

Self-assembly of a Biocompatible Prodrug with High Drug Loading Content for the Antitumor Therapy

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Abstract. Endosomal pH-responsive micellar nanoparticles were prepared by self-assembly of amphiphilic polyethylene glycol-Schiff base bond-doxorubicin (PEG-Schiff-DOX) prodrug prodrugs, and free DOX can be encapsulated within the hydrophobic cores of the nanoparticles. These nanoparticles exhibited excellent storage stability under normal conditions for more than a week but were rapidly disassembled in a slightly acidic environment. The DOX loading within the nanoparticle can be up to 61%. The CCK-8 assay showed that the nanoparticles exhibited better antitumor activity against HeLa cells than free DOX. Consequently, we are optimistic about the potential of these prodrug-based nanomedicines in developing translational DOX formulations for cancer treatment.

Keywords: Nanomedicines; dox; pH-responsive; antitumor.

1. Introduction

In the past ten years, nanomedicine has become a research direction that has attracted widespread attention in the field of biomedicine and drug molecular design, because they have excellent properties such as avoiding glomerular filtration to extend drug blood circulation time, improving drug kinetic properties, improving drug solubility, enhance drug permeability, maintain (EPR) effect, and enhance tumor cell accumulation. Compared to chemotherapy, nanomedicines offer many advantages, including higher drug efficiency, better biocompatibility and tolerability, possible drug aggregation effects, and access to more effective drug delivery technologies that can help improve the effectiveness of cancer treatment. In a range of nanomedicines, including polymer nanoparticles, prodrugs, micelles, vesicles, nanogels, and liposomes [1-8], prodrug-based nanoparticles have garnered significant attention because their morphology is more stable than polymer nanoparticles and can provide timely release ability, which can release drugs on demand.

Doxorubicin (DOX) is a broad-spectrum anthracycline antibiotic, with good water solubility and tolerability, the structure of this compound is analogous to daunorubicin, differing solely by the substitution of a hydroxyl group for a hydrogen atom at the 14-position carbon of the side chain. Encompassing both fat-soluble anthracycline ligands and water-soluble soft rubigosamine, it features acidic phenolic hydroxyl and basic amino groups, imparting it with potent anticancer pharmacological activity.

In the treatment of cancer, the main advantage of doxorubicin is that it can suppress tumors by inhibiting cancer cell division and growth, and its dose is lower than other antitumor drugs. In addition, doxorubicin can also improve the quality of life of patients by improving the symptoms of depression and CNS disease. However, doxorubicin also has some disadvantages of superior water solubility and small molecular weight, which can lead to hepatotoxicity, cardiotoxicity, and other side effects such as nausea, vomiting, and skin reactions. Hence, it is imperative to devise a tumor-specific drug delivery mechanism that minimizes adverse effects and enhances drug potency.

Doxorubicin liposome stability is not enough, the drug is easy to leak out and will cause interference in the targeted effect. The decomposition of lipase and the uptake of reticuloendothelial tissue in plasma by PEG-modified liposomes are inhibited, so that its efficacy is more long-lasting and effective. However, while these PEGDOX prodrugs have their unique advantages, they also have some drawbacks, such as relatively low DLC and potentially incomplete drug release.

In order to effectively release drugs into cells, it is critical that the junction between the drug and the polymer must be able to break down in the context of tumor cells. pH-sensitive chemical bonds

mainly include Schiff base bonds [9-13], hydrazone bonds [14-18], cis-acetyl group [19,20], polyhistidine and imidazole [21-24], amide bonds [25], acetal and ketal bonds [26-31], due to abnormalities such as high glutathione concentration and low pH in tumor cells, prodrugs containing these bonds can be broken by breaking chemical bonds in an acidic environment to achieve the purpose of releasing a large number of anticancer drugs at the tumor site to enhance cancer treatment [32]. In 2016, Wang et al. reported that polymeric prodrugs of benzaldehyde groups and doxorubicin (DOX) on polymers (CPOFs) obtained by covalent bonding of Schiff base bonds showed superior drug loading and better release properties than CPP-DOX single-molecule prodrug micelles obtained in their previous work. The importance of DLC and drug release kinetics to drug delivery systems cannot be underestimated. Once a new nanocarrier with higher DLC is developed, the cost will be reduced because less carrier material is required, and the risk of toxic side effects will be reduced accordingly, so many researchers are sparing no effort to explore [21-23]. To consolidate the effect of inhibiting tumor growth, drug release kinetics must be used to maintain the concentration and duration of effective drugs in cells.

