Leakage-free DOX/PEGylated chitosan micelles fabricated via facile one-step assembly for tumor intracellular pH-triggered release

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Abstract

A general strategy was developed to fabricate the chitosan-based micelles as smart drug delivery system (DDS) for anti-cancer drug doxorubicin (DOX), via modulating the feeding ratio of PEG (PEG₂₀₀₀-CHO) and molecular weight of chitosan. Typically, benefiting from the solubility of chitosan in media with different pH values as an “off-on” switch, the DOX/CS_{100K}-PEG₂ micelles possess spherical core with a narrow dispersed diameter of 64 nm and regular shell with a thickness of 14 nm in pH 7.4 PBS. Interestingly, the DOX/CS-PEG micelles exhibited drug leakage-free behavior in a physiological environment, with a cumulative release ratio of only 2.32% under pH 7.4 PBS, while achieving rapid drug release and a cumulative release ratio of 72.76% in the intracellular microenvironment. Moreover, the micelles transformed into the water-soluble CS_{100K}-PEG₂ after the pH-triggered intracellular release of

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DOX, it makes them easy to be biodegraded and eliminated by metabolic system. As revealed by MTT assays and CLSM images, DOX could be efficiently and rapidly transported into cell nuclei of tumor cells and showed significant cell inhibition. The simple fabrication and excellent properties make them intelligent and favorable on-demand DDS in cancer treatment.

Keywords: drug delivery system; drug leakage-free; chitosan micelles; pH-triggering; controlled release

1. Introduction

Cancer is still one of the murderers endangering human health, and either the incidence or mortality of cancers is ever-increasing. Chemotherapy has attracted extensive attention as a highly efficient approach in cancer treatment. However, severe side effects and limited therapeutic efficiency in field of clinical current chemotherapy are two main obstacles existing for cancer therapy, which has restricted therapeutic effect as well as aroused abominable status in patients [1]. To reduce severe side effects and improve therapeutic efficiency, the physicochemical differences (such as acidity [2], overexpressed proteins and enzymes [3] and abnormal temperature gradients [4]) between the normal tissues and microenvironment or endosomes and lysosomes of the tumor tissues have been utilized to design intelligent and favorable drug delivery platform.

Unfortunately, most DDSs showed the uncontrollable drug leakage in the circulatory system before the nanocarriers have entered the tumor tissues [5]. Taking doxorubicin (DOX) as an example, it has been mostly loaded onto the drug delivery system (DDS) via the non-covalent interaction, such as electrostatic interaction, hydrogen bonds, hydrophobic interaction, π - π stacking, or combinations of more than one ways above. In the simulated normal physiological media, 20-40% or even higher of DOX could be released from the DOX-loaded DDS with those non-covalent interactions, due to the certain solubility of DOX in pH 7.4 media [6].

To further reduce severe side effects and improve therapeutic efficiency, recent efforts have been turned to the drug loading method, namely the interaction between drugs and DDS. DOX has been covalently conjugated onto the DDS via the acid-labile [7-11] or reductant-cleavable [12-14] linkages. The prodrugs are relatively stable during blood circulation, but quickly dissociate to achieve burst release of DOX toward exposing acidic lysosomes in tumor tissues. But even so, the DOX leakage from the prodrugs was more than 5%, and in some case > 20% [14,15], and up to 40% [16]. Furthermore, for some prodrugs conjugated via acid-labile linkage [9] and all those with reductant-cleavable disulfide linkage, DOX was released in the form of its derivatives [12-14].

In comparison, designing the smart DDS with more accurate response seems to be a more efficient strategy to reduce severe side effects, by reducing the drug leakage in circulation system to the greatest extent. Tang et al coated pH-responsive polymer shell chitosan/poly (methacrylic acid) (CS-PMAA) on mesoporous silica

nanoparticles (MSN) through the facile in situ polymerization method to control the DOX release in different pH media [17]. Chen et al designed bioresponsive polymer-mesoporous silica nanoparticles (PAA-PCL-MSNs) with function akin to an “AND” logic gate for targeted drug delivery with <5% of final DOX leakage after 50 h incubation [18]. Luo et al designed a new type of peptide-functionalized MSN with a plug-gate nanovalve (PGN) for targeted drug release in cancer cells without obvious release of DOX at pH 7.4 [19]. Most recently, Dai et al designed novel gold-shell nanoparticles (pH-GSNPs) exhibiting drug leakage-free behavior in a physiological environment [20].

