The preparation of pH-sensitive doxorubicin prodrug for enhanced antitumor efficacy

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Abstract. We have produced a micelle nanoparticle with intrinsic pH response, which is formed by self-assembled amphiphilic polyethylene glycol-Schiff-doxorubicin (PEG-Schiff-DOX) prodrug. These nanoparticles can maintain stability well under normal conditions and last for more than a week, but they will rapidly decompose in slightly acidic environments. The concentration of DOX in the nano solution is high with the drug loading ratio of 49.4%. This pH-responsive drug release behavior may lead to higher intracellular drug concentrations and prolonged action times. CCK-8 assays have shown that the nanosuspension exhibits superior anti-tumor activity against HeLa cells compared to free DOX. It is believed that these nanoparticle-based prodrug have great potential for developing DOX formulations for cancer therapy.

Keywords: doxorubicin, pH-sensitive, Prodrug, Polymeric nanoparticles.

1. Introduction

In the field of cancer treatment, the research on polymer-based nanomedicines has been conducted for decades. We not only hope to greatly improve the solubility of drugs and the therapeutic effect on the disease, but also hope to reduce the harm of drugs to patients [1-3]. Nanobody drugs have many advantages and are significantly superior to small molecule anticancer drugs. For example, in order to prolong the residence time of drugs in the body, it is necessary to avoid glomerular filtration, improve pharmacokinetic properties, and promote tumor accumulation through enhanced permeability and retention (EPR) effects [4-5].Out of the polymer nanoparticles, prodrugs, micelles, vesicles, nano gel, liposomes, and other nano drugs [6-8], the prodrug-based nanoparticles stand out as having great potential for clinical transformation because of its clear and simple structure.

Among the many anti-tumor antibiotics, doxorubicin is a very good drug. Firstly, at the genetic level, it can block the synthesis of RNA and DNA, and its inhibitory effect on RNA is the most significant. Secondly, it has a wide range of anti-tumor effects and can play a certain role in many types of tumors.Aminoglycosides have strong hydrophilic properties, which allows them to distribute throughout the body. Additionally, as a chemotherapeutic drug, they possess significant toxicity. Therefore, it is crucial to develop an alternative DOX formulation that selectively targets cells and reduces adverse drug reactions. Most of the reported PEG-doxorubicin drugs had good antitumor activity through EPR effect, but these drug carriers still have two shortcomings: low concentration of the loading drug and uncontrollable release of the drug.

In order to achieve our goal of releasing drugs immediately in the cell, it is crucial to determine whether the connection between the drug and the polymer can be cleaved in the tumor cell environment. However, due to the sometimes-abnormal behavior of tumor cells, for example the concentration of glutathione exceeding the range or the pH value being too low, disulfides that are sensitive to oxidation-reduction reactions and Schiff bonds that are unstable in acidic environments are used to create stimuli-responsive polymers for loading DOX prodrugs [9].Chen et al. reported a polymer-DOX conjugate including disulfide bonds that exhibited superior anti-tumor activity compared to free DOX and analogous conjugates without disulfide linkages [10].Zhang et al. synthesized D- α- The prodrug of adriamycin, which is sensitive to the oxidation of tocopherols and polyethylene glycol, has a significant inhibitory effect on the growth of multidrug-resistant tumors [11].Another very important point is that for drug delivery systems, DLC and drug release kinetics have extraordinary significance. For example, higher DLC allows us to use less carrier materials

during the preparation stage, which reduces costs and the risk of adverse reactions. Therefore, in order to explore new nano-carriers with high DLC, people have invested a lot of time, money, and effort.The kinetics of drug release is inextricably linked to two factors: first, the concentration of the drug after it reaches the cell; The second is the retention time of drugs in the body.

