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Fecile Preparation of pH-responsive PEGylated Prodrugs for Intracellular Drug Delivery

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Abstract

A new class of acid-sensitive doxorubicin prodrug nanoparticles (PEG-Schiff-DOX nanoparticles) were synthesized and characterized. Acid-sensitive PEG-Schiff-DOX polymer was synthesized by Schiff base reaction, and PEG-Schiff-DOX nanoparticles were prepared by self-assembly. The polymer structure was characterized by dynamic light scattering (DLS) and nuclear magnetism. The microscopic morphology of the nanoparticles was observed by transmission electron microscopy (TEM). The release behavior of PEG-Schiff-DOX nanoparticles under acidic conditions was detected by HPLC sampling analysis. Cck-8 assay was used to determine the in vitro cytotoxicity of nanoparticles on breast cancer cells (McF-7). The results show that PEG-Schiff-DOX polymers can self-assemble into nanoparticles with diameters of about 20nm. PEG-Schiff-DOX nanoparticles can release DOX rapidly under acidic conditions. Adriamycin prodrug has ideal antitumor efficacy, and compared with small molecule adriamycin, it has important advantages such as improved pharmacokinetic characteristics in vivo, significantly reduced toxicity and side effects, and greatly improved safety. It is concluded that PEG-Schiff-DOX nanoparticles has a good anti-tumor effect in vitro. This study has laid an important theoretical foundation for the development of novel doxorubicin nanoprodrug with clinical application prospect. The synthesized PEG-Schiff-DOX nanoparticles has many advantages and It is worth further study.

Keywords

Pegylated Prodrugs; Intracellular Drug; PEG-Schiff-DOX Nanoparticles.

1. Introduction

Malignant tumor is now one of the most important diseases harmful to human health, and the incidence is also increasing year by year. Chemotherapy is a treatment for a variety of cancers, using anticancer drugs to stop or slow the growth of cancer cells. Traditional antineoplastic drugs have some disadvantages such as short half-life and lack of selectivity. while treating tumors, they also cause great side effects on normal tissues, thus limiting the clinical therapeutic effect of antineoplastic drugs. In the treatment of cancer in recent years, nano-drugs have been widely studied in cancer treatment. they have small particle size and narrow distribution, which can increase the water solubility of hydrophobic drugs and prolong the circulation of drugs in the blood. and inhibit or eliminate rapid renal excretion and other advantages. In addition, nano-carriers can also improve

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pharmacokinetic properties and enhance tumor accumulation by enhancing osmotic and retention (EPR) effects. So far, many types of drug carriers have been developed, including polymer nanoparticles, magnetic nanoparticles, dendrimers, liposomes, polymer micelles and so on.[1-7] Among them, prodrug nanoparticles have high drug loading and relatively low side effects. Pre-drug-based nano-drugs have great potential in the development of cancer treatment.

Doxorubicin (DOX) is an anti-tumor antibiotic, which can inhibit the synthesis of RNA and DNA. It has the strongest inhibitory effect on RNA and a wide anti-tumor spectrum. It has an effect on many kinds of tumors. It belongs to a cycle-nonspecific drug and has killing effect on tumor cells of various growth cycles. Its mechanism is mainly due to its intercalation of DNA and inhibition of nucleic acid synthesis. Doxorubicin can produce a wide range of biochemical effects on the body, with strong cytotoxic effects, such as myelosuppression, heart/liver dysfunction and gastrointestinal reactions. All these have seriously affected its clinical application. [1, 2, 4, 8, 9]Therefore, it is necessary to reduce the adverse reactions of doxorubicin and improve the therapeutic efficiency by combining with drug carriers.

The enhanced permeability and retention effect (EPR effect). It refers to the nature that molecules or particles of certain sizes tend to aggregate in tumor tissue compared to normal tissue. The microvascular endothelial space in normal tissue is dense and intact, and macromolecules and lipid particles are not easy to pass through the blood vessel wall, [2]while in solid tumor tissue, the blood vessels are rich, the vascular wall space is wide, the structural integrity is poor, and the lymphatic reflux is absent. As a result, macromolecules and lipid particles have selective high permeability and retention.[3, 7, 10] The EPR effect promotes the selective distribution of macromolecules in tumor tissues, which can increase the efficacy and reduce the systemic side effects.