In the protocol outlined in this study, we have meticulously designed and successfully synthesized an amphiphilic polymer prodrug, designated as PEG-Schiff-DOX, PSD, which is capable of spontaneously forming unstable acid micelle nanoparticles and can be used as a drug carrier for free DOX. As shown in Figure 1, the nanoparticles first penetrate into tumor tissue through the EPR effect, and then are engulfed by tumor cells through endocytosis. Under the action of the low pH environment of the intracellular/lysosomal region, the nanoparticles undergo decomposition as a result of pH-triggered cleavage of Schiff base bonds. This leads to the prompt liberation of encapsulated DOX. Subsequently, the previously concealed Schiff base bonds are exposed to an acidic milieu, ultimately resulting in the complete release of the conjugated DOX.

This paper presents a comprehensive study on the preparation of PSD prodrugs and DOX-loaded nanoparticles, along with an examination of pH-responsive drug release, cell uptake, and antitumor activity against HeLa cancer cells *in vitro*. The aim was to gain a deeper understanding of these processes and their potential applications in cancer therapy.

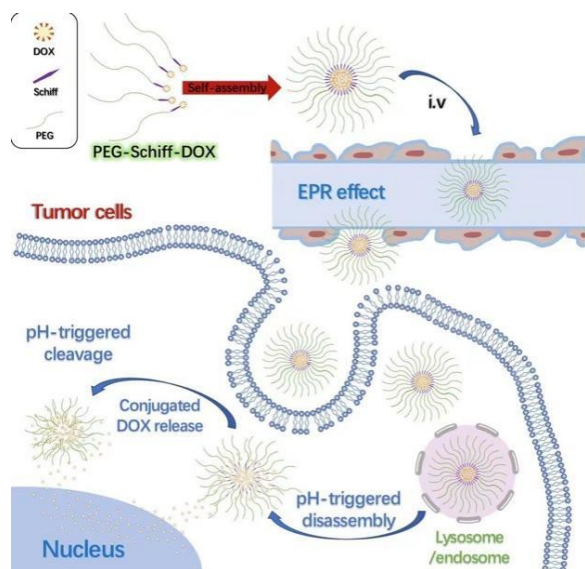


Fig 1. Schematic Representation of the Formation and Delivery Process of PSD-Encapsulated Micelles.

2. Materials and Methods

2.1. Raw Materials and Reagents

P-carboxy benzaldehyde, 4-dimethylamino pyridine, doxorubicin hydrochloride, anhydrous N, N-dimethylformamide (DMF), and anhydrous dimethyl sulfoxide (DMSO), purchased directly from

Aneji Chemical. The experimental water is ultrapure water, and other chemical reagents used are purchased from the Beijing Chemical Plant and used directly.

2.2. Characterization and Testing

Nuclear magnetic resonance hydrogen spectroscopy (^1H NMR): Avance Hockey 400 (400 MHz) spectrometer. Deuterated reagents were deuterated chloroform (CDCl_3), deuterated acetone (Actone- D_6), and deuterated dimethyl sulfoxide (DMSO-D_6), TMS as internal standard, 25°C measurement.

Transmission electron microscopy (TEM) was tested on a JEM-2200FS (JEOL, Japan) electron microscope with an accelerating voltage of 100 kV. A 3 μL sample was dropwise applied to a copper mesh (300 mesh) covered with a carbon film, excess liquid was sucked out with filter paper and allowed to dry naturally before observation. EM images were recorded using Gatan multiscan CCD and processed with Digital Micrographs.

Dynamic Light Scattering (DLS): Malvern Zetasizer Nano ZS Dynamic Light Scattering Particle Size Analyzer. Equipped with a 633 nm helium-neon laser, the detection angle is 173° , and the particle size test cell is a quartz cuvette.

UV-Vis spectroscopy: Shimadzu TU1901 UV-Vis spectrophotometer.