Recently, chitosan and its derivatives in the forms of nanoparticles and micelles have been widely investigated for tumor chemotherapy and diagnosis due to their unique characteristics, such as biocompatibility, biodegradability, remarkable cell membrane penetrability, pH-dependent therapeutic unloading, ease to multi-functionalization, and so on [21], especially the PEGylated chitosan copolymers [22]. And for most of the chitosan and its derivatives-based nanoparticles and micelles, the drug carriers were fabricated at first, and then the drugs were loaded [23-26]. Because of its nature of polycation, the targeted pH-mediated intracellular release of methotrexate disodium (MTX) has been reported with the crosslinked chitosan microgels as vehicle, benefiting from the swelling of chitosan in different media [27].

In the present work, a facile strategy was developed to fabricate the drug leakage-free DDS by means of the solubility of chitosan in different pH media as an

“off-on” switch (Scheme 1). As a natural biocompatible weak polybase with pKa of approximately 6.5 [28], chitosan could deprotonate in slightly basic media (pH 7.4), resulting from the increase in its hydrophobicity. On the contrary, the water-solubility of chitosan is enhanced because of protonation in slightly acidic media, especially pH value < 5.0 [29]. Herein, chitosan was PEGylated via the reduction of Schiff’s base between PEG and chitosan to form well-defined core-shell structure for compactly encapsulating anticancer drug DOX due to the hydrophobicity of chitosan and DOX [30], and prolonged blood circulation of doxorubicin [25]. This amphiphilic copolymer is a reasonably amphiphilic precursor for molecular self-assembly by hydrophobic interaction in PBS. More importantly, the typical DOX/CS_{100K}-PEG₂ micelles possess the “off-on” switchable release properties in the different pH media. The micelle dissociates to burst release DOX in response to acidic lysosomes. Meantime, the DOX/CS_{100K}-PEG₂ micelles transform into the water-soluble CS_{100K}-PEG₂, which could be eliminated by metabolic system, after the pH-triggered intracellular release of DOX.

2. Experimental

2.1. Materials and reagents

Three chitosan (CS, degree of deacetylation: 95%) samples with different viscosity-average molecular weights (3.0×10^5 , 1.0×10^5 or 5×10^4) were provided by Weifang Sea Source Biological Products Co., Ltd. Single aldehyde-terminated poly(ethylene glycol) (PEG₂₀₀₀-CHO) was purchased from Beijing Kaizheng Bioeng.

Doxorubicin hydrochloride (DOX·HCl) was obtained from Beijing Huafeng United Technology Co., Ltd. Furthermore, NaCNBH₃, and acetic acid were analytic reagent grade from Tianjin Chem. Co. Ltd. and used without further purification. Deionized water was used throughout

2.2. PEGylation of chitosan

To regulate morphology and endow biocompatibility of the DOX delivery system, different amounts of PEG₂₀₀₀-CHO were used to modify chitosan with different viscosity-average molecular weight (3.0×10^5 , 1.0×10^5 or 5×10^4) (Scheme 2), as summarized in Table 1. In a typical procedure,³⁰ 0.165 g of chitosan (1.0×10^5) was dissolved in 20 mL of 1 M acetic acid aqueous solution. 20 mL of methanol and 1.300 g of PEG₂₀₀₀-CHO were added into the solution under magnetic stirring at room temperature overnight. Subsequently, the pH value of the mixture was adjusted from original 4.90 to 6.24 with 1 M NaOH aqueous solution and continually kept for 30 min. Then 0.102 g of NaCNBH₃ (1.625 mM) was added into the solution by dropwise, and the reduction reaction was performed under vigorous stirring at nitrogen atmosphere for 8 h. The obtained pre-product was concentrated under reduce-pressure state by vacuum pump, and transferred into dialysis tube (MWCO: 12 000) against abundant deionized water for 72 h to remove the unreacted PEG₂₀₀₀-CHO. Finally, the obtained product was lyophilized and stored at 4 °C.

2.3. Drug loading and triggered release

The DOX-loading was conducted via a facile *in-situ* micellization approach. Typically, 40.0 mg CS_{100k}-PEG₂ was introduced into 1 mg/mL of DOX aqueous solution (40.0 mL), and then the pH value of solution was adjusted to 6.8 for drug-loading with the aid of ultrasound. Subsequently, the obtained mixtures were transferred into dialysis tube (MWCO: 12 000) against abundant PBS (pH 6.8) for 7 days to remove the excess DOX. Finally, the DOX/CS_{100K}-PEG₂ was lyophilized and stored at 4 °C. Other DOX-loaded micelles were prepared by the same procedure (Table 1). Next, the UV-vis spectrophotometer was determined the drug concentration in the supernatant solution at 480 nm to assess the encapsulation efficiency and the drug-loading capacity, by comparing the drug concentrations before and after loading.

