Ph-Responsive Drug Delivery System Based on Polymeric Prodrug for Efficient Antitumor Therapy

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Abstract. Ph-responsive micellar nanoparticles in nuclear endosomes were prepared by self-assembling amphiphilic poly (ethylene glycol)-Schiff-doxorubicin (PEG-Schiff-DOX, PSD) predrugs. Self-assembly of an amphiphilic poly (ethylene glycol)-Schiff-doxorubicin (PEG-Schiff-DOX, PSD) prodrug was adopted to prepare endosomal pH-responsive micellar nanoparticles, and the encapsulation of free DOX could be made in the hydrophobic core of the nanoparticles by Schiff base bond. These nanoparticles showed great storage steadiness for above 1 week under normal circumstances but disassembled rapidly to answer faintly acidic environment. Thus, we are able to obtain a simple nano-polymer prodrug material that enables efficient and accurate delivery of tumor drugs and can maintain stability in neutral environments and rapidly degrade in the acidic context of tumor cells. In addition, it benefits from the good stability of PSD with high drug loading concentration and long drug release behavior. According to CCK-8 assays, the nanoparticle showed great antitumor activity over free DOX against HeLa tumor cells. These prodrug-based nanomedicines show excellent potential for the development of translational DOX formulations for cancer therapy.

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

1. Introduction

Cancer is a disease with a very high worldwide fatality rate and is extremely difficult to treat. Its morbidity and mortality have increased very rapidly, and it poses a very great threat to human health and life. The cause of cancer is cancer cells, which are the body's own cells and are a symptom of its own genetic mutations. The effectiveness of cancer treatment has improved with the development of modern medical technology and treatment methods, but millions of people still die from the disease every year [1].

Traditional cancer therapies, such as surgery, radiation and chemotherapy, and immunotherapy, while effective for some types of cancer in different settings, have little effect on advanced metastatic cancer. In addition, both conventional chemotherapy and radiation therapy can cause myelosuppressive conditions. Conventional chemotherapy can kill tumor cells in a non-specific manner, and the toxicity and side effects in patients are obvious. The effects of chemotherapy drugs on the whole body can affect other normal organs, such as the nephrotoxicity of carboplatin and cisplatin, and the cardiotoxicity of doxorubicin. After radiotherapy and chemotherapy, some patients are prone to drug resistance, resulting in significant systemic toxicity and adverse reactions during treatment [2,3]. At the same time, individual tumor cells may self-evolve and develop a tolerance to high-energy radiation and chemotherapy drugs, and tumor multi-drug resistance mainly causes failure of clinical tumor chemotherapy [4-6]. Therefore, there is an urgent need to further research new anticancer drugs, develop efficient anticancer therapies, and improve the effectiveness of cancer treatment to greatly alleviate the suffering of patients.

Advances in nanotechnology over the past three decades have given new hope to cancer treatment. Polymer-based nanomedicines are even more emerging in cancer treatment because of their higher bioavailability and lower toxicity, improving the distribution and steadiness of the drug in the body, thus improving the therapeutic effect. Moreover, nanomedicine can target specific tumor cells, enabling accurate targeted therapy and reducing damage to normal cells. In addition, nanomedicine can also induce an immune response by altering the microenvironment of tumor cells and enhance the body's ability to clear cancer cells [7-12]. Nanomedicine has shown many advantages over small-

molecule anticancer drugs. Nanomedicine, with its higher loading capacity and longer drug release time, can achieve sustained therapeutic effects and reduce the frequency and dose of medication. At the same time, nanomaterials can overcome various obstacles encountered by drugs in vivo, such as lipid solubility constraints, protein binding rates, etc., and improve drug bioavailability. Moreover, nanomedicine can also achieve targeted therapy, reduce toxicity and side effects on normal cells, and can exert an effect on various ways [13-16]. Therefore, with many advantages in cancer treatment, nanomedicine will be an important measure of cancer treatment in the future. Meanwhile, prodrug-based nanoparticles have also been extensively studied due to their well-defined structure, simple preparation, convenient application and great clinical translation potential.

