European Journal of Pharmaceutics and Biopharmaceutics 128 (2018) 247–258

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Acid-degradable lactobionic acid-modified soy protein nanogels crosslinked by ortho ester linkage for efficient antitumor in vivo

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ARTICLE INFO

Keywords:
Soy protein
Ortho ester
Nanogels
Lactobionic acid
Antitumor

ABSTRACT

It remains a crucial challenge to achieve efficient cellular uptake and intracellular drug release in tumor cells for the nanoscale drug delivery systems. Herein, acid-degradable nanogels were prepared by cross-linking methacrylated soy protein with an acid-labile ortho ester cross-linker (NG1), and then modified with lactobionic acid (LA) to give tumor-targeted nanogels (NG2). Both NG1 and NG2 displayed excellent stability in neutral environment, while showed pH-triggered degradation behaviors under mildly acidic conditions resulting from the breakage of ortho ester bonds. Doxorubicin (DOX) was successfully loaded into nanogels, which exhibited an accelerated release at low pH. In vitro cell studies demonstrated that LA-modified nanogels could effectively improve cellular internalization, show higher cytotoxicity and apoptosis toward asialoglycoprotein receptor (ASGPR) over-expressed HepG2 cells. In vivo antitumor experiment proved that LA modification could significantly enhance the tumor-targeting ability of nanogels and increase DOX concentration in tumor site, leading to better therapeutic efficacy. Histological analysis further demonstrated that soy protein-based nanogels did not cause any damage to normal organs. Overall, these pH-sensitive and tumor-targeting soy protein-based nanogels can be potential drug carriers for efficient tumor treatment.

1. Introduction

In the past few decades, biocompatible drug delivery systems (DDS) including micelles, vesicles, liposomes and nanoparticles have substantially contributed to the progress of tumor diagnosis and therapy owing to their desirable advantages [1,2]. Compared with other nanoscale carriers, nanogels have been considered as one of the most potential drug delivery platforms because of their attractive properties such as high water content, hydrophilic three-dimensional networks, excellent colloid stability, and high drug loading [3,4]. Furthermore, nanogels can also prolong drug circulation and passively deliver drugs to tumor site by the enhanced permeability and retention (EPR) effect, thus augment antitumor efficiency and reduce side effects [5–9]. Based on these advantages, various kinds of nanogels have been investigated as anticancer drug carriers to improve chemotherapy effects [10–13]. Generally, nanogels can be prepared via synthetic or natural polymers based on the physical or chemical cross-linking interactions [14]. Compared with synthetic materials, natural biopolymers have lower systemic toxicity and excellent biodegradability, which make them attract more and more attention on the preparation and application of nanogels [15–18].

Soy protein (SP) as a renewable plant protein, has been widely used in food production and tissue engineering owing to their natural abundance, high-nutrition, excellent bioactivity, and low immunogenicity [19–21]. Besides, there is a balanced composition of nonpolar, polar, and charged amino acids on its molecular structure, thus being able to provide lots of functional sites for further modification, which endows SP with great potential as nano-carriers for antitumor drug delivery [22,23]. For example, Jin et al. reported a core–shell structured nanogel via self-assembly of soy protein and dextran, which displayed a desirable stability against various environmental conditions [24]. Ding prepared soy protein/soy polysaccharide complex nanogels using a high-pressure homogenization method, and the resultant nanogels could successfully protect and deliver folic acid [25].

Although soy protein has so many advantages, there are still some restrictions in the design and development of nanogels using SP. Commonly, SP nanogels are prepared mainly through heat or ultrasound treatment, which lacks good particle dispersion and uniform size distribution, thus leading to inadequate stability and particle aggregation [26,27]. Besides, drug release from these nanogels was mainly dominated by passive diffusion due to the swelling of nanogels, resulting in a non-specific release in vitro [25]. Furthermore, the absence

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https://doi.org/10.1016/j.ejpb.2018.05.011
Received 21 November 2017; Received in revised form 28 April 2018; Accepted 3 May 2018
Available online 04 May 2018
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of targeting ability makes nanogels inefficient cellular uptake in tumor site and eventually leads to insufficient drug accumulation in cancer cells, which may hamper cancer chemotherapy to a large extent [28,29]. Therefore, there is a strong incentive to develop intelligent SP-based nanogels, which will be stable in blood circulation, release upon arrival at the tumor site, and accomplish efficient intracellular drug accumulation to maximize the therapeutic efficacy of anti-cancer drugs.

Herein, we aimed to prepare a type of intelligent soy protein-based nanogels with acid-degradable and tumor-targeting moieties. As well known, there are distinct pH gradients in the tumor micro-environment (6.5–7.2 in extracellular, 5.0–5.5 in endosomes and 4.0–5.0 in lysosomes) [30]. Based on this biological feature, the acid-responsiveness of SP-based nanogels can be easily realized by co-polymerizing methacrylated soy protein and acid-labile ortho ester cross-linker because ortho ester bonds hold good stability in neutral environment while rapidly hydrolyze under mildly acidic conditions [31–33]. Lactobionic acid (LA) as a tumor-targeting ligand was grafted on the surface of SP nanogels to further improve intracellular drug concentration, which was probably because LA could efficiently interact with tumor cells over-expressing the asialoglycoprotein (ASGP) receptors , and resulted in a high selectivity and binding affinity [34,35]. Besides, all operations were performed in aqueous solution without any surfactants or toxic organic solvents. The prepared SP and LA-modified SP nanogels were characterized by particle size, size distribution, and morphology. The acid-triggered degradation and drug release behaviors of these nanogels were measured at different pH values in vitro. The cellular uptake and growth inhibition of DOX-loaded SP nanogels in monolayer cell models and three-dimensional multicellular spheroids (MCs) were qualitatively and quantitatively evaluated by using confocal laser scanning microscope (CLSM) and flow cytometer (FCM). Histological staining was used to confirm the biocompatibility of these nanogels in vivo. Finally, the accumulation and antitumor activity in tumor tissue of SP nanogels with or without LA modification were also investigated using H22-bearing mice.