In this experiment, we conceived and successfully developed a pH-sensitive amphiphilic polymer drug conjugate, polyethylene glycol-Schiff base doxorubicin (PSD), which can independently form unstable micelle nanoparticles in acidic environments without the help of external factors.In neutral environments, these nanoparticles are protected by the low critical aggregation concentration (CAC) of PSD and the external PEG corona, so they can have very good stability. However, once in the acidic environment of the endosome/lysosome compartment within the nucleus, the pH-triggered exposure and lysis of the Schiff base leads to the disintegration of the nanostructure, resulting in the rapid and complete release of DOX encapsulated in the nanomaterial into tumor cells, ultimately achieving our experimental goal (Figure 1).This study evaluated the preparation of PSD prodrugs and adriamycin-loaded nanoparticles, pH-responsive drug release, cellular uptake, and in vitro anti-tumor activity of nanoparticles against HeLa cells.

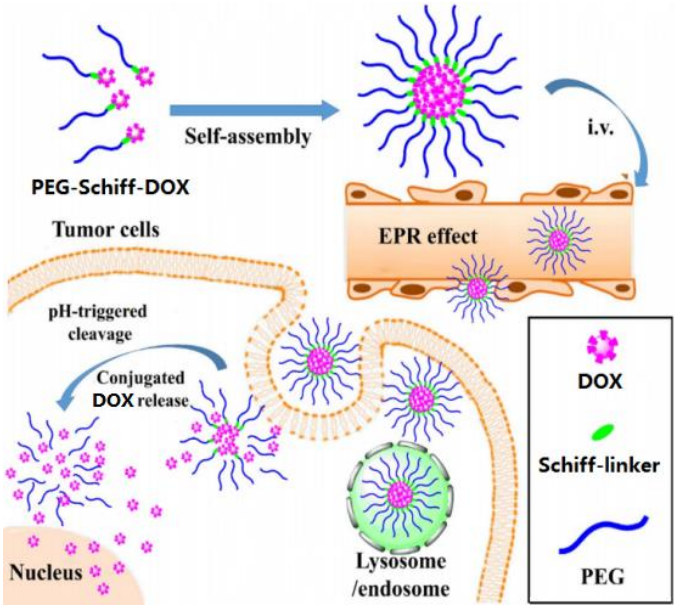

Figure 1. Synthetic fabrication of PSD prodrug and its antitumor therapy.

2. Materials and methods

2.1. Materials

Table 1. The main reagent lists in this study

Name	Abbreviation	Manufacturer	Remark & Purity
Polyethylene glycol methyl ether	PEG-OH	Alfa Aesor	Mn = 750
4-carboxybenzaldehyde	-	J&K	98%
4-(Dimethylamino)-pyridine	DMAP	Aldrich	99%
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride	EDCI	Energy Chemistry	99%
Triethylamine	TEA	Beijing Chemical	99%
Hydrochloric acid	HCl	Beijing Zhongshuo Pharmaceutical Technology.	37%
Sodium sulfate	Na_2SO_4		98%
Sodium bicarbonate	$NaHCO_3$		98%
n-Hexane	-		AR
Ether	-		AR

All other reagents are purchased from Energy Chemistry and used directly without any further purification. All the used cells came from the Chinese cell line resource infrastructure (Table 1).

2.2. Characterization and testing

We can use tetramethylsilane (TMS) as an internal reference factor to analyze the NMR spectrum on a Bruker 400 MHz spectrometer. To obtain transmission electron microscope (TEM) images, we used a JEM microscope for the purpose. Then, a laser light scattering spectrometer equipped with a multi-tau digital time correlation and a 22 mW cylindrical helium-neon single-mode laser were used to obtain dynamic light scattering (DLS) spectra.The laser scattering cells were stored in a large container with a temperature index matching pure, dust-free toluene. When performing fluorescence experiments, we use a xenon lamp as the light source and operate with a Hitachi F4600 photoluminescence spectrometer.All data represent the average of three doses.