Doxorubicin belongs to the class of tetracyclic antibiotics, whose chemical structure consists of two amino acids and three purine rings. It can be embedded in the DNA double helix structure of the tumor cell, causing topoisomerase II to disrupt the tertiary structure of the DNA and inhibit RNA polymerase activity, thus inhibiting protein synthesis in the tumor cell and thus acting to kill the tumor cell [17,18]. Doxorubicin has the advantages of broad antibacterial spectrum and strong antitumor activity. At the same time, doxorubicin acts as a water-soluble anti-tumor drug, which is soluble in water or blood and easily reaches various tissues and cells in the body, thus acting as an anti-tumor drug. However, due to its good water solubility, when doxorubicin enters the blood, it will be distributed to various parts of the body along with the blood flow area, which not only acts on tumor tissues, but also affects the normal tissues of the body, especially the tissues that proliferate rapidly, such as bone marrow and digestive tract mucosa. This can lead to severe myelosuppression and reactions such as kidney and heart failure [19-22]. Based on this, it is necessary to encapsulate doxorubicin in the form of nanoparticles and change its internal processes before clinical use. PEG is a non-toxic water-soluble polymer material that can be covalently bonded to doxorubicin to form a PEG-DOX copolymer, which can then be assembled and prepared as a PEG-DOX nanoparticle with a diameter of approximately 100-200 nm. The PEG outer layer protects doxorubicin from direct exposure and also reduces the clearance of the drug by the body, extending its half-life. Nanoparticles can alter drug processes in the body and typically accumulate in tumor tissue, reducing damage to normal tissue [23-24]. As a result, PEG-DOX nanoparticles enable tumor-oriented aggregation of doxorubicin and decrease its toxic side roles in normal tissues.

Nevertheless, the declared PEG-DOX prodrugs have several obvious shortcomings, such as low relative drug loading capacity and inadequate drug release. The existing covalent linking method used for PEG-DOX is that only one doxorubicin drug molecule is attached to each PEG chain, resulting in a large amount of PEG usage and drug waste, as well as an extremely low drug loading rate. In addition, covalent bonding prevents doxorubicin from being effectively released from the carrier, and there is a problem of "blind targeting". The enzymes in the blood are difficult to efficiently hydrolyze the link to release the drug, and the blood drug concentration cannot be maintained.

In order to achieve an efficient intracellular drug delivery process, it is necessary to link drugs and carriers through dynamic chemical bonding. Also, to guarantee the successful release of the drug within the cell, it is important that the connection between the drug and the polymer in the tumor cell context be cleaved. Due to the abnormal presence of high glutathione concentration and low pH in tumor cells [25], acid-labile Schiff base bonds are adopted for fabricating stimuli-responsive polymer-DOX prodrugs.

Accurate control of DLC can improve drug efficacy and stability, and proper regulation of drug release dynamics can improve drug efficacy and reduce side effects. Therefore, for the design and optimization of drug delivery systems, the effects of DLC and drug release dynamics should be fully considered and properly regulated and controlled. Higher DLC not only leads to lower costs because less carrier material is required, but also reduces the risk of toxic side effects. This is because by increasing the load of the drug, unnecessary doses can be reduced during administration, thereby reducing the amount of the drug that can cause toxic side effects. As a result, a lot of efforts has been put into exploring and investigating novel nanocarriers with high DLC in the expectation of developing more effective and safe drug delivery systems [26-27]. There is a close relationship

between drug release dynamics and intracellular drug concentrations and drug action times. The concentration and time of action of a drug can be described by the maximum effect, half maximum effect concentration, and the half-life of the drug. Understanding the release dynamics of drugs can help rationalize drug use and predict drug efficacy and safety.

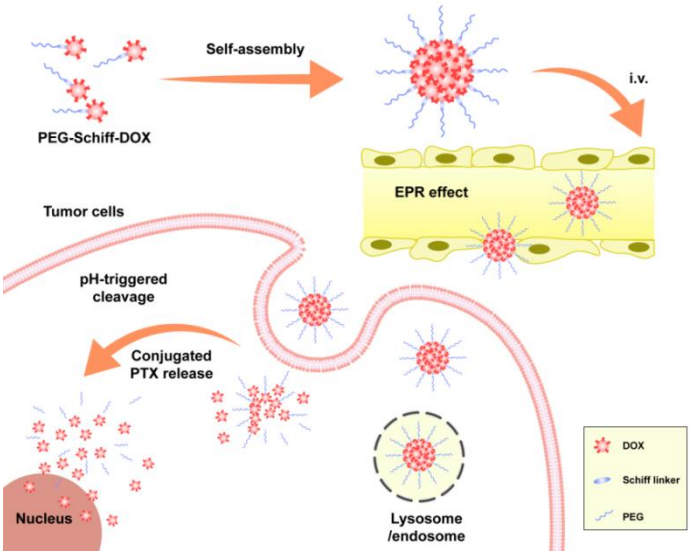

Fig. 1. Schematic description of generation and delivery of the DOX-loaded micelles.

