

Self-assembly of pH-responsive Prodrugs for Effective Antitumor Therapy

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Abstract. A novel class of endosomal pH-responsive micellar nanoparticles was created by employing the self-assembly of an amphiphilic poly(ethylene glycol)-Schiff-doxorubicin (PEG-Schiff-DOX) drug. Under normal circumstances, these nanoparticles had excellent storage durability for at least 7 days, but they immediately disintegrated in a mildly acidic environment. Furthermore, a planned drug release behavior was seen, taking advantage of the different drug release mechanisms, which could lead to a higher intracellular drug concentration and longer action duration. The nanoparticles outperformed free DOX in anticancer efficacy against Hela cells, according to CCK-8 assays. Therefore, these prodrug-based nanomedicines hold significant promise for developing translational DOX tumor therapy formulations.

Keywords: doxorubicin; pH-responsive; prodrug; polymeric nanoparticles.

1. Introduction

Polymer-based nanomedicines have become popular with extensive research recently because of their potential to boost the solubility of the drug, improve curing efficacy, and lessen adverse effects in cancer therapy [1-8]. Nanomedicines have many advantages over small molecular anticancer medications, including increased tumor accumulation through the increased permeability and retention effect, lengthened circulation time by refraining from glomerular filtration, and improved pharmacokinetic features [9-14]. Prodrug-based nanoparticles have garnered greater attention than other types of nanomedicines, including polymeric nanoparticles, prodrugs, and liposomes, due to their structure and great clinical translation potential [15-23].

Doxorubicin (DOX), an anthracycline-based chemotherapeutic drug derived from a strain of *Streptomyces*, demonstrates considerable efficiency against several malignancies, including bladder and breast cancer [24]. Due to its excellent water solubility (around 10 mg/ml) [25], it is commonly given intravenously as a bolus injection after aqueous reconstitution in traditional clinical use.

In addition to nausea, side effects at a total dose of 550 mg/m² include hair loss and more frequent heart damage. In early medication trials, heart damage, which can be caused by oxidative stress, was a substantial cause of morbidity and mortality [26].

Therefore, creating new DOX formulations that will target the tumor cells and produce fewer side effects is desirable. The observed PEG-DOX prodrugs do, however, have certain intrinsic disadvantages, including the low drug loading capacity and insufficient drug release [27-29]. The bonds that connect drugs and polymers should be cleaved when the prodrugs have entered the cancer cell environment so that DOX can be released rapidly in the cancer cells. To create stimuli-responsive polymer-DOX prodrugs, redox-sensitive disulfide and acid-labile Schiff linkages were used since tumor cells contain aberrant environment, including a higher glutathione concentration and lower pH [30-34].

This study uses poly (ethylene glycol)-Schiff-doxorubicin (PEG-Schiff-DOX), an amphiphilic polymer-drug conjugate capable of self-assembly into acid-labile micellar nanoparticles, was developed and produced. As a result of the outer PEG corona's protection, these nanoparticles had good storage stability. In the acidic environment of the tumor cells, Schiff bonds were cleaved, and the conjugated DOX was released completely. Investigations were conducted on the DOX-loaded nanoparticle synthesis, drug release response to pH, and in vitro anticancer efficacy against HeLa cancer cells (Fig.1).

2. Materials and Methods

Poly (ethylene glycol) methyl ether (PEG-OH, Alfa Aesor), carboxybenzaldehyde, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 4-(dimethyl-amino)-pyridine, 1,4-dithiothreitol, hydrochloric acid, anhydrous magnesium sulfate, and ether were bought from Beijing Chemical Works.

On a Bruker 400 MHz spectrometer, nuclear magnetic resonance spectra were captured using deuterated chloroform as the solvent. Transmission electron microscopy images were acquired on a JEM-2200FS microscope. Using a Malvern Zetasizer Nano, dynamic light scattering (DLS) measurements were done.

Synthesis of PEG-CHO: PEG-OH (1 eq), EDCI (3 eq), carboxybenzaldehyde (3 eq), and DMAP (4-Dimethylaminopyridine) (0.2 eq) were dissolved in the THF (Tetrahydrofuran) solution. After continual stirring at normal condition, the PEG-CHO polymer was purified by extraction and precipitation.