In this study, PEG-Schiff-DOX (PSD), a hydrophilic polymer drug conjugated with PSD, was designed and synthesized, which can self-assemble into acid-responsive micellar nanoparticles. Benefitting from chemical conjugation, the nanoparticles have high concentration of DLC and DOX, and show programmed drug release behavior. As illustrated in Fig. 1, nanoparticles entered tumor tissue from blood vessels through EPR effect and were internalized by tumor cells via endocytosis. Then, in the acidic environment of the intracellular endosome/lysosome compartment, pH-triggered cleavage of the Schiff bond led to disintegration of the nanoparticles and rapid release of the encapsulated DOX. In this study, the preparation of PSD prodrug and DOX loaded nanoparticles, pH responsive drug release, cellular uptake and MCF7 cancer cell activity in vitro were investigated.[5, 9].

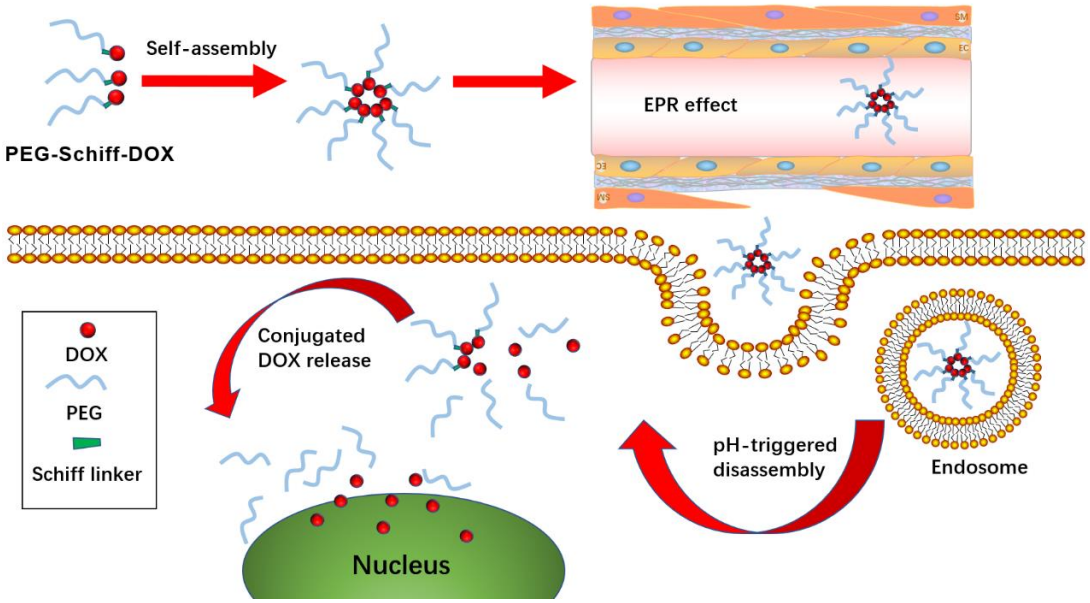

Fig.1 Schematic illustration of formation and delivery of the DOX-loaded micelles.

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2. Materials and Methods

2.1 Materials

4-carboxybenzaldehyde, 4-dimethylaminopyridine, doxorubicin hydrochloride, anhydrous N-dimethylformamide (DMF) and anhydrous dimethyl sulfoxide (DMSO) were purchased directly from Energy Chemical. The water used in the experiment is ultra-pure water, and other chemical reagents are purchased from Beijing Chemical Plant and used directly.