An amphiphilic polymer drug conjugate, poly (ethylene glycol)-Schiff-doxorubicin (PEG-Schiff-DOX, PSD) were designed and synthesized. The excellent storage stability of these nanoparticles is attributed to the safeguarding of the external PEG corona and through the introduction of dynamic chemical bonds in the PEG chain. The breaking of dynamic chemical bonds can be controlled by pH, reducing agents, and other stimuli, so that the release rate of PEG-doxorubicin can be regulated, enabling accurate control of the drug release process. Moreover, through dynamic chemical bonding, it has excellent environmental responsiveness and drug load concentration, thus further reducing the toxicity of targeted drugs and further improving the therapeutic effect for patients. In the acidic context of the intracellular endosomal/lysosomal compartment, the pH-triggered cleavage of the Schiff base bond resulted in the disassembly of the nanoparticle, resulting in the quick release of the encapsulated DOX, followed by the exposure of the previously hidden Schiff bond to the acidic context, and eventually the DOX released entirely. Here, the production of PSD prodrugs and DOX-loaded nanoparticles, cellular uptake, pH-responsive drug release, and in vitro antitumor activity of the nanoparticles against HeLa cancer cells are examined (Fig. 1).

2. Materials and methods

2.1. Materials

Polyethylene glycol methyl ether (PEG-OH, Mn = 750, Alfa Aesor), 4-(dimethylamino)-pyridine(DMAP,99%,Aldrich), triethylamine (TEA, 99%, Beijing Chemical), 4-carboxybenzaldehyde (98%,J&K),1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, 99%, Energy Chemistry), hydrochloric acid (HCl, 37%, Beijing Zhongshuo), sodium sulfate (Na_2SO_4, 98%, Pharmaceutical Technology), sodium bicarbonate ($NaHCO_3$, 98%, Pharmaceutical Technology), n-hexane (AR, Pharmaceutical Technology), ether (AR, Pharmaceutical Technology). All other reagents are offered by Energy Chemistry and adopted directly with no any further purification. All the used cells came from the Chinese cell line resource infrastructure.

2.2. Characterization and testing

Nuclear magnetic resonance hydrogen spectroscopy (1H NMR) is also known as Avance hocky 400 (400 MHz) spectrometer. The reagents were deuterated acetone (Actone-D6), deuterated

chloroform (CDCl3), and deuterated dimethyl sulfoxide (DMSO-D6), with TMS as the internal norm and measured at 25 ℃. Nuclear magnetic resonance (NMR) spectroscopy is derived from Bruker's 400 MHZ spectrometer (internal reference tetramethylsilane, TMS). Dynamic Light Scattering (DLS) is also known as Malvern Zetasizer Nano ZS Dynamic Light Scattering particle size meter. Dynamic Light Scattering (DLS) spectroscopy is from a laser scattering spectrometer with a multi-τ digital time correlator, which requires the utilization of a cylindrical 22 mW single-phase helium-neon laser. The laser-scattered cells were stored in a vat with a thermostat index matched to that of the purified dust-free toluene. A Hitachi F4600 photoluminescence spectrometer was used for the fluorescence experiments and the light source was a xenon lamp. All data were averaged over three doses.

2.3. Synthesis of PEG-Schiff-DOX

The composition of the pH sensitive prodrugs is shown in Fig.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 into a 50 mL bottom containing a magnetic stirring bar. The organic phase can be easily collected by adding 10 mL of fresh dichloromethane to dissolve the reactive solids and then stirring the system. Subsequently, the collected organic solution was washed three times using a solution divided into HCL aqueous solution, NaCl solution, bicarbonate solution, and deionized water, and dried by the solid Na_2SO_4 after washing. The precipitation of product was made in ether to provide a white powder with a calculated yield of 89.1%.

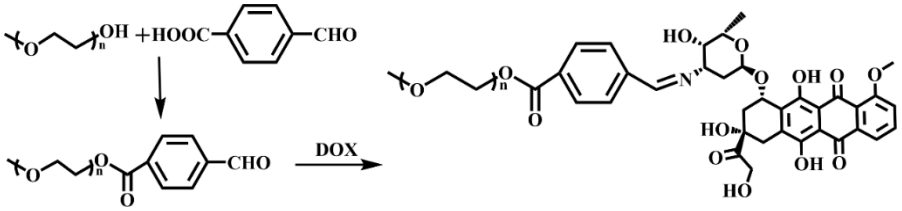

Fig. 2. Synthetic approach of the PEG-Schiff-DOX prodrug.

