassembled from PEG polymer and released to express anti-cancer effect. The pH responsiveness of the nanomaterials was determined by comparing the particle size distribution of nanoparticles after two hours of shock at 37 ^{o}C in solution with pH = 7.4 and pH = 5.0 PBS solutions. Fig. 4B shows after acid incubation, smaller molecules and bigger aggregates were detected, suggesting that the nanoparticles disintegrated under the action of acidic condition, releasing free drug molecules.

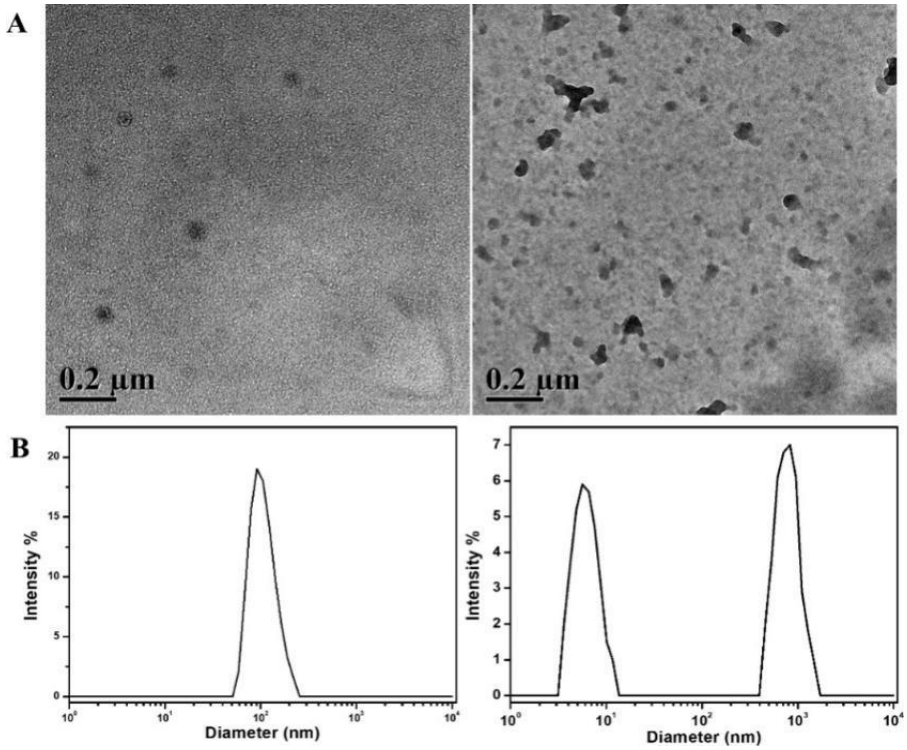

Figure 4. Morphology (A) and particle size (B) of PEG-Schiff-DOX nanoparticles under pH = 7.4 and pH= 5.0 PBS solutions.

As seen from the ultraviolet detection results, the result of drug release behaviour test is visualized. On account of the molecular calculation of PEG-Schiff-DOX polymer, the drug loading content can be precisely calculated to be 39.5%, far beyond the most reported literatures. As shown in Fig. 5, PEG-Schiff-DOX nanoparticles were associated with constant and almost no drug release at pH 7.4, indicating the most Schiff bonds have remained stable under this pH environment and DOX molecules are still conjugated to PEG polymers. While after being incubated under pH = 5.0 for 2 hours, the drug release behaviour was significantly enhanced, especially during the first 12 hours. This graph demonstrated the superior acid liable property of PEG-Schiff-DOX nanoparticles, allowing decreased drug leakage under neutral pH when being transferred within bloodstream, and fast drug release under acidic conditions such as in tumor tissues and intracellular endosomal and lysosomal compartments of tumour cells. These above results indicated that these PEG-Schiff-DOX particles possessed high drug loading contents and controlled drug release behaviours for its antitumor applications.

