Schiff-Linked Pegylated Doxorubicin Prodrugs Forming Ph-Responsive Nanoparticles with High Drug Loading Capacity and Improved Drug Consignment

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Abstract. A new pH-responsive micellar nanopolymers was manufactured through self-assembly of PEG-Schiff-DOX prodrug in aqueous solutions. These nanoparticles show good biocompatibility and storage fixity for more than one week under normal conditions, and the advantage of rapid degradation under weak acid conditions. Under this circumstance, the in vitro experiments show that compared with the dissociative DOX drug, the PEG-Schiff-DOX nanoparticles exhibit the lower cytotoxicity and higher anti-tumor activity against the Hela tumor cells, indicating its great potential in various nanomedicine fields.

Keywords: Doxorubicin, nanopolymers, pH-responsive, anticancer drugs.

1. Instruction

In recent years, with increasing level of science and technology, the research on cancer treatment methods is more and more in-depth. Among them, nanomedicines based on polymers have gradually become an important means of cancer therapy. Compared with ordinary small molecular drugs which can fight against cancer, nanomedicines have many advantages in treatment. Due to the polymerization of nanomedicines can change the original properties of drugs, thus changing their solubility and fixity. Besides that, they can not only reduce the loss in drug circulation and transportation to improve the curative effect, but also can reduce the biotoxicity and by-effect of drugs on human body [1-4]. Above all, they can better adapt to the enhanced permeation and retention (EPR) effect [5]. Because of the well-defined and concise construction and great potential in medical mutation, the variety of nanomedicines have caused widespread concern. For example, nanoparticle polymers, prodrugs, micelles, vesicles and bangosome [6-10].

Doxorubicin (DOX), the types of tumors that can be resisted by it are very wide and diverse and it is suitable for acute leukemia (lymphocytic and granulocytic), malignant lymphoma, breast cancer, bronchogenic lung cancer (undifferentiated small cell and non-small cell), osteosarcoma, rhabdomyosarcoma, Ewing sarcoma, neuroblastoma, thyroid cancer, prostate cancer, testicular cancer, gastric cancer, liver cancer, etc. Even if it can deal with a wide range of cancer types, it has some shortcomings. Due to its very strong hydrophilicity, the drug is uncontrollable and easy to penetrate rapidly, resulting in non-selective side effects, such as cardiomyopathy including infarction, nausea and vomiting, hair loss etc. [11-14]. Therefore, researching and developing the new dosage forms of DOX with strengthened selective biotoxicity and less by-effects is very important.

DOX is too hydrophilic and so small in size to target tumor cells, which will cause great toxicity. Therefore, it is necessary to change the size of DOX through chemical conjugation to match the size required by the EPR effect (80-200 nm). Based on this, there are many studies on PEG-DOX prodrugs, but it is reported that there are still many inherent defects, including the low rate of drug loading and uncontrolled release of drugs. If the drug is to be released successfully, it is necessary to pay attention to the dynamic linkers between the drug and the polymer. Using the differences between the environment of tumor cells and that of normal cells, the linkers can be cut in tumor cells. For example, the pH of tumor cells is lower than that of normal cells, the concentration of metal ions in tumor cells is different from that of normal cells, and the concentration of glutathione in tumor cells is higher. These conditions can be used as the reason why the linkers can be cut off when they sense

environmental changes. Schiff bonds with Oxidation-reduction sensitivity and acid infixity have been widely applied to assemble into the irritant-responsive polymer-DOX prodrugs.

During this research, we devised and conflated an amphiphilic polymer, PEG-Schiff-doxorubicin (PEG-Schiff-DOX), which could assemble into hydrophilic nanoparticles. The resulted nanopolymers could be used as drug load bearing to carry dissociative DOX. As shown in Fig. 1, nanopolymers first penetrate into tumor tissue through EPR effect, then close to tumor cells, enter tumor cells through endocytosis, and then disintegrate due to pH difference. For tumor cells, the environment of the intracellular endosomal/lysosomal compartments is acidic, and the Schiff bonds keep steady in neutral environment, but disintegrate rapidly in acidic environment, so as to achieve the accurate delivery and targeted release of drugs. The targeted delivery and efficient release of this pH-responsive drugs have great research prospects for fighting against tumor cells such as Hela cells.