Subsequently, to assess release behavior of the DOX-loaded CS_{100k}-PEG₂ micelles in stimulating both normal tissues and microenvironment of tumor tissues, the DOX-loaded CS_{100k}-PEG₂ micelles were dispersed into 10 mL PBS (pH=7.4, pH=6.5, and pH=5.0). In the coming step, the suspension of the DOX-loaded CS_{100k}-PEG₂ micelles was transferred into dialysis tubes (MWCO of 12 000) and immersed in 120 mL of related PBS at 37 °C for controlled release. The DOX concentration in the dialysate was detected with a UV spectrophotometer at certain time intervals.

2.4. Cell toxicity and cellular uptake

MTT assay was carried out to assess the biocompatibility of the CS_{100K}-PEG₂ with HepG2 cells, and the cytotoxicity of the DOX/CS_{100K}-PEG₂ micelles and free DOX for HepG2 cells were evaluated by incubation of HepG2 cells in nutrient solution

supplemented with 10% FBS (fetal bovine serum).

The cellular uptake was conducted with confocal laser scanning microscopy (CLSM) (LYMPUS FV-1000) using HepG2 cells after 12 h and 24 h of incubation.

The location of intracellular fluorescence was validated with excitation wavelengths of 480 nm for DOX and 405 nm for Hoechst. These specific steps were referred to previous report [6].

2.5. Instruments and characterization

Elemental analysis of C, N, and H of CS-PEG was performed on an Elementar vario EL instrument (Elementar Analysensysteme GmbH, Munich, Germany).

A Bruker IFS 66 v/s infrared (IR) spectrometer (Bruker, Karlsruhe, Germany) was used for the Fourier transform IR spectroscopy analysis in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} , with KBr pellet technique.

The morphologies of the DOX/CS-PEG micelles were characterized using a JEM1200 EX/S transmission electron microscope (TEM), with the samples dispersed in water and then deposited on a copper grid covered with a perforated carbon film.

The hydrodynamic diameters and distributions of the DOX/CS-PEG micelles were determined using the dynamical mode [dynamic light scattering (DLS)] on a Light Scattering System BI-200SM device (Brookhaven Instruments) equipped with a BI-200SM goniometer, a BI-9000AT correlator, a temperature controller, and a coherent INOVA 70C argon ion laser at 20°C. DLS measurements are performed using a 135-mW intense laser excitation at 514.5 nm at a detection angle of 90° using

the emulsion directly at 25 °C.

All fluorescence spectra were obtained with a PerkinElmer LS 55 luminescence spectrophotometer equipped with a 1.0 cm quartz cell. The fluorescence spectra of the DOX/CS-PEG micelles were conducted in the wavelength range of 500–650 nm upon excitation at 495 nm at different pH values. Typically, 1.0 mL of 10 µg/mL DOX/CS_{100K}-PEG₂ micelles aqueous dispersion was added into the color comparison tubes, and the synchronous fluorescence spectra were recorded in the wavelength range of 480–680 with $\Delta\lambda$ of 50 nm.

The controlled release behaviors of the DOX/CS_{100K}-PEG₂ micelles were measured with a PerkinElmer Lambda 35 UV–vis spectrometer (PerkinElmer Instruments, USA) at room temperature.

3. Results and discussion

3.1. Synthesis of CS-PEG

Both excellent biocompatibility and controllable morphology are extremely important for DDS. Therefore, PEG was utilized to modify the backbone of chitosan to enhance hydrophilicity. And chitosan with different average molecular weights was chosen to regulate size of the DDS for obtaining a general method. The synthetic approach of the CS-PEG is shown in Scheme 2: PEGylation of chitosan via Schiff's base formed between the amino group of chitosan and the aldehyde group of PEG₂₀₀₀-CHO, and the reduction of the Schiff's base [30]. The PEG segments were introduced onto the skeleton of chitosan to make the PEGylated chitosan favorable

morphology-controllable in the range of pH 7.4 to 5.0. In the IR spectrum of the typical CS_{100K}-PEG₂, after the PEGylation with PEG₂₀₀₀-CHO, the C–O–C stretching vibration of PEG appeared at 1108 cm⁻¹, while the characteristic peak at 1596 cm⁻¹ of the N-H in-plane deformation and vibration in amide group of chitosan was still remained (Figure 1A). Furthermore, the characteristic peak at 2900 cm⁻¹ of C–H stretching enhanced obviously. It demonstrated that the PEG brushes have been effectively conjugated onto the skeleton of chitosan via reduction of Schiff's base. This result also indicated partial amine groups of chitosan were retained, which are contributed to preserve the pH-response of chitosan.