2.3. Synthesis of pH-responsive prodrugs

The synthesis of pH-responsive prodrug PEG-Schiff-DOX is performed in two steps, as shown in Figure 2.

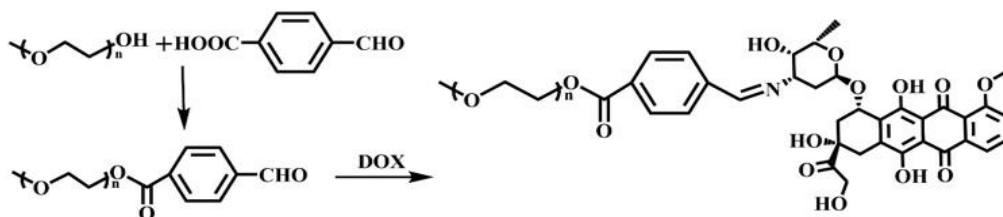


Fig 2. Synthesis Scheme of PEG-Schiff-DOX.

2.4. Synthesis of PEG-CHO

Parecarboxylbenzaldehyde (150 mg, 1 mmol), EDCI (191.7 mg, 1 mmol), and DMAP (61 mg, 0.5 mmol) were dissolved in ultra-dry DCM, and then PEG-OH (375 mg, 0.5 mmol) was added under the protection of nitrogen. The whole system was stirred at 37°C for 24 hours and then washed three times with 1 M HCl, saturated NaHCO_3 , and saturated saline. Collect the organic direction, dry with anhydrous magnesium sulfate, and filter rotary steaming. The final yield is 86%.

2.5. Synthesis of PEG-Schiff-DOX

PEG-CHO (100 mg, 110 μmol), doxorubicin (50 mg, 90 μmol), and TEA (70 μL , 500 μmol) were dissolved in 3 mL of anhydrous DMF and oscillated overnight. After rotary evaporation to remove the reaction solvent, it is dissolved with a large amount of DCM, extracted three times with saturated saline, and precipitated in cold ether. The final yield is 78%.

2.6. Stability and pH Responsiveness Studies of Nanomedicines

The nanodrugs were dissolved in PBS buffer solution with $\text{pH}=7.4$ (0.5 mg/mL) and their particle size distribution was tested by dynamic light scattering. The nanodrugs were dissolved in PBS buffer at $\text{pH}=5.0$ (0.5 mg/mL), and placed in a constant temperature water bath at 37°C for 2 h, and their particle size distribution was tested by dynamic light scattering.

3. Results and discussion

3.1. Analysis of Synthetic Results

The ^1H NMR spectra of PEG-CHO are shown in Figure 3, and it is evident that the benzene cyclohydrogen within the synthesized PEG-CHO aligns with the methyl peak ratio present at the PEG terminus. Consequently, it can be reasonably concluded that the PEG-CHO has been successfully synthesized. The ^1H NMR spectra of PEG-SCHIFF-DOX showed that the benzene cyclohydrogen aligns with the methyl peak ratio at the PEG terminus, which suggests that the successful synthesis of PEG-CHO. On the other hand, the ^1H NMR spectra of PEG-SCHIFF-DOX exhibit a more complex and shortened peak pattern for the drug molecule. Using the methyl peak at the PEG terminus as a reference, it is observed that the main chain's methyl peak area is generally comparable, which postulated that the peaks in the low-field region originate from a combination of the benzene ring and Schiff base bond, accounting for a total of 8H, and the area after integration basically corresponded, indicating the successful preparation of PEG-Schiff-DOX.

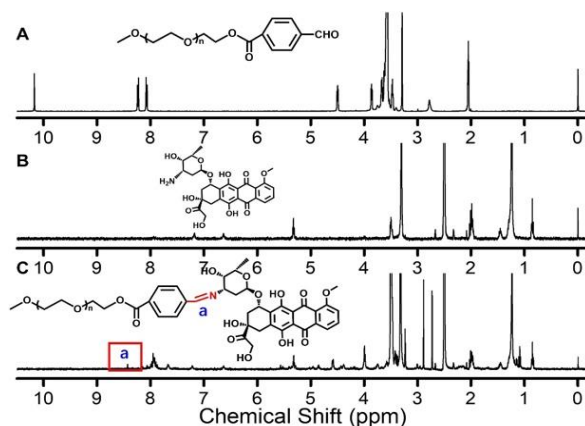


Fig 3. ^1H NMR Spectra of PEG-CHO.