2.3. Synthesis of PEG-Schiff-DOX (PSD)

The composition of the pH-sensitive prodrug is shown in Figure 2. 1 mmol of PEG-OH, 1.2 mmol of 4-carboxybenzaldehyde, 1.2 mmol of EDCI, and 0.3 mmol of DMAP were mixed in a 50 mL bottom solution containing a magnetic stirring rod. After adding 10 mL of fresh dichloromethane to dissolve the active solid, the organic phase was collected by a stirring system. We washed the collected organic solution with HCl aqueous solution, carbonate solution, NaCl solution, and deionized water for 3 times, respectively. After washing, the solution was dried over solid Na2SO4. Finally, precipitating the product in ether yields a white powder with a calculated yield of 89.1%.

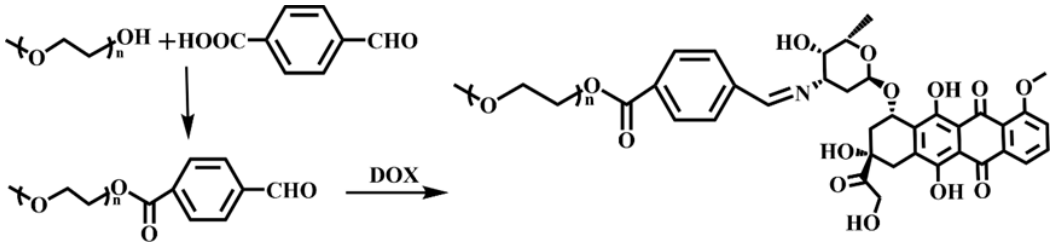

Figure 2. Synthetic approach of the PSD prodrug.

2.4. Synthesis of the PSD polymer

We mixed 0.31 mmol of polyethylene glycol, 0.25 mmol of DOX, and 1 mmol of TEA in 20 mL of DMF solution at room temperature. The system was refluxed under vigorous stirring for half a day, and the product was extracted and dissolved in DCM. After washing several times, the solid was dried with Na_2SO_4. The final product was precipitated in cold ether and yielded a red powder with a yield of 62.4%.

2.5. Stability and pH-responsive degradation of the nanoparticles

We used DLS technology to detect the particle size distribution of nanoparticle solutions before and after storage at room temperature for one week to evaluate whether the nanoparticle solution remained stable.To investigate the pH-responsive degradation behavior of nanoparticles, we re-dispersed the lyophilized powder of nanoparticles in phosphate buffer solution with a pH value of 5.0 and a concentration of 1 mg/mL. In short, we dissolved 5 mg of PSD in DMF solution (1 mL), and then added it to aqueous solution (4 mL) at a slow drop rate. During the self-assembly process, the colloids were kept stirring in the solution for 2 hours. By using a dialysis process with a 2 kDa cutoff value, we removed deionized water from the DMF solution for 5 days. These PSD nanoparticles can be formed and characterized by TEM and DLS.

2.6. pH-triggered polymeric degradation in various pH solutions

We measured the size distribution changes of PSD nanoparticles in PBS solution at pH 7.4 to assess their stability and resistance to adsorption at room temperature. After replacement in different

pH solutions (pH 5.0), the size distribution and changes were recorded using DLS at predetermined times (e.g. 4 h). Similarly, TEM images were used to document the formation and changes in morphology.

2.7. DOX release from the PSD nanoparticles in vitro

pH-induced DOX release: First, we added PEG-Schiff-DOX nanoparticles to dialysis tubes with a molecular weight cutoff of 1 kDa, and oscillated them in 30 mL PBS solutions with pH 5.0 and 7.4 at 37 °C. Then, the dialysis tubes were placed in a water bath. In order to measure the UV/VIS absorbance of the solution, a pH-triggered DOX release curve was obtained at 480 nm. We conducted a total of 3 experiments, and finally calculated the standard deviation of the results as the average value.

2.8. CCK-8 assay

The cells were inoculated into 200 μL of DMEM in a 96-well plate, with 10% FBS and 1×10^5 cells per well, to evaluate the cytotoxic effect of PSD nanoparticles on Hela cells. Ninety microliters of fresh DMEM containing 10% FBS were used to replace the cell culture medium, followed by the addition of different concentrations of surfactant suspension to PBS solution at pH 7.4. After incubating for 24 h, the culture medium was removed from the plate, and immediately fresh medium and CCK-8 reagent were added to the wells and mixed evenly. The plate was then incubated in an incubator for 6 hours. Finally, 100 μL of the solution was taken out and placed in a 96-well plate, and the microplate reader was used to read the optical density at 450 nm for each well.