The PEG content in CS_{50K}-PEG, CS_{100K}-PEG₁, CS_{100K}-PEG₂, CS_{100K}-PEG₃, and CS_{300K}-PEG are assessed from its N elemental content as 16.20 wt%, 15.14 wt%, 27.12 wt%, 38.00 wt%, and 13.21 wt%, respectively. With the increasing of feeding molar ratio of PEG from 25% to 45% and 65%, the PEG content in the CS_{100K}-PEG₁, CS_{100K}-PEG₂, and CS_{100K}-PEG₃ increased from 15.14 wt% to 27.12 wt%, and 38.00 wt%. This phenomenon is attributed to the increase in the PEG₂₀₀₀-CHO feeding ratio. Meantime, in comparison with the high PEG contents of the CS_{50K}-PEG of 16.12 wt%, the CS_{100K}-PEG₁ and CS_{300K}-PEG showed lower PEG contents of 15.14 wt%, and 13.21 wt%, respectively. It's worth noting that increasing of the average molecular weight of chitosan led to decreasing of PEG contents in CS_{100K}-PEG₁ and CS_{300K}-PEG. The reason might be that increasing of molecular chain of chitosan enhances the viscosity of mixture and further weak the activity of polymer chain in solution as well as then increased the steric hindrance effect to reduce reactivity of between the

aldehyde group of the PEG₂₀₀₀-CHO and the amine groups in chitosan. Taken together, a series of the CS-PEG are efficiently synthesized through precise control of PEG₂₀₀₀-CHO feeding ratio and viscosity-average molecular weight of chitosan.

3.2. Preparation of DOX/CS-PEG micelles

To achieve the less leakage of anticancer-drug in normal tissues, DOX is encapsulated into the CS-PEG micelles via hydrophobic interaction by a facile *in-situ* micellization approach. In the DOX/CS-PEG micelles, DOX is utilized as hydrophobic section to form core, the pH-responsive skeleton of chitosan is used as a pH-modulated shell to prevent leakage of DOX, and the hydrophilic PEG brushes are utilized to endow biocompatibility of the DDS and regulate the morphology of the nanoplatform. The hydrophobic properties of the chitosan can form a hydrophobic interaction with DOX. Therefore, DOX could be noncovalently encapsulated on the CS_{100K}-PEG₂ micelles by a hydrophobic interaction in aqueous solution at related pH values with the aid of slight ultrasonication. Considering the enhanced water-solubility of DOX [6] and the deprotonation of chitosan (pK_a of approximately 6.5) [28], the pH value of mixture of DOX and CS_{100K}-PEG₂ was adjusted from pH 5.0 to pH 6.8 to enhance the hydrophobicity of DOX and CS_{100K}-PEG₂. At the same time, the PEG moieties keep hydrophilic from beginning to end at the adjusted process of pH value of the mixture. Thus the DOX/CS_{100K}-PEG₂ micelles were synergistically formed in aqueous solution via hydrophobic interaction between DOX and the PEGylated

chitosan, and then the encapsulation of DOX has been efficiently realized without any additional organic solvent at vigorously magnetic stirring.

After DOX-loading, the characteristic peak at 1596 cm^{-1} and 1108 cm^{-1} appeared in the IR spectra of the DOX/CS_{100K}-PEG₂. And the characteristic peaks at 1730 cm^{-1} and 1073 cm^{-1} were ascribed to the carbonyl asymmetric stretching and C=C of benzene stretching vibration of DOX in the DOX/CS_{100K}-PEG₂ [31]. Owing to the introduction of DOX molecules in the CS-PEG, the DOX/CS-PEG possessed strong UV-vis absorption and fluorescence emission under excitation of certain wavelength. As a result, the product became dark red compared to the original white one. The UV-vis absorption of DOX in various DOX/CS-PEG micelles was detected at 490 nm to 510 nm (Figure 1B). The shifts in the peaks of various DOX/CS-PEG micelles might be resulted from the hydrogen bond interaction between DOX molecule and polymer. Excitingly, the DOX/CS-PEG micelles exhibited maximum fluorescent emission at around 560 nm and 590 nm with 495 nm excitation (Figure 1 C), indicating the presence of DOX in these micelles. Furthermore, double fluorescence channel of DOX in the DOX/CS-PEG micelles is observed [32]. Taken above analysis into consideration, it could be concluded that DOX molecule has been successfully encapsulated into the CS-PEG micelles, and the physical encapsulation didn't influence the UV-vis absorption and fluorescence emission of DOX to conveniently monitor tumor tissues by real-time fluorescent imaging.