Synthesis of the PEG-Schiff-DOX polymer: Under a nitrogen environment, PEG-CHO (1 eq), DOX (1 eq), and TEA (Triethanolamine) (1 eq) were dissolved in freshly dried DMF (Dimethylformamide). After continual stirring at normal condition, PEG-Schiff-DOX was produced after the products were purified using extraction and centrifugal precipitation.

Self-assembly of the polymeric nanoparticles: PEG-Schiff-DOX was dissolved in DMF, and water was added drop by drop using a syringe pump at 0.05 mL per minute. To produce nanoparticles, the colloidal dispersion was stirred for an additional eight hours and then filtered by dialysis.

Stability and pH-responsive degradation: To determine the pH-sensitive destabilization of nanoparticles, in a shaking water bath, a specific quantity of phosphate-buffered saline with pH 7.4 and pH 5.0 was introduced to polymeric nanoparticles. TEM and DLS measurements documented morphological and size changes after many treatments.

In vitro drug release: A dialysis membrane tube (MW cut-off, 1 kDa) was filled with dispersed PEG-Schiff-DOX nanoparticles and then incubated at 37°C in 30 mL of PBS with pH 7.4 and pH 5.0 solutions. UV-vis at 480 nm was used to assess pH-triggered DOX release patterns.

Activity analyses: The cytotoxicity of polymeric micelles was investigated in vitro using the CCK-8. 1×10^4 HELA cells were planted into each well of a 96-well plate, and different doses of micelle suspensions were treated with the cells for 24 hours. We used cells grown in a DMEM medium containing 10% FBS, which has no exposure to micelles, as controls.

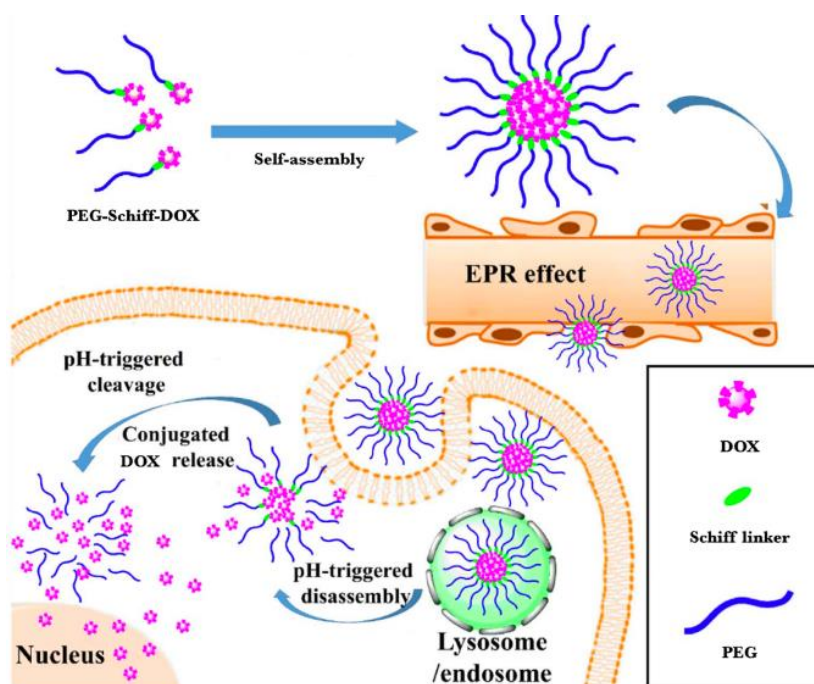


Figure 1. Schematic illustration of formation and delivery of the DOX-loaded micelles

3. Results and Discussion

3.1. Synthesis of PEG-Schiff-DOX Prodrugs

The synthesis process used for the PEG-Schiff-DOX prodrug was depicted in Fig.2. By observing the ^1H NMR spectrum (Fig. 3), the structures of PEG and DOX were shown clearly in structures 1 and 2. Then the formation of the prodrug was verified by the ^1H NMR spectrum. In Figure 3, new peaks at $\delta=8.1$ ppm, representing Schiff groups, were observed in C, demonstrating that the Schiff bonds had formed. Therefore, it confirmed the syncretization of the PEG-Schiff-DOX prodrug.