2.2 Characterizations

Nuclear magnetic resonance (1HNMR): Avancehocky400 (400MHz) spectrometer. The deuterated reagents are deuterated chloroform (CDCl3), deuterated acetone (Actone-D6) and deuterated dimethyl sulfoxide (DMSO-D6). TMS is the internal standard and 25oC is used. Transmission electron microscope (TEM) is tested on JEM-2200FS (JEOL, Japan) electron microscope, and the acceleration voltage is 100kV. The 3 μ L sample was dripped on the copper mesh (300 mesh) covered with carbon film, and the excess liquid was absorbed by filter paper. The electron microscope photos were recorded by Gatan multiscan CCD and processed by Digital Micrograph. Dynamic light scattering (DLS): Malvern Zetasizer Nano ZS dynamic light scattering particle size analyzer. Equipped with 633nm he-ne laser, the detection angle is 173 °, and the particle size test sample pool is a quartz colorimetric dish. UV-vis spectrum (UV-Vis): Shimadzu TU1901 UV-vis spectrophotometer.

2.3 Synthesis of the PEG-CHO Polymer

PEG-OH (7.5 g, 10 mmol), 4-carboxybenzaldehyde (1.8 g, 12 mmol), EDCI (2.4 g, 12 mmol) and DMAP (250 mg, 2 mmol) were added to a 150 mL round-bottom flask fitted with a magnetic stirring bar. Then add 50 mL of fresh dried DCM to completely dissolve all solids. After stirring at room temperature for 24 h, the organic phase was collected and washed several times with HCl aqueous solution, saturated sodium bicarbonate solution, saturated NaCl aqueous solution and deionized water, and dried on anhydrous MgSO4. The final product was precipitated into ether/hexane for three times to afford the white powder with 82.2% yields.

2.4 Synthesis of the PEG-Schiff-DOX Polymer

PEG-CHO (2.7 g, 2.1 mmol), DOX·HCl (1.5 g, 2.5 mmol) and TEA (700 μL, 5 mmol) were dissolved in 100 mL of DMF solution under a nitrogen atmosphere. The mixture was refluxed with vigorous stirring for 12 h. After removing the solvent under the vacuum, the crude products were resolved in DCM and washed with saturated NaCl aqueous solution and DI water for several times and dried over anhydrous MgSO4. The final product was precipitated into ether/hexane for three times to afford the dark-red powder with 42.4% yields.

2.5 Formation and Self-Assembly of the Polymeric PEG-Schiff-DOX Nanoparticles

A typical self-assembly solution was prepared as follows: first, the PEG-Schiff-DOX (10 mg) was dissolved in DMF (2 mL), and then deionized water (8 mL) was added drop by drop to the solution through an injection pump at a rate of 0.05 mL/min. During the self-assembly process, the colloidal dispersion was further stirred at room temperature for 8 hours. PEG-Schiff-DOX nanoparticles were obtained by deionized water dialysis (MW cutoff, 1 kDa) for 7 days to remove organic solvents.

2.6 Stability and pH-Responsive Degradation of the Nanoparticles

The storage stability of the nanoparticle solutions was investigated by determining their size distributions using DLS before and after being stored at room temperature for 6 months. In order to investigate pH-responsive degradation behavior of the nanoparticles, the lyophilized powder of the nanoparticles was re-dispersed in phosphate buffer at pH = 5.0 with a concentration of 1 mg/mL. After 1 h, their morphologies were observed by TEM and size distributions were characterized by DLS.

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2.7 In Vitro Drug Release of the Nanoparticles

The release of DOX from nanoparticles was studied by using a dialysis tube (MWCO 3500 Da) at 37 °C (100 rpm) in phosphate buffer with pH 7.4 and acetate buffer with pH 5.0, respectively. Usually, 2mL nanoparticle solution is dialyzed with 30mL release medium. The 10mL release medium is extracted at predetermined intervals and updated with the same number of fresh media. the concentration of DOX in the medium was de-ermined by HPLC measurements.

2.8 CCK-8 Assay

MCF-7 cells were used to study the cytotoxicity of PEG-Schiff-DOX by CCK-8 assay. Cells were seeded onto a 96-well plate at a density of 1×105 cells per well in 200 μL of DMEM containing 10% FBS and further incubated for 24 h (37 °C, 5% CO2). The medium was replaced by 90 μL of fresh DMEM medium containing 10% FBS, and then various concentrations of suspensions in PBS (pH 7.4) solutions were added. After incubation for another 24 h and removal of culture media from cell culture plates, 100 μL of fresh culture media and 10 μL of CCK-8 kit solutions were immediately added and homogeneously mixed and then incubated for 4 h in a CO2 incubator. Finally, put 100μL solution into a 96-well plate. The optical density of each well at 450 nm was read by a microplate reader.