The DOX-loading capacity (%) and encapsulation efficiency (%) of the CS_{50K}-PEG, CS_{100K}-PEG₁, CS_{100K}-PEG₂, CS_{100K}-PEG₃, and CS_{300K}-PEG micelles, which are

determined with standard curve by UV-vis spectrometer at 485.00 nm, were 0.4821 mg/mg $\pm$ 0.0130 mg/mg, 0.4423 mg/mg $\pm$ 0.0096 mg/mg, 0.3214 mg/mg $\pm$ 0.0231 mg/mg, 0.1470 mg/mg $\pm$ 0.0043 mg/mg, and 0.1681 mg/mg $\pm$ 0.0044 mg/mg, and 48.21% $\pm$ 1.30%, 44.23% $\pm$ 0.96%, 32.14% $\pm$ 2.31%, 14.70% $\pm$ 0.43%, and 16.81% $\pm$ 0.44%, respectively. Interestingly, the DOX contents in various DOX/CS-PEG showed inverse correlation in comparison with the PEG content because of increasing of hydrophilic capability of PEG segments to decline hydrophobic interaction between chitosan and DOX molecules. In addition, it is strict that this above DOX contents are obtained by means of dialysis method under abundant deionized water to remove adsorbent DOX through hydrogen bond interaction between drug molecules and segments of polymer to decline leakage of DOX during long-term cycle of blood circulation.

As shown in Figure 2 (a, b and c), with the increasing of average molecular weight of chitosan, the diameter of the DOX/CS_{50k}-PEG micelles, DOX/CS_{100k}-PEG₁ micelles and DOX/CS_{300k}-PEG micelles increased from 54.67 $\pm$ 11.43 nm to 101 $\pm$ 18.76 nm and 158 $\pm$ 14.52 nm. Furthermore, the hydrodynamic diameter of the DOX/CS_{50k}-PEG micelles, DOX/CS_{100k}-PEG₁ micelles and DOX/CS_{300k}-PEG micelles also increased from approximately 111 nm to 175 nm and 295 nm with increasing of average molecular weight of chitosan. More importantly, the morphology of the DOX/CS-PEG micelles exhibited spherical shape in PBS (pH = 6.8). Both the TEM and DLS analysis demonstrated that the size of the DOX/CS-PEG micelles could be regulated by altering the average

molecular weight of chitosan. Meantime, different amount of PEG-CHO was utilized to modify the backbone of chitosan for investigating the micelle morphology. By increasing of PEG from 15.14 wt%, to 27.12 wt% and 38.00 wt%, the morphology of the DOX/CS_{100k}-PEG micelles changed from spheres with protuberance, to spheres with the contrast of uniformly, and to vesicle.

Furthermore, the N elemental contents of the various CS-PEG were determined for evaluating the PEG contents and the results are presented in Table 1. The surface structure of the DOX/CS_{100k}-PEG₂ and DOX/CS_{100k}-PEG₃ became smooth and orderliness in comparison with the DOX/CS_{100k}-PEG₁, with the increase of contents of PEG brushes. In particular, the DOX/CS_{100k}-PEG₂ micelles might preferably encapsulate DOX by comparison of DOX/CS_{100k}-PEG₁ with protuberance of structure and DOX/CS_{100k}-PEG₃ with vesicle of structure.

Actually, these differential presentations are attributed to different contents of hydrophobic DOX and hydrophilic PEG on the skeleton of chitosan. The DOX/CS_{100K}-PEG₃ micelles showed “bubbly” shape with the average diameter of 96.89 ± 38.43 nm due to the hydrophilic PEG brushes distributing both sides of skeleton of chitosan, with increasing amount of PEG to 38.00 wt%. Furthermore, high content of the hydrophilic PEG brushes could weak the hydrophobic interaction of DOX in the DOX/CS_{100K}-PEG₃ micelles resulting from hydrophilicity of both sides of skeleton of chitosan. More importantly, the fuzzy boundaries between hydrophobic and hydrophilic domains easily form different self-assembly structure, as well as resulting in morphological transition as

previously reported [33].

Additionally, DLS technique was employed to evaluate the particle dispersion index (PDI) of all DOX/CS-PEG micelles as shown in Figure 3. The DOX/CS-PEG micelles have excellent PDI, besides the DOX/CS_{100K}-PEG₃ because of hydrophilic increasing of both sides of the skeleton of chitosan. This hydrophilic increasing of skeleton of chitosan might lead to fuzzy boundaries between the hydrophobic and hydrophilic domains resulting in “bubbly” shape with unfavorable PDI.