3.2. Stability and pH-responsive degradation of the nanoparticles

The storage stability of pharmaceutical formulations is critical. To evaluate the alterations in the nanoparticles during storage, their size distribution was measured via Dynamic Light Scattering (DLS) prior to and following one week of storage at room temperature, as depicted in Figure 4A. The minimal changes observed across all three nanoparticles suggest their remarkable long-term storage stability.

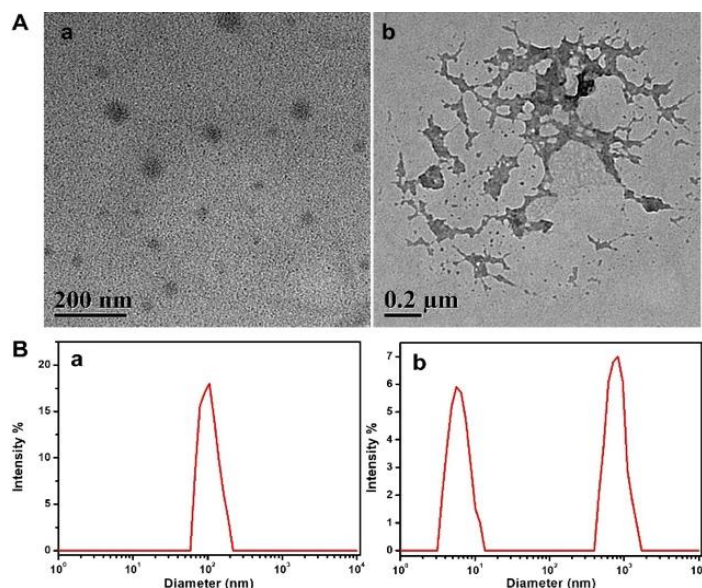


Fig 4. The Changes in the Nanoparticles during Storage.

The excellent stability exhibited by micellar nanoparticles can be partially attributed to the low CAC characteristics of PSD prodrugs. In addition, according to reports on polyethylene glycolates prodrug nanoparticles, because the surface of these nanoparticles is negatively charged, they can effectively prevent aggregation and agglomeration through electrostatic repulsion and resistance protein absorption, thereby providing them with good stability.

As the acid-unstable Schiff base bond between DOX and PEG is susceptible to slight acidic conditions, leading to the decomposition of the nanoparticles, we employed DLS to investigate the pH-responsive degradation behavior of these nanoparticles at pH=5. The observed phenomenon was then summarized and presented in Figure 4B.

After 1h of incubation at pH=5, we detected the presence of small molecules and aggregates, implying the decomposition of nanoparticles; The small chunks and big-picture collectives we also observed in the TEM images (Figure 4A) also further confirm the pH responsiveness of the nanoparticles.

3.3. In vitro drug release of the nanoparticles

In the slightly acidic environment of tumor tissue, PSD prodrugs release DOX by breaking the acid-unstable junction connecting DOX and PEG, and to verify the complete release of conjugated DOX, HPLC was employed to detect the degradation components of PSD NPs. As depicted in Figure 5, after PSD NPs were cultured at pH=5 for 2 h, the HPLC trace of PSD NPs was the same as the peak of free DOX, indicating the release of the original DOX from the PSD NPs. Moreover, we conducted in vitro drug release experiments on acetate buffer at a pH level of 5.0 and phosphate buffer at a pH level of 7.4, both at a temperature of 37°C, and compiled a comprehensive analysis of the resulting drug release curves.

The results show that in a neutral environment, only the encapsulated DOX can be released by the diffusion mechanism. At a pH level of 5.0, the cumulative release of DOX escalated notably to 83.8%, suggesting the liberation of the majority of conjugate DOX drugs. In the initial stages of liberation, DOX is expelled promptly, as previously mentioned primarily, attributed to the dissociation of Schiff base bonds.