3. Results and discussion

3.1. Analysis of structure

Figure 3 is the 1H NMR spectrum of PEG-Schiff-DOX. The peak shape of the drug molecule is relatively irregular and short. We take the methyl group at the end of PEG as the starting point for analysis, and the methyl peak basically corresponds to the peak area of the main chain. In particular, the appearance of the c peak at 8.1 ppm indicates the formation of a Schiff base bond, which can prove that PEG-Schiff-DOX can be successfully prepared.

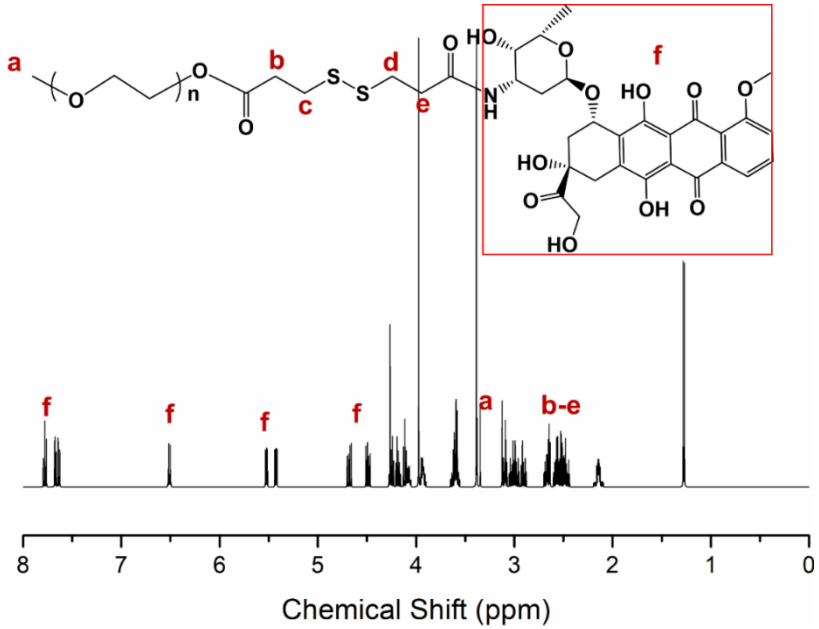

Figure 3. ^{1}H NMR spectrum of PEG-Schiff-DOX

3.2. Nanomedicine Testing

The amphiphilic prodrug PSD can independently (without the help of external factors) assemble into micelles to form nanoparticles.The content of DOX is maximized, and DOX is encapsulated into self-assembled PSD micelles in the nano-particle solution, while the free DOX is wrapped within the micelles. The pH responsiveness of the nanomedicine is given by the results of particle size changes after 24 h of shaking at 37 ºC in pH 5.0 buffer, as shown in Figure 4 and Figure 5. After acid treatment, the morphology and size of nanoparticles undergo significant changes, and some nanoparticles completely disintegrate. The narrow distribution of nanoparticle size indicates that heterogeneous nanoparticles undergo fragmentation and release drugs under the action of acid.