2.4. Synthesis of the PEG-Schiff-DOX polymer

0.31 mmol polyethylene glycol, 0.25 mmol DOX, and 1 mmol TEA were dissolved in a 20 mL DMF solution at room temperature. The system was refluxed under heavy stirring for half a day, the product was extracted and dissolved in DCM, washed several times, and after washing was further dried by solid Na2SO4. The final result was put into cold ether and precipitated some times to obtain a red powder with a calculated yield of 62.4%.

2.5. Self-assembly of the polymeric PEG-Schiff-DOX nanoparticles in mixed solutions

Briefly, the dissolution of 5 mg PEG-Schiff-DOX was made in the DMF solutions (1 mL), which was then added into the aqueous solutions (4 mL) via the slow dropwise rate. During self-assembly, the colloids were held in solution for 2 h stirring. Remove deionized water from the DMF solutions by the dialysis (MW cut-off, 2 kDa) process for 5 d. The TEM and DLS measurements were adopted to form and characterize these PEG-Schiff-DOX nanoparticles.

2.6. pH-triggered polymeric degradation in various pH solutions

We measured the shift in the size distribution of PEG-Schiff-DOX nanoparticles in the PBS solutions at pH 7.4 to assess their stability and resistance adsorption at room temperature. After displacement into the various pH solutions (pH 5.0), we recorded the size distribution and variation by using DLS measurement at the schedule time (e. g. 4 h). Similarly, the morphological formation and change were employed using the TEM images.

2.7. DOX release from the PEG-Schiff-DOX nanoparticles in vitro

Ph-induced DOX release was performed by adding PEG-Schiff-DOX nanoparticles to a MWE-cut-cut, 1 kDa dialysis membrane tube and shaking them at 37 ℃ in 30 mL of two PBS with pH 5.0

and 7.4, followed by a water bath. The measurement of UV/VIS absorbance of the solution was made at 480 nm to obtain the pH-triggered DOX emission curve. The experimental procedure was repeated three times and the normative deviation of the experimental results was averaged.

2.8. CCK-8 assay

Cytotoxicity of PEG-Schiff-DOX nanoparticles by measuring Hela cells by CCK-8: seeded on a 200 μL of DMEM 96-well plate at 10% FBS with 1×105 cells per well. Replace the cell medium by 90 μL of fresh DMEM including 10% FBS, then add different concentration of micellar suspension to the pH 7.4 PBS solution, respectively. Incubate for another 24 h, remove the medium from the dish and immediately add fresh medium and CCK-8 kit solution and mix well, then incubate in an incubator for 6 h. Eventually, take 100μL of solution and place it into a 96-well plate. The optical density of every trap was read out at 450 nm with a microplate reader.

3. Results and discussion

3.1. Structural analysis

Fig. 3 shows the ^{1}H NMR spectrum of PEG-CHO, and the discussion showed that the proportion of benzene to cyclic hydrogen in the PEG-CHO relates to the methyl peak at the end of PEG, which could be concluded to indicate that the synthesis of PEG-CHO was successful.

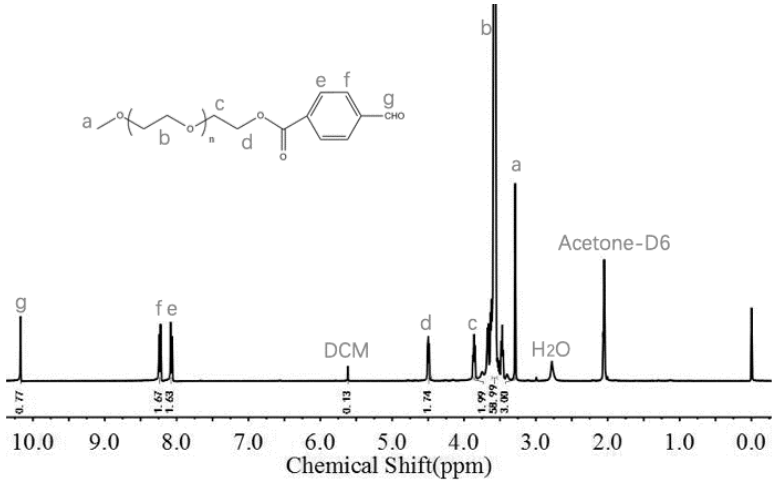

Fig. 3. ^{1}H NMR spectrum of PEG-CHO polymer.