Owing to the favorable surface structure and diameter, and relatively high encapsulation efficiency and drug-loading capacity, the DOX/CS_{100K}-PEG₂ micelles were chosen as the desired DDS for further exploration. With changing the media pH value from 6.8 to 7.4, the core-shell structure of the DOX/CS_{100K}-PEG₂ micelles could be observed in Figure 3. The DOX/CS_{100K}-PEG₂ micelles have spherical core with a diameter of around 64 nm and regular shell with a thickness of approximately of 14 nm. The well-defined structure was attributed to the hydrophobic skeleton of chitosan, hydrophobic DOX molecules and hydrophilic PEG brushes in the pH 7.4 media. The DOX/CS_{100K}-PEG₂ micelles could keep their core-shell structure in pH 7.4 PBS, whereas in pH 5.0 PBS, their well-defined structure was destroyed as shown in Figure 2f. The reason is that the protonation of the amine groups of chitosan in slightly acidic media (pH 5.0), resulting in an increase of the hydrophilicity of chitosan. This is consistent with observation of DLS for dissociation of the DOX/CS_{100K}-PEG₂ micelles, implying that most DOX/CS_{100K}-PEG₂ micelles are successfully dissociated in the slight acidic media. On the basis of above analysis and

discussion, the well-defined core-shell structured DOX/CS_{100K}-PEG₂ micelles with pH-response have been successfully prepared.

To further preferably understand the pH-responsive behavior of the DOX/CS_{100K}-PEG₂ micelles, the responsive behavior of the CS_{100K}-PEG₂ was evaluated by TEM in desired pH, such as pH 7.4 and 5.0. As shown in Figure 4, the dissociation of CS_{100K}-PEG₂ micelles were observed with the pH value mixture of CS_{100K}-PEG₂ from 7.4 to 5.0 by adjusted with the diluted HCl solution. This phenomenon revealed that the DOX/CS_{100K}-PEG₂ micelles possessed desirable pH-responsive behavior in the range of pH 7.4 to 5.0.

3.3. *In vitro* release performance

As a pH-responsive chitosan based drug delivery system, the DOX/CS_{100K}-PEG₂ micelles not only exhibited a relatively stable structure in normal tissues but also a disassociated structure in response to typical conditions in tumor tissues because of the protonation of amine group of chitosan in slightly acidic pH 5.0. Meantime, therapeutically on-demand release of DOX from the DOX/CS_{100K}-PEG₂ micelles is predicted to be accelerated under acidic medium in the cytoplasm and endolysosomes of tumor tissues. Considering this difference, therefore, the *in vitro* release performance of DOX from DOX/CS_{100K}-PEG₂ micelles was evaluated under certain conditions such as pH 7.4 (stimulating normal tissues), pH 6.5 (stimulating microenvironment of tumor tissues), and pH 5.0 (stimulating cytoplasm and endolysosomes of tumor tissues), as shown in Figure 1 (D).

The cumulative release ratio from the DOX/CS_{100K}-PEG₂ micelles was only 2.32% at pH 7.4 and 9.16% at pH 6.5 for 60 h. These results indicated that the DOX/CS_{100K}-PEG₂ micelles possessed highly stability at physiological pH media. More importantly, this tiny release of DOX from the DDS, which is also much less than those from the pro-drugs with acid-liable or reduction-cleavable conjugations, attributes to reduce the severe toxic side effect of anticancer drugs to normal tissues of patients. To imitate the intracellular trafficking process of tumor tissues, drug cumulative release experiments were performed at pH 5.0 (stimulating the acidic endosomal compartments). In comparison with the drug leakage-free behavior at pH 7.4, the cumulative release of DOX from the DOX/CS_{100K}-PEG₂ micelles was conducted at pH 5.0. Notably, the cumulative release of DOX from the DOX/CS_{100K}-PEG₂ micelles was apparently accelerated in elevated pH values (pH 5.0) as well as cumulative release ratio reached 73.35% for 60 h. These results revealed that the premature release of DOX was obviously reduced because of the highly stability of the DOX/CS_{100K}-PEG₂ micelles at pH 7.4 to realize safely long-term cycle during blood circulation. Furthermore, rapid and burst release of DOX from the DOX/CS_{100K}-PEG₂ micelles at pH 5.0 came true. A key feature of the CS_{100K}-PEG₂ micelles is the protonation of amine group of chitosan upon exposing to at acidic environment. In acidic media, the amino groups of both CS_{100K}-PEG₂ and DOX protonate, and the enhanced repulsive force between them should contribute to the increase in the cumulative release of DOX. Besides, after the pH-triggered release of DOX at slight acidic media, the DOX/CS_{100K}-PEG₂ micelles transformed into the

water-soluble CS_{100K}-PEG₂, that could be degraded, and then eliminated by metabolic system. On the basis of above analysis, the engineered DOX/CS_{100K}-PEG₂ micelles with pH-responsive structure, drug leakage-free characteristic in a physiological environment, and burst release properties at stimulating tumor tissues, namely, the tumor microenvironment-responsive triggered release performance, were achieved.