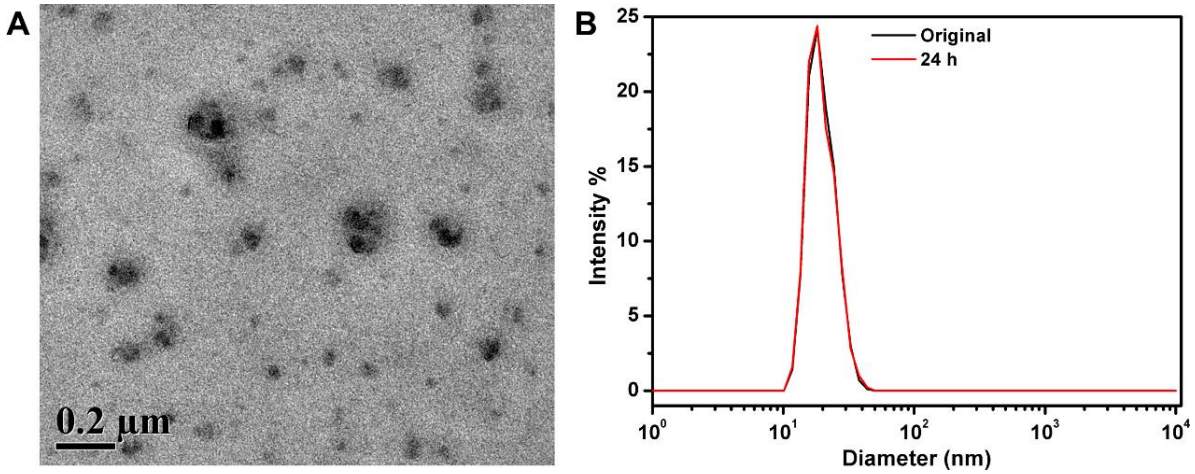

Figure 4. The morphology and size of PEG-Schiff-DOX at pH 7.4 conditions

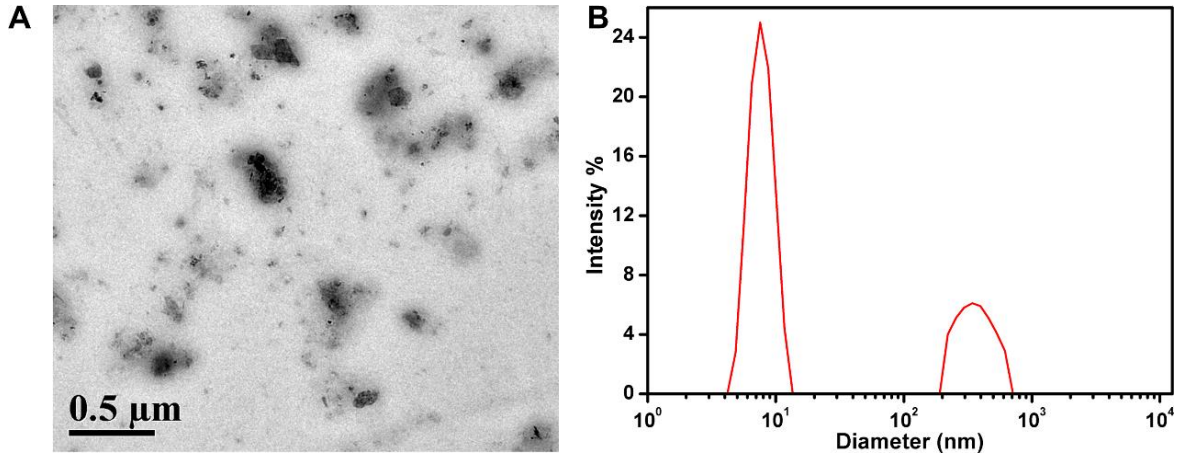

Figure 5. The morphology and size of PEG-Schiff-DOX at pH 5.0 conditions

3.3. Release of drugs

The test results of gel drug-loaded nanoparticles were more intuitive. The results of the ultraviolet spectrophotometer showed that the nanoparticles containing the drug were very stable at pH=7.4, with almost no release of the drug. However, at pH=5.0, this release activity changed significantly, becoming more intense and releasing a significantly higher amount of drug (Figure 6).

