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Preparation and evaluation of folic acid modified succinylated gelatin micelles for targeted delivery of doxorubicin

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ABSTRACT

In this study, novel folic acid modified succinylated gelatin (FA-SG) micelles were prepared and used as drug carrier in folate-receptor (FR) targeted antitumor therapy. FA-SG copolymer was synthesized and characterized by ^{1}H NMR and fluorescence spectroscopy. Doxorubicin-loaded FA-SG (DOX-FA-SG) micelles were prepared by a dialysis method. The shape of DOX-FA-SG micelles was almost spherical by transmission electron microscopy analysis. The mean diameter of DOX-FA-SG micelles was 277 nm, and the DOX-loading content was 12.4%. *In vitro* drug release of DOX-loaded micelles showed sustained release behavior for 168 h. DOX-FA-SG demonstrated significantly greater cellular uptake and cytotoxicity against FR-positive MCF-7 cells than the non-targeted DOX-loaded micelles. Importantly, DOX-FA-SG micelles exhibited higher antitumor activity in MCF-7 tumor-bearing mice than doxorubicin hydrochloride (DOX·HCl) and DOX-loaded micelles without FA modification. Taken together, FA-SG micelles could be an effective carrier for targeted drug delivery.

1. Introduction

Gelatin is a natural and hydrophilic material derived from collagen, which is the most abundant component of the extracellular matrix in the body tissue [1,2]. And it has been widely used in food, pharmaceutical and medical purposes because of its biocompatibility and biodegradability [3,4]. Further, gelatin has the reactive amino groups in the backbones. This chemical structure can be modified with various organic molecules to form amphiphilic conjugates. For example, an amphiphilic gelatin macromolecule was synthesized by grafting hydrophobic hexanoyl anhydrides to the amino groups of primitive gelatin, and was capable of self-assembling to form micelle-like nanoparticles [3]. The antitumor agent camptothecin was released from the amphiphilic gelatin nanoparticles in a combined effect of diffusion and degradation. These nanoparticles permit an intracellular nanotherapy effectively operated for anti-cancer purposes [5].

For the past few decades, polymeric amphiphiles have received tremendous attention owing to their potential applications in drug delivery systems [6–8]. These amphiphiles can form micelles in aqueous media and be used as carrier for hydrophobic drug delivery. Polymeric micelles can prolong the half-life time of encapsulated chemotherapy drugs *in vivo* and accumulating in the tumor regions via the enhanced permeability and retention (EPR) effect [9–11]. Furthermore, active targeting polymeric micelles can be achieved by conjugating targeting moieties or ligands onto the outer surface of the conjugates [12–14]. The targeting molecules can specifically bind to receptor which was either uniquely expressed or overexpressed on the tumor cell surface, but was low or negligible expression in normal cells. As our previous study, glycyrrhetinic acid (GA) modified gelatin (GA-GEL) conjugates with three substitution degrees were synthesized and characterized. GA could specially bind with GA receptor, and GA-modified micelles could be effectively transported into hepatic cells by endocytosis. Doxorubicin-loaded GA-GEL micelles exhibited better antitumor activity in H22 orthotopic mice than DOX·HCl. These results indicated that GA-GEL could be used as carrier of hydrophobic drug for targeting hepatocellular carcinoma [15]. In addition, folic acid (FA) is a low-molecular-weight vitamin, and can selectively bind to folate receptor (FR) overexpressed in human cancer cells and highly restricted in most normal tissues. Then FA has been conjugated to polymeric micelles and liposomes for the purpose of active targeting against

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tumors [16–18].

In this present work, the objective was to synthesize FA-modified succinylated gelatin (FA-SG) conjugate and construct drug-loaded micelles for targeted delivery in FR-overexpressed tumors. Doxorubicin (DOX) is a widely used antitumor drug in the clinical field. Then DOX-loaded FA-SG micelles were prepared by a dialysis method. The physicochemical properties of DOX-loaded micelles were investigated, and drug release behavior *in vitro* was studied. Moreover, the cellular uptake and cytotoxicity of DOX-FA-SG micelles were evaluated in FR-negative A549 and FR-positive MCF-7 cancer cells. *In vivo* antitumor efficacy of DOX-loaded micelles was also investigated in MCF-7 bearing nude mice.

2. Materials and methods

2.1. Chemicals, cell lines and animals

Gelatin (type A), succinic anhydride and 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma-Aldrich (St. Louis, OM, USA). Doxorubicin hydrochloride (DOX·HCl) was from Beijing Huafeng United Technology Co. Ltd (Beijing, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 3-(4, 5-dimethyl-thiozol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were obtained from Aladdin Industrial Corporation (Shanghai, China). Pyrene was provided by Acros Organics (Beijing, China). RPMI1640 medium and trypsin-EDTA were purchased from Jinuo Biotechnology Company (Hangzhou, China). Fetal bovine serum (FBS) was provided by Sijiqing Biological Co. Ltd (Hangzhou, China). All other chemical reagents were of analytical grade.

A549 and MCF-7 cells were provided by the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China). The cells were routinely cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin and grown at 37 °C in 5% CO_2 atmosphere. Female BALB/c nude mice (20 ± 2 g) were purchased from Hunan SLAC Jingda Laboratory Animal Co. Ltd (Changsha, China) and maintained in a pathogen-free condition. All animal procedures were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The protocol of the study was approved by the Animal Ethics Committee of Jiujiang University (approval number: 20170302). The European Community guidelines as accepted principles for the use of experimental animals were adhered to.