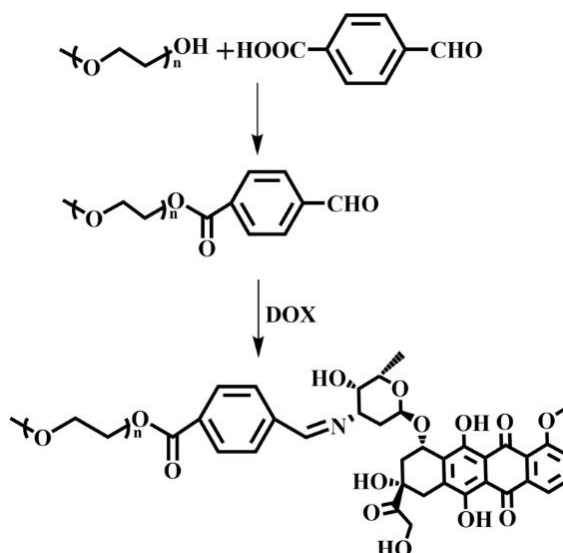


Figure 2. Synthetic route of the PEG-Schiff-DOX prodrug

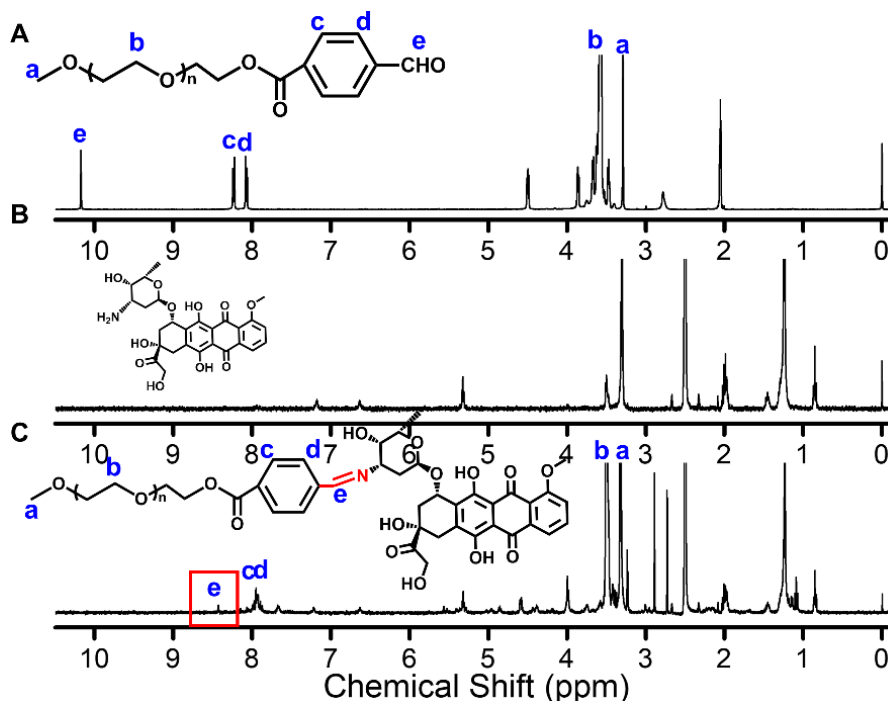


Figure 3. ^1H NMR spectra of (A) PEG-CHO, (B) DOX, and (C) Prodrug

3.2. Nanoparticle Stability and pH-responsive Drug Release

The water-soluble amphiphilic prodrug could form micellar nanoparticles by itself. For drug formulations, storage stability is crucial. The TEM pictures (Fig. 4A) showed big aggregates and small pieces, further demonstrating the pH-responsive breakdown of the nanoparticles. By comparing the size distributions of these nanoparticles before and after 24 hours at room temperature, DLS was used

to examine the stability of these nanoparticles during storage. As shown in Fig. 4B, the first image showed the original size of the nanoparticles and the size after storing them at room temperature for 24 hours. The figure showed that the size of the particles had minimal alterations, proving they had exceptional long-term storage stability.