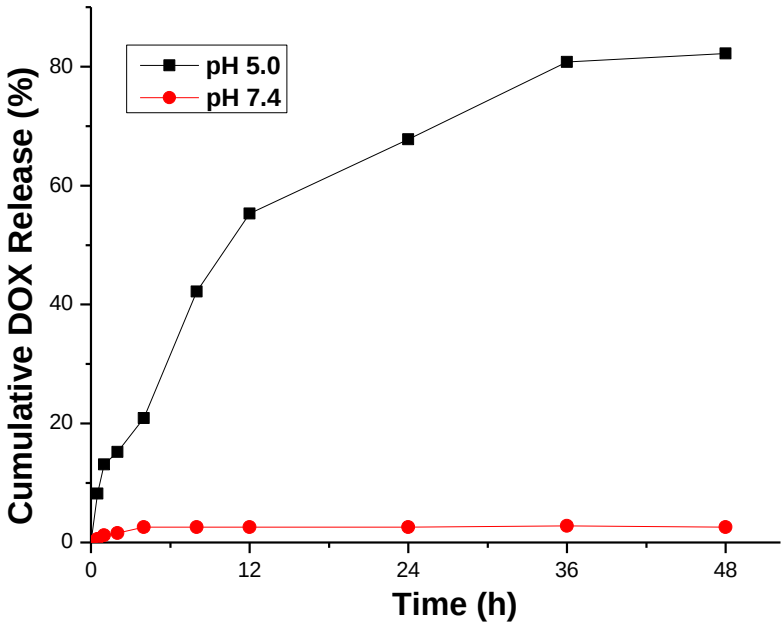

Figure 6. In vitro drug release behavior of PEG-Schiff-DOX at various pH solutions

3.4. Antitumor activity

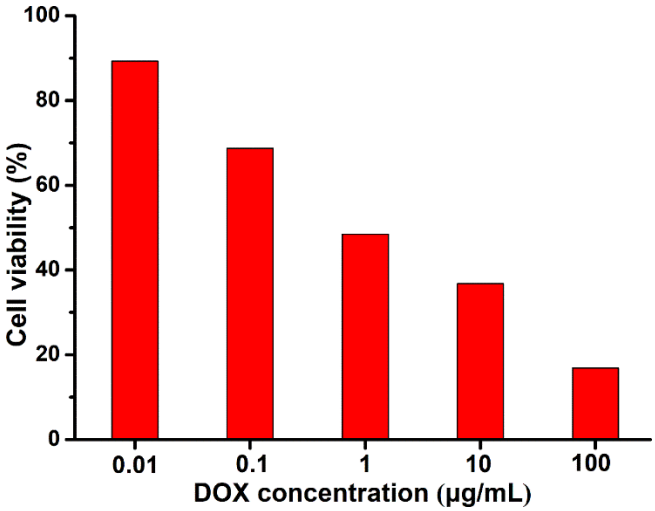

Figure 7. The in vitro antitumor therapy of PEG-Schiff-DOX against the HeLa cells

As can be seen from Figure 7, PEG-Schiff-DOX nanodrugs have excellent anti-tumor effect, especially when the concentration of DOX loaded reaches 100 μg/mL, it shows a very high inhibitory effect on tumor growth, and the cell survival rate is less than 8%. The results indicate that PEG-Schiff-DOX is a promising targeted agent for clinical application. These experimental results further indicate that these nanoparticles can achieve rapid release of drugs in the acidic environment of tumor tissues and the endosome/lysosome compartment within cells, while maintaining stability and reducing leakage during blood circulation in neutral environments.

4. Conclusion

All in all, PEG-Schiff-DOX polymeric pro-drugs were prepared for pH-responsive doxorubicin loading, and their release behavior was tested under different pH conditions. Through a multi-stage release system, it was successfully demonstrated that these drugs could extend their activity duration and provide timely response to acidic environments. These drug-loaded nanoparticles have several advantages: (1) their chemical structure is regular and clear, and the preparation process is easy; (2) they can carry the amount of drugs we want to achieve; (3) their internal structure is very strong under

most conditions; (4) they are very stable under neutral conditions, almost no drugs are released or released very little, and in acidic tumor microenvironment, they release active drugs quickly through rapid hydrolysis to maintain the efficacy of the drug and reduce the damage of the drug to the body; (5) they have high drug concentration in specific sites and long retention time. Therefore, in the research and development of new drug delivery systems, these polymer prodrugs provide us with more options, and also give us a little more hope in treating cancer in the future.

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