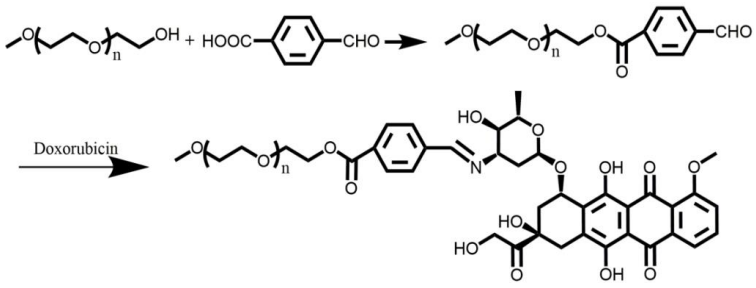

Fig.2 Synthetic route of PEG-Schiff-DOX polymer

3. Result and Discussion

3.1 Synthesis of PEG-Schiff-DOX, A Precursor of Adriamycin

The synthesis of PEG-DOX, the doxorubicin precursor, is shown in Figure 2. In brief, the polymer PEG-Schiff-DOX with Schiff base bond (-RC=N-) is obtained by the reaction of the amino (-NH2) of DOX with the aldehyde group (-CHO) of PEG under mild and efficient conditions.

3.2 Structural Identification of PEG-Schiff-DOX, A Doxorubicin Prodrug

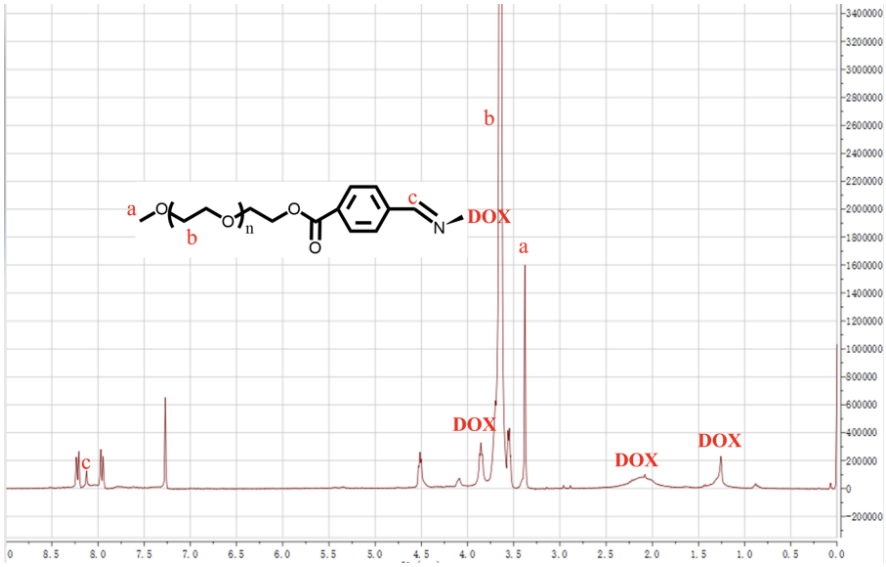

Fig. 3 1H NMR spectrum of PEG-Schiff-DOX polymer.

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The nuclear magnetic spectrum of PEG-Schiff-DOX is shown in Figure 3. The signal of a, b, c and DOX drug represent the formation of different characteristic peaks. The peak at 8.2 ppm proves the formation of Schiff base bond and the successful synthesis of PEG-Schiff -DOX polymer.

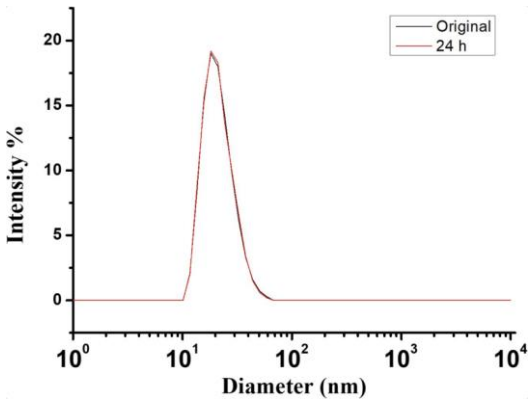

Fig. 4 Size variations of PEG-Schiff-DOX nanoparticles after incubation in PBS (pH = 7.4).