3.4. Cellular toxicity and uptake

To assess the engineered rationality of the DOX/CS_{100K}-PEG₂ micelles, it is crucial to evaluate cytocompatibility via a general acceptable MTT for HepG2 cell (a general model of parenchymal cells in the liver). The viability of the HepG2 cells exceeded 89% at the all tested concentrations (0–80 µg/mL) after 24 h of incubation (Figure 5), revealing that the CS_{100K}-PEG₂ micelles have good biocompatibility toward HepG2 cells.

To realize clinical application, an excellent drug delivery system should possess not only favorable biocompatibility in normal tissues but also a rapid release upon exposure to tumor tissues. To determine the therapeutic effect of the pH-responsive DOX/CS_{100K}-PEG₂ micelles, the in vitro inhibition growth of HepG2 was evaluated the by MTT assays, compared with free DOX. As shown in Figure 5, the DOX/CS_{100K}-PEG₂ micelles presented apparently cytotoxic effects in all tested concentration range. For comparison, free DOX was introduced to as control group. Obviously, introduction of DOX pronouncedly inhibited viability of HepG2 cells with increasing DOX concentration up to 10.0 µg/mL. The inhibition growth degree of free

DOX was obviously higher than the DOX/CS_{100K}-PEG₂ micelles, resulting from the lower cumulative release ratio of DOX (53.36 wt% for around 24 h). Furthermore, the DOX/CS_{100K}-PEG₂ micelles possessed the same killing capacity for HepG2 cell as free DOX in all the tested concentration range.

As a model system, HepG2 cells were incubated with the DOX/CS_{100K}-PEG₂ micelles in the different time intervals. The confocal laser scanning microscopy (CLSM) images presented that strong DOX green fluorescence from the DOX/CS_{100K}-PEG₂ micelles, which was located in cytoplasm after 12 h incubation as shown in Figure 6A. Additionally, partial DOX green fluorescence was found in cell nuclei, indicating the DOX/CS_{100K}-PEG₂ micelles could be able to diffuse in cell nuclei in cytoplasm of the live cells after 12 h incubation. It is worth noting that intracellular DOX fluorescence of the DOX/CS_{100K}-PEG₂ micelles mainly located at the cell nuclei after 24 h incubation, compared to that of 12 h (Figure 6A). This result revealed the nucleus localization of DOX in tumor cells. Meantime, the morphologies of HepG2 cells treated with the DOX/CS_{100K}-PEG₂ micelles after 12 h incubation showed survived cell morphology, while most cells died after 24 h incubation with the DOX/CS_{100K}-PEG₂ micelles, as shown in bright field of Figure 6A and B. This demonstrated that the DOX/CS_{100K}-PEG₂ micelles presented obvious cytotoxicity toward the cells and showed significant cell inhibition for live cells [34,35], as revealed by the MTT assays. The finding is in accordance with well-known DOX's antitumor activity, which forwardly inserts double helix structure in DNAs and induce single-and double-strand breaks to disrupt the replication and transcription processes

[36]. Taking above exploration and analysis into consideration, the “off-on” switchable DOX delivery system with pH-responsive properties has been realized owing to the protonation of amine group of chitosan in response to the slightly acidic media.

4. Conclusions

In summary, a facile strategy was developed to fabricate the drug leakage-free DDS by means of the solubility of chitosan in different pH media as an “off-on” switch. The PEGylated chitosan was used as an amphiphilic precursor for molecular self-assembly by hydrophobic interaction in PBS. The resulting DOX/CS_{100K}-PEG₂ micelles possess well-defined core-shell structure and a narrow size distribution. More importantly, they also own highly stability at physiological pH with ultra-low drug leakage of only 2.32% within 60 h, and burst release properties in a stimulated microenvironment of tumor tissues, realizing the “off-on” switchable release properties. With the help of biocompatible PEGylated chitosan, the DDS possesses remarkable biocompatibility, and hydrophobic anticancer DOX could be efficiently and rapidly transported into cell nuclei of tumor cells and showed significant cell inhibition. Meantime, after the pH-triggered intracellular release of DOX at the tumor microenvironment, the DOX/CS_{100K}-PEG₂ micelles could transform into the water-soluble CS_{100K}-PEG₂, which could be degraded, and then eliminated by metabolic system. So the site-specific delivery of anti-cancer drug could be efficiently achieved in the tumor tissues through the proposed easy-to-prepare biocompatible

drug leakage-free chitosan-based DDS.

