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Chitosan-*g*-poly(*N*-isopropylacrylamide) based nanogels for tumor extracellular targeting

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ABSTRACT

The principle objective of this research was to develop and characterize pH-responsive and biocompatible nanogels as a tumor-targeting drug delivery system. The nanogels were self-assembled from chitosan-based copolymers, chitosan-*graft*-poly(*N*-isopropylacrylamide) (CS-*g*-PNIPAm). The copolymers were synthesized via free radical copolymerization and characterized for their chemical structure by FT-IR and ^{1}H NMR. These copolymers could be efficiently loaded with oridonin (ORI) and the characteristics of ORI-loaded nanogels were evaluated. Drug release researches indicated that the ORI-loaded nanogels displayed pH-dependent release behaviors. Based on MTT assay and cellular morphological analysis, the anti-tumor activity of ORI-loaded nanogels was higher at pH 6.5 than at pH 7.4. In conclusion, the obtained nanogels appeared to be of great promise in tumor extracellular pH targeting for ORI.

1. Introduction

Oridonin (ORI, Fig. 1), extracted from the Chinese traditional medicine *Rabdosia rubescens*, is a potent anticancer agent in Chinese traditional medicine. Both pharmacological experiments and clinical trials have demonstrated that ORI is effective against a variety of tumors and cancer cell types including: liver, prostate, breast and cervical cancer cells, non-small cell lung cancer cells, acute promyelocytic leukemia, and glioblastoma multiforme (Li et al., 1985; Zhang et al., 2003; Zhang and Ren, 2003). However, its clinical application against cancers has been impeded by its low therapeutic index induced by the poor solubility and nonspecifically systemic distribution. Hence, it is of great necessity to develop an alternative carrier of ORI for its referable application.

Recently, pH-responsive nanoparticles have been proposed as promising anti-cancer drug devices. Compared with other nanoparticles, these pH-sensitive nanoparticles create desirable switching carriers in release kinetics, from slow release while circulating to rapid release once target sites have been reached (Na et al., 2003; Lee et al., 2007; Yin et al., 2008; Garbern et al., 2010; Jiang et al., 2010). Amongst these particles, nanoparticles based on chitosan have been paid more and more attention due to their biocompatibility and non-toxicity (Cai et al., 2005; Fan et al., 2008; Li et al., 2009; Wu et al., 2010). Previous studies indicated that nanoparticles composed of NIPAAm and chitosan could achieve a pH-sensitive drug release and an enhanced anti-cancer activity under a slightly acidic environment (Fan et al., 2008; Li et al., 2009).

Therefore, in this research, efforts were devoted to exploit a pH-responsive nanoparticulate delivery system for ORI, intended to improve the therapeutic index. We synthesized CS-*g*-PNIPAm copolymers by free radical copolymerization using APS as an initiator and *N*,*N*-methylenebisacrylamide (MBA) as a cross-linking agent and applied them as the matrices of nanogels. The ORI-loaded nanogels were prepared by a self-assembly method and their physicochemical properties were studied. The in vitro release behaviors of ORI from the nanogels were determined at different pH values. Eventually, the in vitro anti-tumor activities were compared between the ORI solution and ORI-loaded nanogels at different pH values.

2. Materials and methods

2.1. Materials

N-Isopropylacrylamide (NIPAAm, Sigma) was purified by repeated re-crystallization from a mixture of toluene and hexane (1:5, v/v). Chitosan (medium M_w = 200,000, degree of deacetylation: 96%), *N*,*N*-methylenebisacrylamide (MBA) and ammonium

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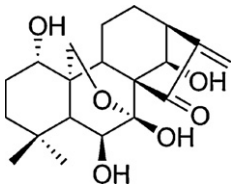

Fig. 1. Chemical structure of ORI.

persulfate (APS) were all purchased from Sigma and used without further purification. Oridonin (98%) was from Nanjing Zelang Pharmaceutical Co., Ltd. MTT and all other chemicals and reagents were obtained from Sigma. Hoechst 33342 and other biological reagents were purchased from Nanjing KeyGen Biotechnology Co., Ltd. All the other chemicals and solvents were of chromatographic and pharmaceutical grade.

2.2. Preparation of CS-g-PNIPAm copolymers

The copolymers of CS-*g*-PNIPAm were synthesized via a free radical copolymerization route (Mahdavinia et al., 2004). The copolymerization was carried out in a three-neck flask equipped with a stirrer, a reflux condenser, and a nitrogen inlet/outlet. An appropriate amount of CS (0.25 g) was first dissolved in 100 mL acetic acid solution (0.60%, v/v) under magnetic stirring in the flask. After CS solution was heated to 80 °C under nitrogen, APS (1.5 mL, 1.0×10^{-2} mol/L) was added. The solution was stirred for 10 min before NIPAAm (1.0 g) and MBA (0.010 g) were added. The mixture was stirred for 3 h under nitrogen. The resulting product was dialyzed against distilled water and further freeze-dried.