3.3 Stability and Ph Responsive Degradation of Nanoparticles

In the amphiphilic target product prodrug PEG-Schiff-DOX, DOX in PEG-Schiff-DOX monomer is hydrophobic, while PEG is hydrophilic [11], thus prodrug nanoparticles can be formed by self-assembly in aqueous solution. Experiments confirmed that under simulated physiological environment (pH 7.4), PEG-Schiff-DOX could form stable nanostructures through solution self-assembly in Figure 4, and the particle size of PEG-Schiff-DOX nanoparticles was about 20 nm by DLS. As shown in figure 5A,C, the morphology is uniform sphere. After PEG-Schiff-DOX nanoparticles was placed in pH 5.0 acetic acid solution for 24 h, its shape showed irregular shape under TEM, and the particle size of PEG-Schiff-DOX nanoparticles was about 5 nm and 500 nm by DLS, indicating aggregation occurred after disintegration as shown in Figure 5B,D.

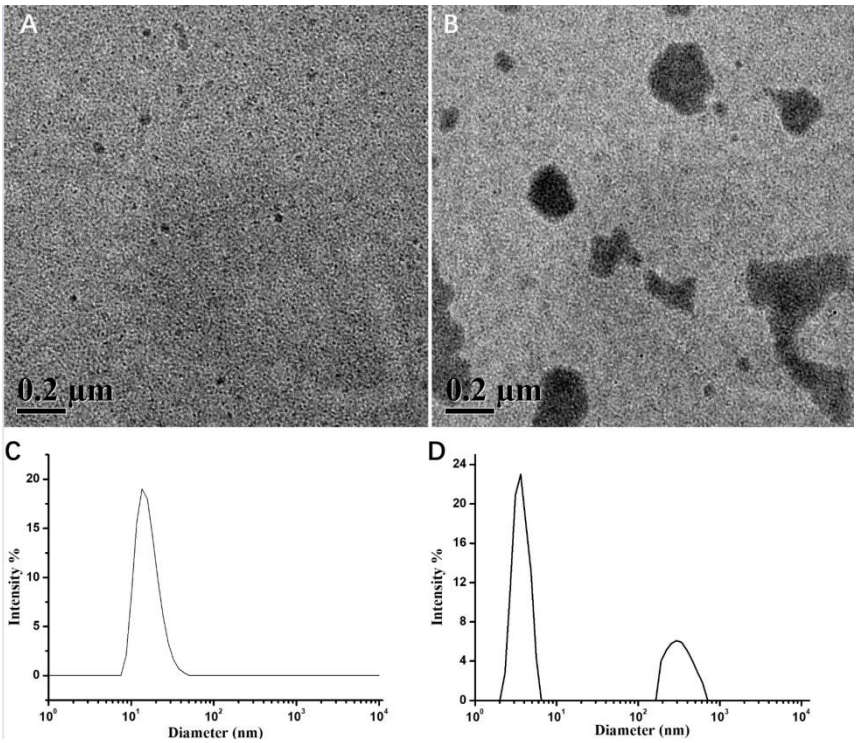

Fig. 5 (A) TEM images and (B) Size changes of the PEG-Schiff-DOX nanoparticles before (a) and after (b) treatment of pH 5.0 PBS solutions for 4 h.

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3.4 In Vitro Release of PEG-Schiff-DOX

In vitro release curve of the doxorubicin prodrug PEG-Schiff-DOX was shown in Figure 6. It can be clearly seen from the figure that The DOX release rate of PEG-Schiff-DOX nanoparticles at pH 5.0 is much higher than that at pH 7.4, mainly because of the acid sensitivity of PEG-Schiff-DOX nanoparticles. The Schiff base bond will break in response under acidic conditions. This results in the disintegration of PEG-Schiff-DOX nanoparticles, leading to the release of the drug. We chose Schiff base bonds to PEG and DOX to enhance the tumor-targeting of the doxorubicin prodrug PEG-Schiff-DOX by accelerating the release of the active agent in the tumor focus (acidic) and hardly releasing the active agent in the blood circulation (weakly alkaline, pH 7.4).