The hydrophilic DOX and hydrophilic PEG were linked by a pH-labile Schiff linker, which leaves the nanoparticles vulnerable to disassembling in acid conditions. We used DLS to track the breakdown characteristics of these nanoparticles in the solutions with a pH of 5.0. In Fig. 4B, the second image showed the size of the nanoparticles when they were put in an environment of pH 5.0 for 4 h. Different from the original image, we can see small molecules were formed, which shows that the nanoparticles had disassembled.

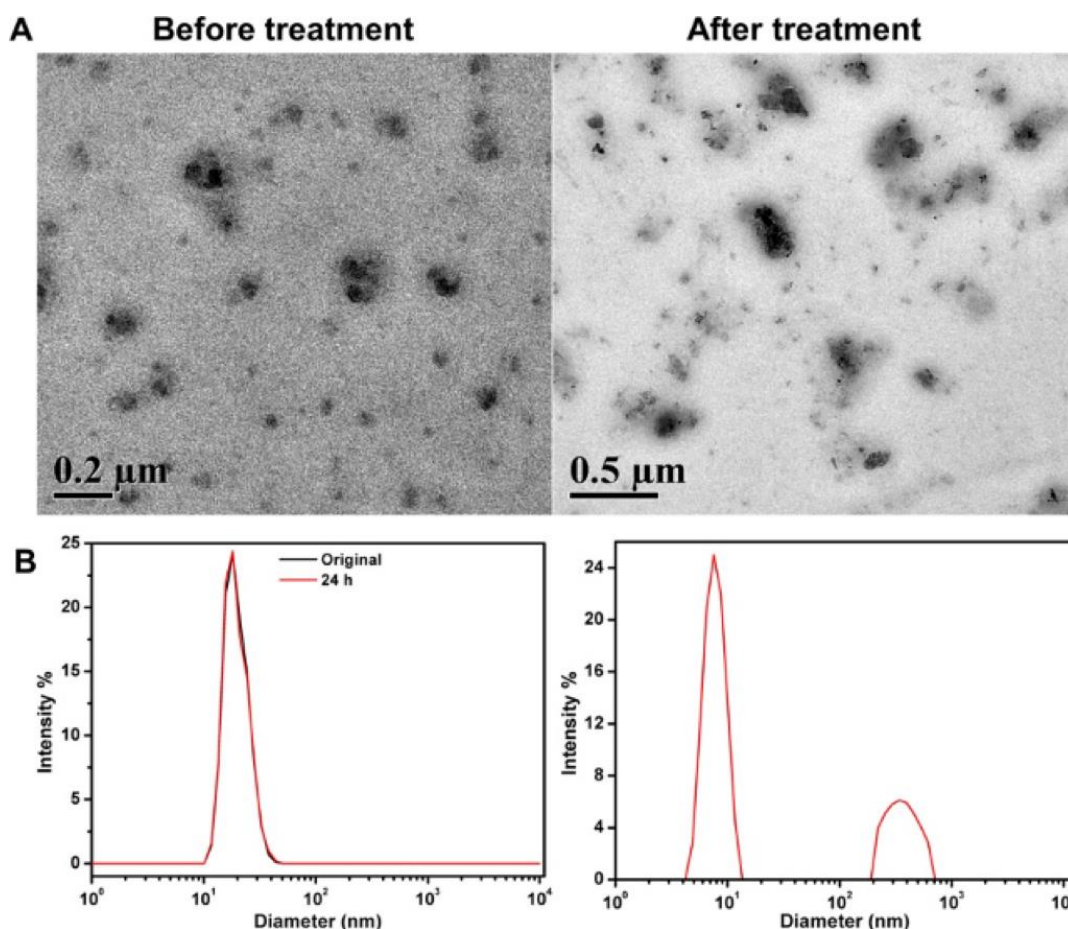


Figure 4. (A) TEM images of the nanoparticles before and after treatment (B) Stability of the nanoparticles at standard conditions and pH = 5.0 measured by DLS.