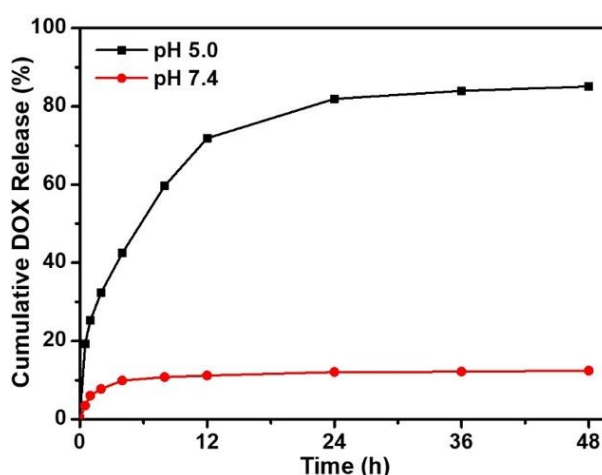


Fig 5. The Cumulative Release of DOX at pH=5.0 and 7.0.

Besides, the release of encapsulated DOX lasts only 24 h, while the release of conjugated DOX can last more than 60 h, meaning that these nanoparticles are designed to minimize drug leakage in the blood-neutral environment, while effectively releasing the drug in the acidic conditions found within tumor tissue and intracellular/lysosomal regions.

Additionally, the PSD nanoparticles loaded with DOX exhibit a two-phase programmed drug release mechanism. Specifically, the encapsulated DOX is rapidly released initially, achieving high drug concentrations to effectively eliminate tumor cells. On the other hand, the conjugated DOX ensures a prolonged release duration, extending the duration of treatment and thereby enhancing its therapeutic efficacy.

3.4. In vitro cytotoxicity of the nanoparticles

Antitumor activity of nanoparticles relative to free DOX against HeLa cervical cancer cells in vitro cytotoxicity assessment by CCK-8 assay. According to Figure 6, these nanoparticles demonstrate comparable antitumor activity against HeLa cells at drug concentrations equal to or lower than 1 $\mu\text{g/mL}$. However, at concentrations exceeding 1 $\mu\text{g/mL}$, the nanoparticles exhibit a superior cell-killing effect compared to free DOX, possibly due to the low solubility of free DOX resulting in its inability to be fully internalized by tumor cells. In addition, PSD NPs exhibit the highest antitumor activity against HeLa cells, possibly because only conjugated DOX is loaded into them, resulting in minimal drug leakage before internalization by tumor cells.

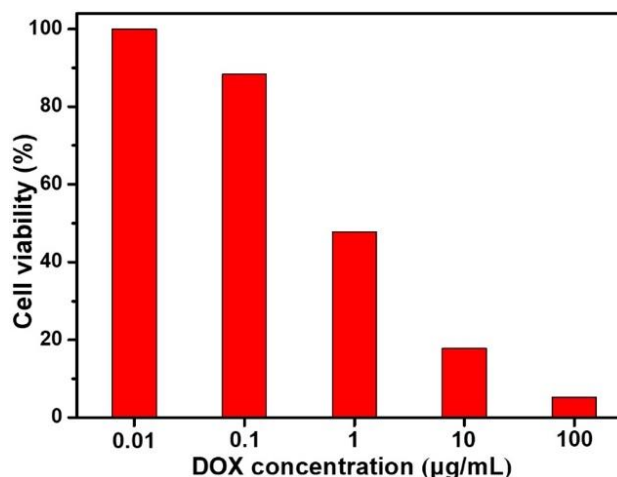


Fig 6. The Viability of Hele Cells under Varying Concentrations of DOX.

4. Conclusion

In brief, we have successfully designed and synthesized an amphiphilic PEG-Schiff-DOX prodrug, and further employed its self-assembly with free DOX to create novel pH-responsive micellar nanoparticles. These prodrug-based nanoparticles possess numerous advantageous features:

(1) The structure is clear and the preparation process is simple; (2) High drug loading can up to 61%; (3) Excellent stability in storage conditions, maintaining stable at room temperature for durations exceeding one week; (4) During the release of primitive PTX triggered by neutral circulation and endosomal pH, drug loss is almost negligible or minimal, which not only maintains the effective therapeutic effect of DOX but also reduces side effects; (5) Tumor suppression is superior to free DOX, so prodrug-based nanoparticles offer the possibility of developing promising DOX preparations.

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