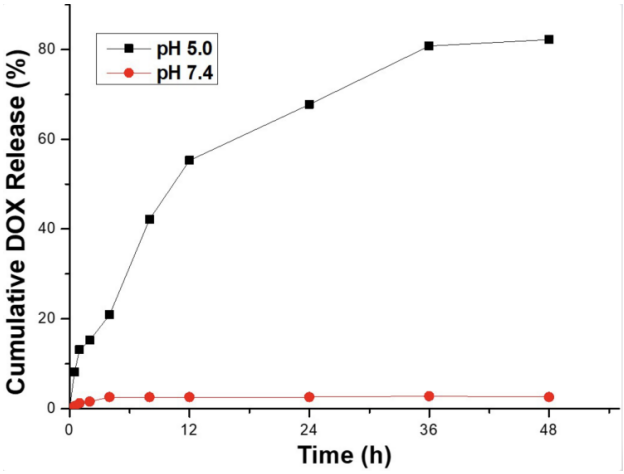

Fig. 6 pH-triggered DOX release from PEG-Schiff-DOX nanoparticles

3.5 Cytotoxicity of PEG-Schiff-DOX

The antitumor activity is derived from cytotoxicity, so it is of great significance to evaluate the antitumor effect in vitro. In this study, the in vitro cytotoxicity of nanoparticles on breast cancer cell MCF-7 was determined by CCK-8 assay using pure DOX as control. As shown in Figure 7, PEG-Schiff-DOX retained the high cytotoxicity of DOX itself and presented a good dose dependence. The survival rate of MCF-7 breast cancer cells decreased with increasing dose, which was consistent with our experimental expectations. The experimental results shown here also show that PEG-Schiff-DOX, the doxorubicin pro-agent, is less cytotoxic than DOX on breast cancer cells, which is consistent with literature indicating that doxorubicin modified by bond is usually less cytotoxic than DOX.[12]

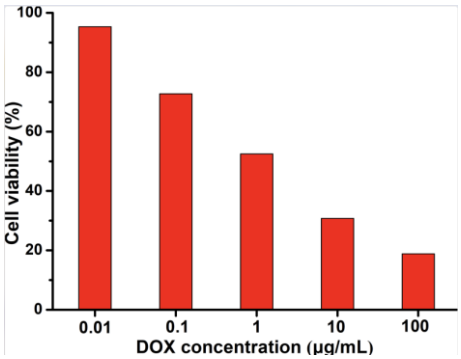

Fig. 7 Cytotoxicity of MCF-7 cells incubation with PEG-Schiff-DOX nanoparticles

4. Conclusion

In summary, prodrug nanoparticles can efficiently improve the water solubility of hydrophobic drugs, extend the cycle time in the body, where unique structure by EPR effect and increase the drug concentration in tumor tissues, and pH response performance enough to significantly reduce the drug

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release in normal tissue, ensure that improve the anti-tumor effect and reduce the body bad reaction. In the PEG-Schiff-DOX polymer synthesized in this study, DOX has hydrophobic properties and PEG has hydrophilic properties. PEG and DOX are connected by Schiff base bond, and prodrug nanoparticles are formed by self-assembly in aqueous solution. The PEG-Schiff-DOX nanoparticles can release DOX rapidly under acidic conditions (pH 5.0). Once the prodrug nanoparticles enter the tumor, due to the acidic microenvironment in the tumor tissue, the chemical bond can be quickly opened and a large amount of DOX can be released, thus improving the killing of the tumor tissue and improving the efficacy. Adriamycin prodrug has ideal antitumor efficacy, and compared with small molecule DOX, it has important advantages such as improved pharmacokinetic characteristics in vivo, significantly reduced toxicity and side effects, and greatly improved safety.

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