3.3. Drug Release of the Nanoparticles

Using Schiff bond that is cleavable in the mildly acidic environment in the tumor cells, DOX was being attached to PEG and DOX can be released rapidly. At 37 $^{\circ}\text{C}$, we conducted in vitro drug release tests in neutral buffers with a pH of 7.4 and acidic buffers with a pH of 5.0. Fig. 5 shows the drug release profiles. At pH = 7.4, about 11.5% of DOX was released from the prodrug after 24 hours, and the amount of the released DOX barely changed from 24 hours to 48 hours. In comparison, at 10 mM DTT solutions, nearly all of the DOX was released while the total release of DOX was considerably increased, which were 79.5%, 85.4%, and 91.3% at 12, 24, and 48 hours, respectively. The findings suggested that while these nanoparticles will achieve rapid drug release in tumor tissues' pH environment, they would limit DOX going into blood, which has a neutral pH.

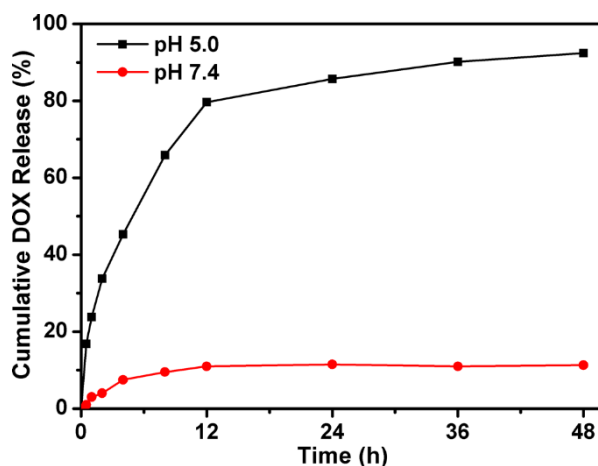


Figure 5. Drug release profile at pH = 7.4 and pH = 5.0

3.4. Cytotoxicity of the Nanoparticles

The nanoparticles' cytotoxicity was assessed against HeLa cervical cancer cells. Fig. 6 displayed the cell viability at various DOX concentrations, which include 0.01, 0.1, 1, 10, and 100 $\mu\text{g/mL}$. The cell viability decreased to less than 20% at DOX concentrations of 10 $\mu\text{g/mL}$ and above, indicating that the prodrug had a relatively high antitumor effect on HeLa cells.

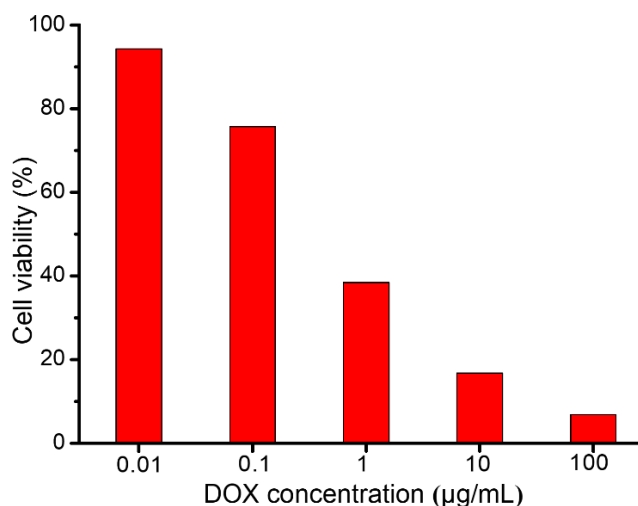


Figure 6. Cytotoxicity of PEG-Schiff-DOX prodrug against HeLa cells after 48 h treatment

4. Conclusion

In conclusion, we designed and synthesized an amphiphilic PEG-Schiff-DOX prodrug and self-assembled the prodrug and free DOX into pH-responsive micellar nanoparticles. The nanoparticles have unique and well-defined shapes, and the prodrugs may have high drug concentrations and outstanding loading capacity. The endosomal pH-triggered release of DOX preserves its substantial therapeutic efficacy, while the prodrug's limited drug leakage in neutral circulation helps decrease side effects. In addition, they suppress tumors more effectively than free DOX. These nanoparticles derived from prodrugs offer a viable option to create innovative DOX formulations.

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