2.2. Synthesis of FA-SG conjugate

Two step procedures were adopted for the synthesis of FA-SG (Fig. 1). Firstly, gelatin (1.0 g) was dissolved in 16 mL of deionized water. 1.6 mL of NaOH (0.1 N) were added and stirred at 37 °C. Then succinic anhydride (0.1 g) was added. After reaction for 24 h, the mixed solution was cooled at room temperature and the pH value was adjusted to 7.4 with diluted sodium hydroxide [3]. Subsequently, the mixture was dialyzed (MWCO:14 kDa) against the excess amount of deionized water for 2 days. The dialyzed solution was freeze-dried. The lyophilized product was dispersed in anhydrous ethanol under the help of ultra-sonication to remove unreacted succinic anhydride. The mixture was centrifuged at 12 000 rpm for 5 min (Sigma laboratory centrifuges 3K18, Germany), and ethanol solution was discarded. The precipitated product was dissolved in water and dialyzed against water for 1 day. The dialyzed solution was filtered through 0.8 μm membrane and lyophilized. The product succinylated gelatin (SG) was obtained. Secondly, SG (300 mg) was dissolved in 10 mL of deionized water and diluted with 8 mL of ethanol. EDC (42.8 mg) and FA (30 mg) were added. The reaction mixture was stirred for 5 h at 70 °C. The cooled mixture was centrifuged at 12 000 rpm for 2 min. The supernatant was dialyzed (MWCO: 14 kDa) against deionized water for 24 h, and followed by freeze-drying. Finally, FA-SG conjugate was obtained.

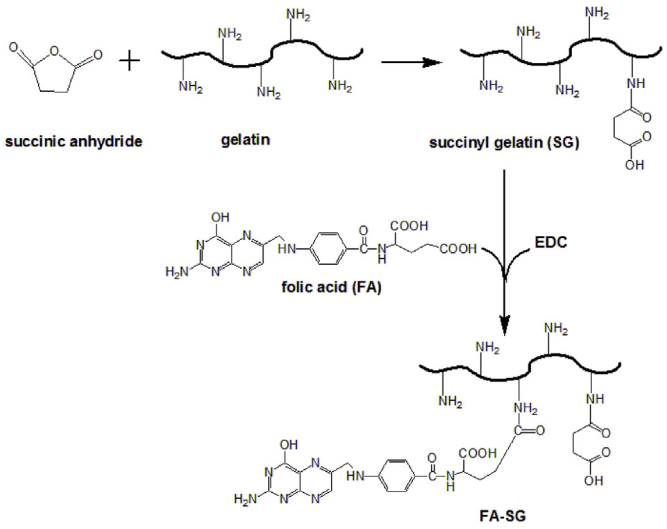

Fig. 1. Synthetic scheme of FA-SG conjugate.

The chemical structure was investigated by ^{1}H NMR (Avance DMX500, Bruker, Germany). D_2O was used as the solvent. The substitution degrees of amino groups in SG were determined by TNBS method [3,15]. Briefly, a serial of gelatin or SG solutions were prepared. Each stock solution was taken out, and deionized water was added to a final volume of 2 mL. Then 2 mL of 4% sodium hydrogen carbonate and 2 mL of 0.1% TNBS were added. The mixed solution was incubated in 37 °C for 2 h. The absorbance was detected by UV spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd, China) in 350 nm. To determine the amount of FA in the conjugate, FA-SG was dissolved in water and diluted with dimethyl sulfoxide (DMSO). The extent of FA conjugation was studied by UV spectrophotometry at 365 nm (TU-1901, Beijing Purkinje General Instrument Co., Ltd, China).

2.3. Preparation of DOX-loaded micelles

DOX-loaded micelles were prepared by a dialysis method. Briefly, copolymer samples (100 mg) were dissolved in deionized water (5 mL). 20 mg of DOX·HCl were stirred with excess triethylamine (mole ratio of triethylamine to DOX·HCl = 3:1) in 5 mL of DMSO overnight under the dark condition. Then DOX solution was added to the above copolymer solution. The mixture solution was magnetically stirred for 6 h, and placed into the dialysis bag (MWCO: 14 kDa) for dialysis against deionized water for 24 h. The dialysis solution was filtered through a 0.8 μm membrane and lyophilized.

2.4. Characterization of blank and DOX-loaded micelles

Fluorescence spectroscopy was adopted to study the critical micelle concentration (CMC) of the conjugate. Pyrene was used as a probe. Briefly, the conjugate suspension was prepared and adjusted to various concentrations. 1 mL of pyrene in acetone (6.0×10^{-7} M) was added to each of a series of 10-mL vials, and the solvent acetone was evaporated. Then, 10 mL of various concentrations of sample suspension were introduced into each vial. The mixed solution was heated at 50 °C for 10 h, and remained undisturbed overnight at room temperature. Fluorescent scan spectra were measured using a fluorescence spectrophotometer (Hitachi F-7000, Japan). The excitation and emission wavelengths were set at 339 and 390 nm, respectively.

The mean diameter and size distribution of blank and DOX-loaded micelles were analyzed by dynamic light scattering (90Plus, Brookhaven Instruments Corp., USA). The morphology of the micelles was observed using a transmission electron microscopy (JEM-1230, Jeol, Japan). A drop of sample solution was placed onto a 300-mesh