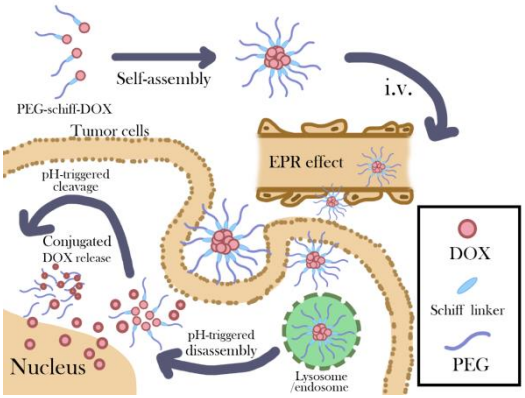

Figure 1. Schematic diagram of nanoparticles delivery

2. Materials and methods

2.1. Raw materials and reagents

Doxorubicin hydrochloride, anhydrous N, N-dimethylformamide (DMF), P-carboxybenzaldehyde, 4-dimethylaminopyridine and dimethyl sulfoxide (DMSO) are directly bought from Energy Chemical. Then water for experiment is ultra-pure water, and other chemical agentias are bought from Beijing Chemical Plant and directly used.

2.2. Characterization and testing

^{1}H NMR: Avance 400 (400 MHz) spectrometers. The deuterated reagents are deuterated acetone (Actone-D_6), deuterated dimethyl sulfoxide (DMSO-D_6), deuterated chloroform ($CDCl_3$), TMS is the internal standard, measured at 25 ^{o}C.

Transmission electron microscope (TEM) is tested on JEM-2200FS (JEOL, Japan) electron microscope, and the acceleration voltage is 100 kV. three μ L sample is dripped onto the copper mesh (300 mesh) covered with carbon diaphragm, the redundant liquid is removed with filter paper, and then it is left to dry naturally and then observed. The electron microscope pictures were recorded with Gatan multiscan CCD and processed with Digital Micrograph.deuterated dimethyl sulfoxide (DMSO-D_6)

Dynamic light scattering (DLS): Malvern Zetasizer Nano ZS dynamic light scattering particle size analyzer. It is outfitted with a 633 nm He-Ne optical maser with a measure angle of 173 °. The specimen cell for granularity dimensions test is a quartz cuvette.

UV-Vis: Shimadzu TU1901 UV-Vis spectrophotometer.

2.3. The Way to Synthesize pH-responsive prodrug

The synthesis of pH-responsive prodrug PEG-Schiff-DOX is carried out in two steps, as shown in Fig. 2.

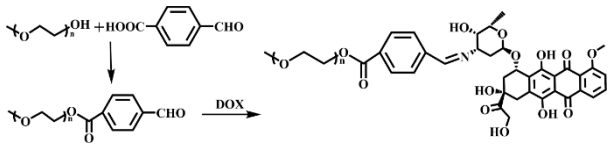

Figure 2. Synthetic route of the PEG-Schiff-DOX prodrug

2.4. The Way to Synthesize PEG-CHO

Dissolve p-carboxybenzaldehyde (300 mg, 2 mmol), EDCI (383 mg, 2 mol) and DMAP (122 mg, 1 mmol) in ultra-dry DCM, and then add PEG-OH (750 mg, 1 mol) under the protection of nitrogen filling and air isolation. At 37 ℃, the whole scheme was agitated for 24 hours, then washed with 1M HCl, and soaked three times with impregnate NaHCO3 solution and impregnate brine. Collect the organic solvent, dry it with anhydrous magnesium sulfate, filter it, and spin it for drying. The final yield is about 86%.

2.5. The Way to Synthesize PEG-Schiff-DOX

Doxorubicin (50 mg, 90 μmol), PEG-CHO (100 mg，110 μmol), TEA (70 μL，500 μmol) and PEG-CHO (100 mg，110 μmol) dissolve in 3 mL of waterless DMF and shake the reaction overnight. After the reaction solvent is eliminated by gyrate evaporation, use a large amount of DCM to made it dissolve, purified with impregnate brine for three times, and then made it form the precipitation in cold ether. The final yield was about 78%.