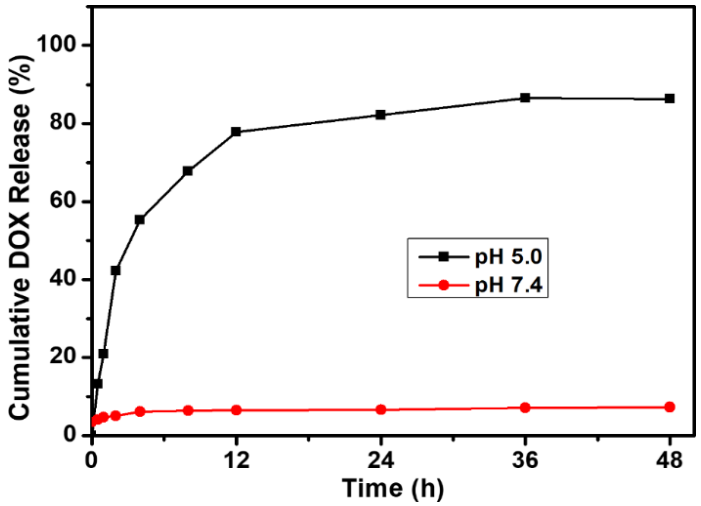

Figure 5. The release curve of PEG-Schiff-DOX nanoparticles (in vitro).

3.3. The cytotoxicity of PEG-Schiff-DOX in vitro environment

Fig. 6 is used to demonstrate the in vitro cytotoxicity effect of the nanoparticles against Hela cervical carcinoma cells. We can see that PEG-Schiff-DOX nanoparticles have excellent anti-tumor effect, especially when the concentration of loaded DOX reaches 100 μg/mL, which shows a very high inhibitory effect on tumor growth, with the cell survival rate being less than 8%. This may be attribute to targeted drug release of the nanoparticles and high cytotoxicity of DOX drug molecules, which demonstrated that the promising future of PEG-Schiff-DOX nanomedicine to be used in clinical application of cancer treatment.

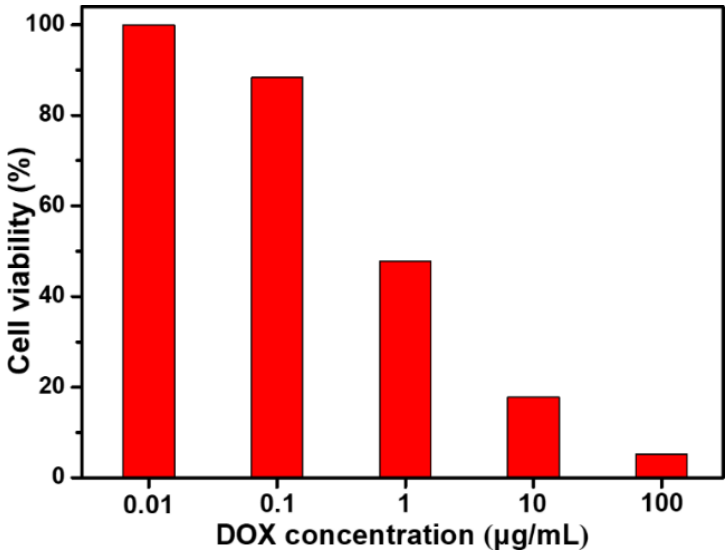

Figure 6. Evaluation of the antitumor effect of PEG-Schiff-DOX nanomedicine against Hela cells.

4. Conclusion

In summary, we designed and investigated a PEG-Schiff-DOX prodrug structure to improve the delivery of DOX drug molecules and reduce its side effect profile. From analysing its properties, a serious of advantages are proved to be processed by those prodrug nanoparticles: (1) simple structure and relatively easy production method; (2) high drug loading capacity; (3) chemical conjugation allowing stable structure under neutral pH with few drug leakage, allowing reduced drug side effects; (4) rapid drug release under acidic condition, improve targeted therapeutic effect against cancer cells to be achieved; (5) superior anti-cancer effect against Hela cancer cells.

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