2. Experimental

2.1. Materials

Soybean protein (SP) was synthesized according to a previously reported method [36]. Doxorubicin hydrochloride (DOX) was purchased from Meilun Biological Technology (Dalian, China). Potassium persulfate (KPS) was purchased from J&K. Lactobionic acid (LA), N-(3-d-imethylaminop-y)-N'-ethylcarbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were bought from Mackin Biochemical Co., Ltd. Human neuroblastom cancer cell line (SH-SY5Y), human liver carcinoma cell line (HepG2), murine hepatic cancer cell line (H22) were obtained from KeyGen Biotech (Nanjing, China). Male ICR mice (18–22 g) were purchased from Anhui Medical University (Anhui, China).

2.2. Synthesis of methacrylated soy protein (MASP)

MASP was synthesized by chemically modifying with methacrylic anhydride [37]. Briefly, 1.0 g of soy protein (SP) was dissolved in 100 mL of phosphate buffer at pH 8.0, and 2 mL of methacrylic anhydride was slowly added into the above solution under ice bath. Then the mixture was stirred, and the pH value was maintained at 8.0 via adding sodium hydroxide for 12 h. After that, the mixture was placed into a dialysis bag (8–14 KDa MWCO) and dialyzed against deionized water for 24 h to remove unreacted molecules. Finally, the pure methacrylated soy protein (MASP) was harvested by lyophilization. The chemical structure of MASP was detected by ^{1}H NMR (Bruker Aavance 400 NMR spectrometer) using D_2O as solvent, and the substitution degree was calculated by the ninhydrin coloring method [36].

2.3. Preparation of SP and LA-SP nanogels

Nanogels were prepared by cross-linking MASP with an acid-labile ortho ester cross-linker (N,N'-((((oxybis(methylene)) bis(1,3-dioxolane-4,2-diyl)) bis (oxy)) bis(ethane-2,1-diyl)) bis(2-methylacrylamide), OEAM), which was synthesized following a reported method [31]. Briefly, MASP (60.0 mg) was dissolved in 20 mL of phosphate buffer at pH 8.5, KPS (100.0 mg) and OEAM (30.0 mg) were then added. The polymerization system was heated to 70 °C under a nitrogen atmosphere and slowly stirred for 45 min. During this stage, nanogels were formed by the polymerization between MASP and OEAM cross-linker. Finally, the formed SP nanogels were collected by centrifugation at 1×10^4 rpm for 10 min to remove any unreacted monomers or cross-linker. Next, lactobionic acid (LA) was introduced to the nanogels' surface via an EDC/NHS-mediated amidation reaction to give LA-modified SP nanogels (LA-SP). And the lactobionic acid content was determined by the phenol/sulfuric acid method [38]. The SP and LA-SP nanogels were named as NG1 and NG2, respectively.

2.4. Characterization of nanogels

NG1 and NG2 were analyzed by Fourier transform infrared (FT-IR, NEXUS-870, Nicolet, USA). To obtain FT-IR spectra, samples were mixed with KBr powder at weight ratio of 1/100 and scanned from 4000 to 500 cm^{-1} for 32 times with 2 cm^{-1} resolution to average signal at 25 °C. Z-average hydrodynamic diameter and distribution of nanogels were evaluated using a Zetasizer dynamic light scattering (DLS) detector (Malvern Zetasizer Nano ZS, 6 mM laser, 633 nm incident beam, 173°scattering angle , United Kingdom). All samples were diluted to proper concentration before measurement, and the measurements were repeated three times. The morphology of NGs was imaged by transmission electron microscopy (TEM, JEM-2100, Japan) and scanning electron microscopy (SEM, HITACHI S-4800, Japan). For TEM observation, one drop of nanogel solution was added onto a copper grid and allowed to air-dry at room temperature, and then observed without any staining at an acceleration voltage of 80 kV. For SEM observation, one drop of nanogel solution was dropped onto the surface of a silicon wafer and air-dried at room temperature. Then all samples were coated with a thin layer of gold before observation.

2.5. In vitro acid-triggered degradation of nanogels

To assess the acid-triggered degradation behavior of nanogels, 2 mL of NG1 or NG2 was dispersed in 0.01 M phosphate buffers (pH 5.0, 6.0 and 7.4), and incubated at 37 °C with a shaken speed of 100 rpm. The size and light intensities were monitored at predetermined time by DLS. Meanwhile, the morphology of each sample was observed by SEM. In addition, the kinetic stability of nanogels was also measured in different physiological conditions such as saline, PBS, FBS, and cell culture medium (RPMI 1640) at room temperature by DLS.

2.6. Drug loading and in vitro release

A certain amount of DOX was dispersed into 3 mL of NG1 or NG2 (2.5 mg/mL) suspensions (phosphate buffer, pH = 8.0), and the mixture was slowly stirred at room temperature for 8 h in the dark. After that, the DOX-loaded nanogels were separated by centrifugation at 1×10^4 rpm for 15 min. The resultant DOX-loaded NG1 and NG2 were named as DOX/NG1 and DOX/NG2, respectively. And the size and zeta potential of loaded nanogels were also measured by the Zetasizer dynamic light scattering detector. The amount of DOX in the supernatant was measured by a microplate reader (Molecule Devices, USA) at an excitation wavelength of 480 nm and an emission wavelength of 590 nm. And the amount of DOX loaded into the nanogels was calculated as formula (1) and (2):

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