2.6. Study on fixity and pH-response of nano-pharmaceuticals

Dissolve the nano drug (0.5 mg/mL) in PBS buffer solution with pH=7.4, measure its particle size distribution by dynamic light scattering, and then test again after two weeks to compare the changes.

Use PBS buffer solution with pH=5.0 to dissolve the nano drug (0.5 mg/mL), place it in a invariant temperature water bath at 37 ℃ for 2 hours, and then measure its fleck size distribution by dynamic light scattering.

3. Experiment Result and Analysis

3.1. Characterization and analysis of synthetic results

The ^{1}H NMR spectrum of PEG-CHO is shown in illustration (Fig. 3). From the analysis of the proportion of the synthesized substances, it can be seen that the methyl spike at the end of PEG can correspond to the analysis of the proportion of benzene ring hydrogen in PEG-CHO, and the successful synthesis of PEG-CHO can be inferred. The ^{1}H NMR spectrum of PEG-Schiff-DOX shows that the spike appearance of the drug molecule is relatively unordered and short. Taking the methyl at the end of PEG as the starting point of analysis, it is found that the methyl spike basically corresponds to the spike area of the main chain. At the same time, it is showed that the spike in the low area region comes from a total of 8 H on the benzene ring and the Schiff bonds, and the area after integration basically corresponds, indicating the successful preparation of PEG-Schiff-DOX. Furthermore, the new spike due to the generating of Schiff group was observed at 8.1 ppm, which was confirmed by the formation of the Schiff linkers.

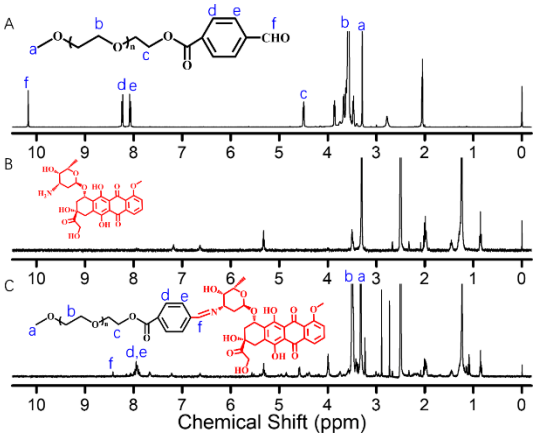

Figure 3. [1]H NMR spectra of PEG-CHO, DOX and PEG-Schiff-DOX polymers

3.2. Fixity and pH-responsive disintegrate of the nanopolymers

The storage fixity of medicamentous preparations is very significant. In order to explore the fixity of these nanopolymers in the storage process, they were stored at room temperature for one week after measuring the particle size, and a comparative test was carried out. PEG-Schiff-DOX can assemble spherical micelles with the size below 200 nm in pH=7.4 aqueous solution (Fig. 4A). From the figure, it can be seen that their morphology and size have no obvious change. When the nanopolymers are placed in pH 5.0 aqueous solution, the morphology and construction of the nanoparticles are significantly damaged, showing an shapeless construction. The pH-response of nano-drugs is given by the particle size change results after shock at 37 ℃ for two hours in a buffer solution with pH=5.0.

Fig. 4B shows that the fleck dimensions of PEG-Schiff-DOX nanoparticles has changed significantly before and after acid treatment, and some nanopolymers have been completely disintegrated. Similarly, the narrow fleck dimensions also confirms that the uneven nanopolymers disintegrate under the action of acid and deliver drugs.

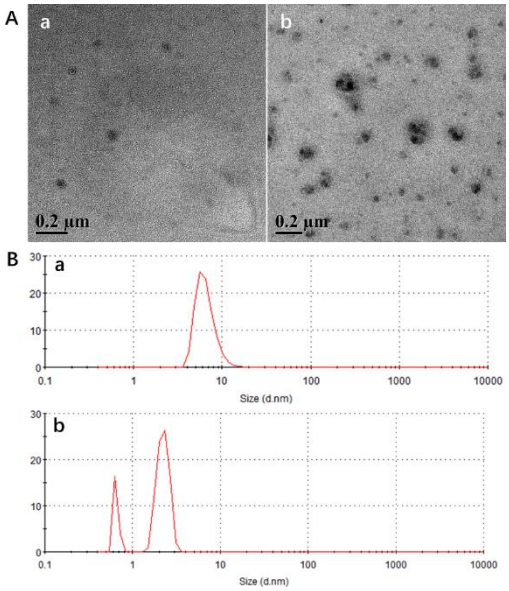

Figure 4. (A) Morphology and (B) Fleck dimensions of PEG-Schiff-DOX nanopolymers at (a) pH=7.4 and (b) pH=5.0