^{1}H NMR spectrum of PEG-Schiff-DOX showed that the peak shape of drug molecules was relatively chaotic and short (Fig. 4). Applying the methyl group at the end of the PEG as a beginning point, the area of the methyl peak essentially related to that of the major chain peak; meanwhile, it was argued that the peak in the low field area originated from the benzene ring and Schiff base bond for 8 H, and the area after integration was basically corresponding. The results suggested that PEG-Schiff-DOX was smoothly prepared.

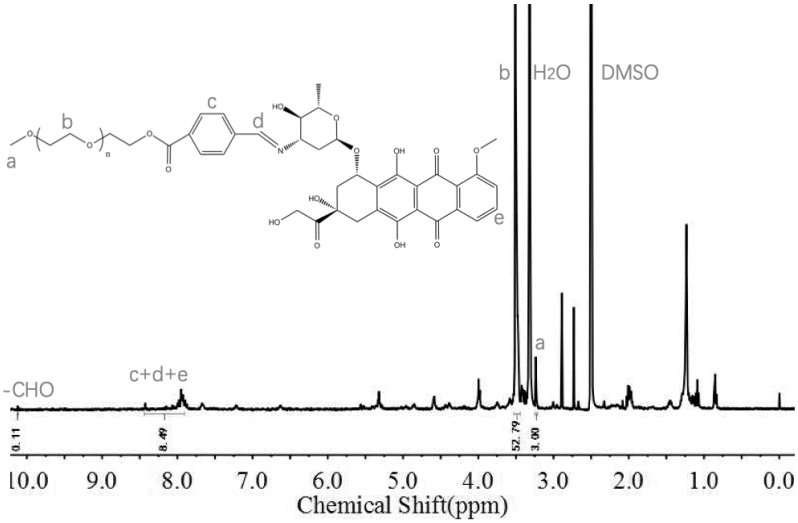

Fig. 4. ^{1}H NMR spectra of PEG-Schiff-DOX.

3.2. Nano drug testing

PEG-Schiff-DOX could assemble spherical micelles with a size below 200 nm in aqueous solution with pH=7.4 (Fig. 5A). While placing and oscillating the nanoparticles in aqueous solution with pH 5.0 and at 37 ℃ for 24 h, it could be seen that the morphology and structure were significantly damaged. It had an amorphous structure (Fig. 5B). The nanoparticles show varied morphology and particle size significantly before and after the acid treatment, and several of the nanoparticles fully disintegrated. The narrow size allocation indicated that the heterogeneous nanoparticles disintegrated under the action of acid (Fig. 5C and 5D) and were able to release drugs in a pH response.

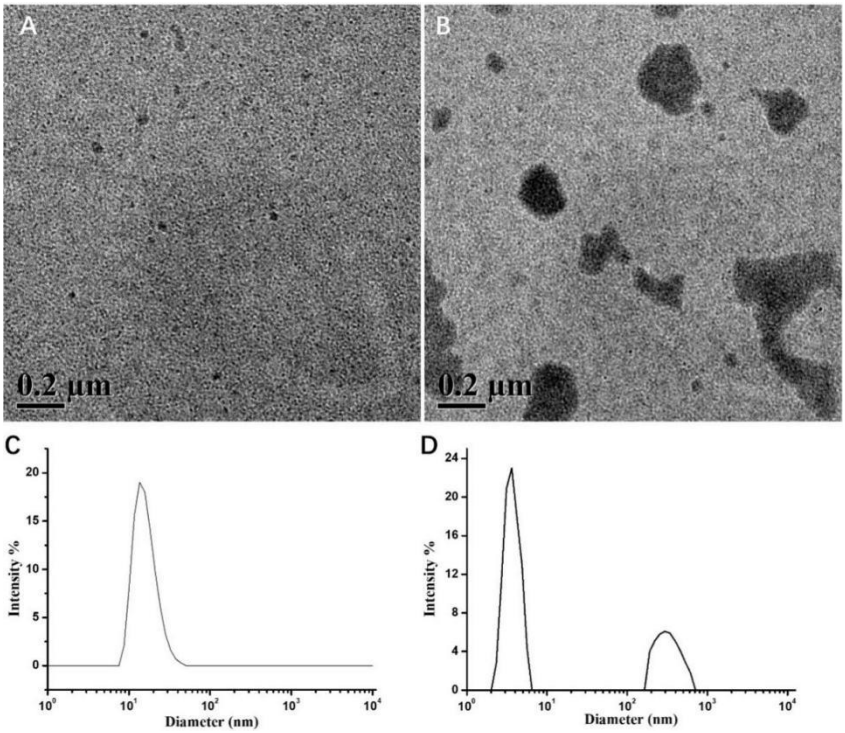

Fig. 5. (A, B) Morphology and (C, D) particle size of PEG-Schiff-DOX drug-carrying nanoparticles at pH=7.4 and pH=5.0.