2.3. Study of chemical structure

Fourier transform infrared (FT-IR, BRUKER VERTEX 70, German) and ^{1}H nuclear magnetic resonance (^{1}H NMR, Inova-600, Varian, USA) measurements were utilized to characterize the chemical structure of CS-*g*-PNIPAm.

2.4. Preparation of ORI-loaded nanogels

ORI-loaded nanogels were prepared by a self-assembly method (Wang et al., 2007). Briefly, the copolymers (500 mg) and ORI (50 mg) were dispersed in 50 mL distilled water. And the dispersion was sonicated using a probe-type sonifier (JY92-Ultrasonic Processor Xinzhi, Linbo, Co., Ltd., China) at 100 W in an ice bath for 2 min. The resultant ORI-loaded nanogels were collected by centrifugation and lyophilized for storage and use.

An exactly weighted amount of the ORI-loaded nanogels was hydrolyzed in 1 mol/L HCl at 60 °C for 1 h and the content of total drug was determined using high-performance liquid chromatography (HPLC) analysis system (Agilent, USA). Free ORI was separated from the ORI-loaded nanogels by ultracentrifugation at 4000 rpm for 20 min and the content was analyzed by the HPLC. The mobile phase, consisting of methanol/water (55:45, v/v), was delivered at a flow rate of 1.0 mL/min. Eluted compounds were detected at a wavelength of 238 nm. The standard curve for the quantification of ORI was linear over the range of 1.04–104 μg/mL with a correlation coefficient of 0.9997. Encapsulation efficiency and drug loading were calculated using the following equations: Encapsulation efficiency (%) = (weight of total drug − weight of free drug found)/weight of total drug × 100; drug loading (%) = (weight of total drug − weight of free drug found)/weight of drug-loaded nanoparticles × 100.

2.5. Characterizations of ORI-loaded nanogels

2.5.1. Morphology and particle size measurement

The particle sizes of the ORI-loaded and unloaded nanogels were estimated by the dynamic light scattering (DLS) method using a Dawn Heleos, Wyatt QELS, and Optilab DSP instrument (Wyatt Technology Co., USA). The morphologies of the unloaded and ORI-loaded nanogels were observed by transmission electron microscope (TEM, Hitachi, Japan).

2.5.2. Crystalline state

The crystalline state of materials was estimated using an X-ray diffractometer (D/max r-B, Rigaku, Japan). Analysis was performed on CS, CS-*g*-PNIPAm, ORI and the freeze-dried ORI-loaded nanogels to investigate modifications of internal structure after drug incorporation.

2.6. Drug release from nanogels

The in vitro release of ORI from the ORI-loaded nanogels was studied at different pH values (pH 7.4, 7.0, 6.5, 6.0 and 5.0). The lyophilized ORI-loaded nanogels (containing 1 mg ORI) were dispersed in 2 mL of different solutions, which was transferred to a dialysis membrane bag (molecular cutoff = 12 kDa). Then, the sample-containing bag was immersed in 30 mL of homogeneous solution and kept at 37 °C with continuously magnetic stirring. At selected time intervals, 1 mL of the release medium outside the dialysis bag was withdrawn and the equal volume of fresh buffer solution was added. The amount of ORI was finally determined using the HPLC method as previously described for the measurement of encapsulation efficiency. Each experiment was repeated in triplicate.

2.7. Cytotoxicity and cell morphology

2.7.1. Cell culture

Human hepatocellular carcinoma cell line HepG2 cells, kindly supplied by the Department of Pharmacology, Shandong University, were cultured in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified incubator with an atmosphere of 5% CO_2.

2.7.2. MTT assay

HepG2 cells were seeded in 96-well culture plates for 12 h and were then exposed to the blank or ORI-loaded nanogels at various concentrations for 24 h. Subsequently, 15 μL of MTT (5 mg/mL) was added to each well. After 4 h incubation, culture media were discarded and 150 μL of DMSO was added. The optical density (OD) was measured at 570 nm with a Microplate Reader. The cell inhibitory rate was calculated as follows: Inhibitory rate (%) = $(A_{570\ \text{control cells}} - A_{570\ \text{treated cells}})/A_{570\ \text{conttol cells}} \times 100$. The IC_{50} value was defined as the drug concentration of 50% inhibition rate relative to controls.

The pH-sensitive cytotoxicities of free ORI and the ORI-loaded nanogels against HepG2 cells were measured by initially plating the cells in 96-well tissue culture dishes for 12 h at pH 6.5 or 7.4 and then exposing them to the free ORI solution or ORI-loaded nanogels at the same pH for 24 h, respectively.

2.7.3. Observation of cell morphology

HepG2 cells were seeded in 6-well plates and allowed to grow overnight at different pH values (pH 6.5 and 7.4). The cells were then treated with the blank nanogels, free ORI solution and ORI-loaded nanogels for 24 h. Finally, the cellular morphologies were estimated using reverse fluorescence microscopy (Nikon, Japan).