3.3. In vitro release of the nanopolymers-drug

In PEG-Schiff-DOX prodrug, DOX and PEG are only connected by an unstable Schiff bond, which will be responsively disrupted in the lower-pH environment of tumor tissue or cells. The test results of drugs release action are more intuitive, which can be seen from the UV test results. Showing

in illustration (Fig. 5), PEG-Schiff-DOX nanoparticles loaded with PEG-Schiff-DOX hardly release drugs at pH=7.4, while the action of release is significantly strengthened at pH=5.0. This is mainly because the Schiff base bond can remain stable in neutral environment and maintain the integrity of micelle morphology, but it can break in acidic environment and cause rapid drug release.

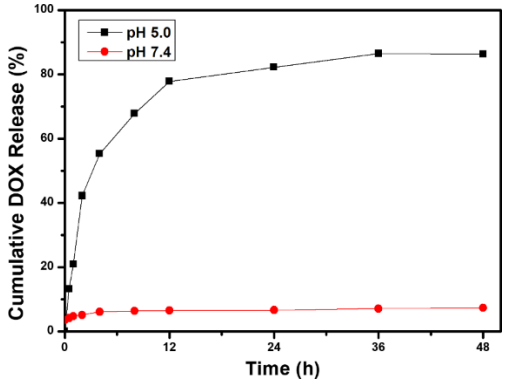

Figure 5. Release in vitro curve of PEG-Schiff-DOX nanodrug

3.4. In vitro cytotoxicity of the nanopolymers-drug

The toxicity of the drug to Hela cells increases with the increase of the concentration of DOX loaded with nanoparticles. From Fig. 6, it can be seen that PEG-Schiff-DOX nanoparticles have excellent anti-tumor effect, especially when the concentration of DOX loaded reaches 100 μg/mL, PEG-Schiff-DOX showed a very high inhibitory effect on tumor growth, and the cell survival rate was less than 10%, indicating that the prepared PEG-Schiff-DOX nanoparticle was a promising target agent of tumors for the clinical application.

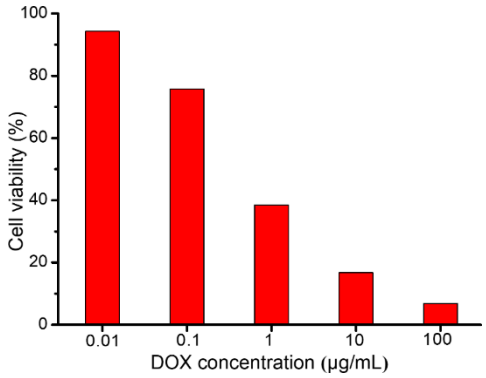

Figure 6. Evaluation of anti-tumor effect of PEG-Schiff-DOX nanodrug

4. Conclusion

All in all, we devised and conflated an amphiphilic PEG-Schiff-DOX prodrug, and obtained a new pH-responsive micellar nanopolymers through self-fabrication of this prodrug and dissociative DOX. In particular, these nanoparticles based on previous drugs have the following advantages: 1) they have clear and concise construction and their preparation process is relatively simple; 2) It has high drug concentration and great advantages in drug loading; 3) It has good fixity during storage (it can be stored for more than one week at room temperature) 4) When circulating in a neutral environment in the body, the amount of drug leakage and the amount of pH-triggered DOX release can be ignored, which greatly improves the therapeutic activity of DOX and reduces the toxicity and side effects of drugs on normal cells; 5) For tumor inhibition, the inhibition effect of this micelle is better than that of dissociative DOX. It can be seen that these prodrug based on the nanopolymers provide a very important idea for the development of new DOX dosage forms.

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