3.3. Drug Release

Fig. 6 shows more intuitive test outcomes of gel drug loading. The UV spectrometer test results showed that at pH = 7.4 the drug-carrying nanoparticles hardly released the drug. However, the release action was greatly strengthened, and the release quantity grew at pH=5.0.

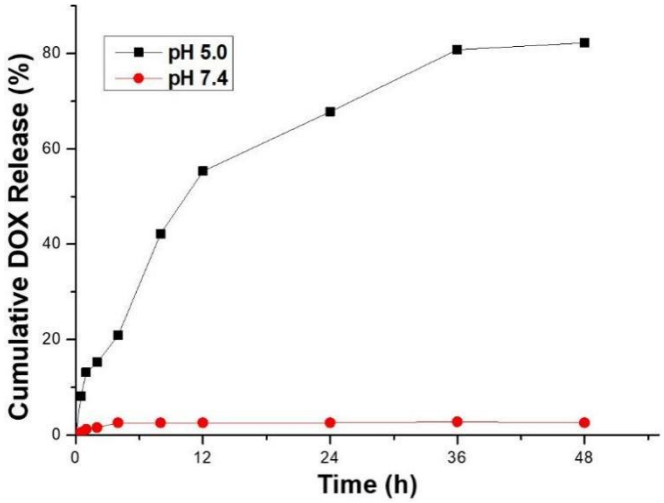

Fig. 6. Release curves of PEG-Schiff-DOX drug-carrying nanoparticles in vitro.

3.4. Antitumor activity

As can be seen from Fig. 7, PEG-Schiff-DOX nanomaterials had excellent anti-tumor effects, especially when the concentration of loaded DOX reached 100 μg/mL, showing a very high inhibitory effect on tumor growth, and the cell survival rate was less than 8%. The results indicated that PEG-Schiff-DOX was a kind of targeted agent that could be used in clinic.

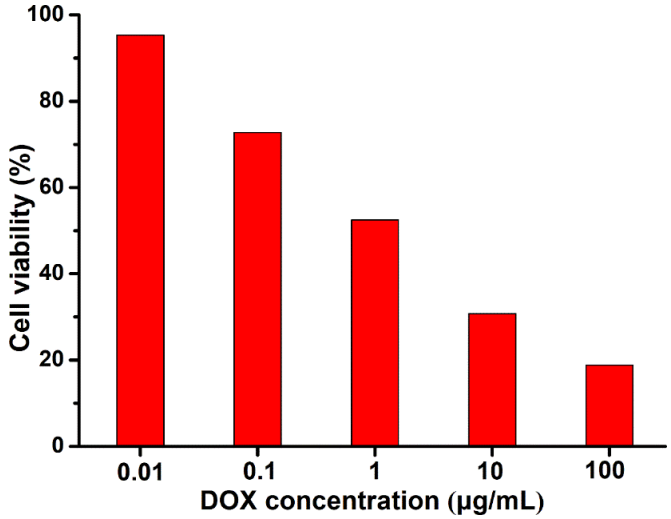

Fig. 7. Evaluation of anti-tumor effects of PEG-Schiff-DOX nanomaterials.

4. Conclusion

In summary, we prepared PEG-Schiff-DOX loaded polymer prodrug nanoparticles containing pH-responsive doxorubicin and tested the release action at various pH values. With the multistage release system, the drug action time can be smoothly extended and can respond to the acid environment in a timely manner, which show some growth potential. These drug-carrying nanoparticles has the merits below: (1) well-defined chemical framework and simple production approach; (2) Heavy drug load; (3) Excellent storage steadiness; (4) Little or no drugs are released under neutral conditions, while DOX agents are released under weakly acidic circumstances in the tumor microenvironment to

maintain drug efficacy and reduce side effects; (5) Efficient anti-tumor therapy. Therefore, these polymer-based prodrug nanomedicines offer options for the growth of novel drug formulations, which will realize promising applications in cancer therapy.

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