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Table 1. DOX/CS-PEG micelles prepared with different feeding amounts of PEG₂₀₀₀-CHO and DOX.

Samples	DOX/CS 50K-PEG	DOX/CS 100K-PEG₁	DOX/CS 100K-PEG₂	DOX/CS 100K-PEG₃	DOX/CS 300K-PEG
PEG (Feeding ratio%)	25.00	25.00	45.00	65.00	25.00
N%^a	2.86	3.01	1.98	1.51	3.26
PEG%^b	16.20	15.14	27.12	38.00	13.21
DOX (Feeding ratio%)	100	100	100	100	100
DOX%^c	48.21	44.23	32.14	14.70	16.81

^a Nitrogen content of the samples determined by elemental analysis;

^b PEG content of the samples calculated from elemental analysis; % means degree of substitution of PEG per constitutional unit of chitosan. For example, in DOX/CS_{100K}-PEG₂ micelles, 27.12% indicates that 27.12 of constitutional unit are reacted with PEG per 100 constitutional unit of chitosan.

^c DOX content of the samples assessed by UV spectrophotometer from standard curve of DOX ; all percentage chemical composition are shown weight ratio. For example, in DOX/CS_{100K}-PEG₂ micelles, 32.14% indicates that 1 mg of CS_{100K}-PEG₂ encapsulated 0.3214 mg of DOX.

Figure Captions

Scheme 1. Schematic diagram of the formation, and DOX release from the

DOX/CS-PEG micelles, triggered by the slightly acidic media in tumor cells.

Scheme 2. The synthesis route of CS-PEG.

Figure 1. FT-TR spectra of CS, DOX, CS-PEG, and DOX/CS_{100K}-PEG₂ micelles (A),

UV-vis spectra of DOX/CS_{50k}-PEG, DOX/CS_{100K}-PEG₁, DOX/CS_{100K}-PEG₂,

DOX/CS_{100K}-PEG₃ and DOX/CS_{300k}-PEG in neutral media (B), fluorescent

emission spectra of DOX/CS_{50k}-PEG, DOX/CS_{100K}-PEG₁, DOX/CS_{100K}-PEG₂,

DOX/CS_{100K}-PEG₃ and DOX/CS_{300k}-PEG in neutral media (C), and cumulative

DOX release from the DOX/CS_{100K}-PEG₂ micelles in PBS (pH=7.4), PBS (pH=6.5)

and PBS (pH=5.0) at 37 °C (D).

Figure 2. The TEM and DLS results of: (a) DOX/CS_{50K}-PEG micelles, (b)

DOX/CS_{100K}-PEG₁ micelles, (c) DOX/CS_{300K}-PEG micelles, (d)

DOX/CS_{100K}-PEG₂ micelles, and (e) DOX/CS_{100K}-PEG₃ micelles, in PBS (pH 6.8);

(f) DOX/CS_{100K}-PEG₂ micelles in PBS (pH 5.0).

Figure 3. The DLS result and TEM image of DOX/CS_{100K}-PEG₂ micelles in PBS (pH

7.4).

Figure 4. The TEM images of the CS_{100K}-PEG₂ micelles in PBS (pH 7.4 (a) and pH

5.0 (b)).

Figure 5. Cytocompatibility of DOX/CS_{100K}-PEG₂, and antitumor capability of

DOX/CS_{100K}-PEG₂ determined by standard MTT assay. Data are presented as the

mean $\pm$ standard deviation (SD; n = 5).

Figure 6. CLSM images of cellular uptake of the DOX/CS_{100K}-PEG₂ micelles after 12

h (A) and 24 h (B) incubation. HepG2 cells were stained by Hoechst, The

concentration of DOX/CS_{100K}-PEG₂ micelles was 20 μ g/mL.

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(a)

(b)

(c)

(d)

(e)

(f)

Figure 2. The TEM and DLS results of: (a) DOX/CS_{50K}-PEG micelles, (b) DOX/CS_{100K}-PEG₁ micelles, (c) DOX/CS_{300K}-PEG micelles, (d) DOX/CS_{100K}-PEG₂ micelles, and (e) DOX/CS_{100K}-PEG₃ micelles, in PBS (pH 6.8); (f) DOX/CS_{100K}-PEG₂ micelles in PBS (pH 5.0).

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7.4).

(a)

(b)

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Graphical Abstract