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Folic acid-modified soy protein nanoparticles for enhanced targeting and inhibitory

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ABSTRACT

Soy protein isolate (SPI) was hydrolyzed by compound enzymes to give water soluble low molecular soy protein (SP). SP and folic acid (FA) modified SP was polymerized with N-3- acrylamidophenylboronic acid (APBA) monomer in aqueous solution to give SP nanoparticles (SP NPs) and FA modified nanoparticles (FA-SP NPs), respectively. These NPs display excellent stability in different conditions, and have a uniform spherical shape with a diameter around of 200 nm. Doxorubicin (DOX) was then successfully loaded into SP and FA-SP NPs with a desirable loading content of 13.33% and 16.01%, respectively. The cellular uptake and cytotoxicity of DOX-loaded SP NPs and FA-SP NPs were investigated using the two-dimensional (2D) monolayer cell model and three-dimensional (3D) multicellular spheroids (MCs). In vivo, tumor accumulation and growth inhibitory were then examined using H22 tumor-bearing mice. All these results demonstrated that conjugation of FA can efficiently enhance SP-based NPs' tumor accumulation and antitumor effect.

1. Introduction

Chemotherapy with small molecular anticancer drugs is a traditional treatment for many types of solid tumor [1–3]. However, the therapeutic effects are always restricted due to rapid eliminate from body and low drug accumulation in tumor sites of anticancer drugs. To achieve better antitumor effects, the development of drug carriers with long-circulating time in body and active tumor accumulating capability are highly required [4,5]. Up to now, various nanoparticles (NPs) as drug carriers based on proteins, including albumin, gelatin, silk, and whey were prepared because they can easily encapsulate drug, food, and nutrients [6–9]. These proteins-based NPs are well-known to possess longer half-life in blood, higher tumor permeability, and improved cellular uptake than the conventional small molecular chemotherapy agent [10–12]. Compared with NPs formed by synthetic polymers, proteins-based NPs have lower systematic toxicity and excellent degradability, which made them a promising candidate for anticancer drug delivery [13–16].

Among them, soy protein isolate (SPI) is one of the most promising candidates for preparing NPs as drug delivery systems [17,18]. SPI, as a renewable plant protein, has been widely used in food production and biomaterial engineering owing to their natural abundance, inexpensiveness, high-nutrition, biodegradability, and biocompatibility in vivo [15,19,20]. SPI is mainly composed of two globular protein fractions: β-conglycinin (7S) and glycinin (11S), with a molecular weight of 141–170 KDa and 300–380 KDa [21,22]. Furthermore, there are plenty of active groups such as amino, carboxyl, and hydroxyl groups in SPI, which provide a lot of functional sites for further modification, which makes SPI an ideal biopolymer for tumor-target drug delivery [23].

Traditionally, SPI-based NPs were prepared by two simple methods: ethanol desolvation and Ca^{2+}-induced cold gelation. Wang group has reported that SPI-based NPs with a diameter of around 150 nm displayed desirable stability and satisfactory drug loading capacity for hydrophobic compounds such as curcumin [23,24]. However, the obtained NPs need to be cross-linked by glutaraldehyde to prevent them from dissociation before drug loading and in vitro using. Otherwise, these methods lack the ability to control the shape and structure of the formed NPs, resulting in un-uniform size and aggregation. In addition, the utilization of organic solvents such as ethanol and dimethyl sulfoxide in preparation of SPI-based NPs has limited their application as drug carriers [8]. Therefore, it is highly desired to develop a secure and non-toxic method to prepare SPI-based NPs without using any organic solvents. Unfortunately, preparation and application of SPI-based NPs is often compromised by its poor solubility in aqueous solution [25]. In order to improve its solubility, a lot of methods are developed, including physical degeneration, chemical modification, and enzyme degradation [14,15].

In present work, SPI was used to preparer tumor-targeted NPs as a drug delivery system. To improve its solubility in aqueous solution, SPI was first enzymatically degraded to give low molecular soy protein (SP), and then modified with folic acid (FA) as a tumor-targeting ligand to give FA-conjugated SP (FA-SP). Non-targeted SP and tumor-targeted FA-SP NPs were prepared in aqueous solution by a previously reported

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biopolymer-monomer polymerization method without any utilize of surfactant, cross-linker, and organic solution [26,27]. SP and FA-SP NPs were characterized in terms of their particle size, zeta potential, and morphology using DLS, TEM, and SEM. The kinetic stability of these SP-based NPs were also evaluated in different conditions. Doxorubicin (DOX), as a therapeutic drug, was encapsulated into SP and FA-SP NPs. Next in vitro release profiles from DOX/SP and DOX/FA-SP NPs were measured within different pH values. Thereafter, the accumulation and growth inhibition of these DOX-loaded NPs were investigated in two-dimensional cell models and three-dimensional multicellular spheroids (MCs). Finally, H22 tumor-bearing mice were used as in vivo model to assess tumor accumulation and anti-tumor efficiency of DOX-loaded SP and FA-SP NPs.

2. Experimental

2.1. Materials

Soybean proteins isolate (SPI) was purchased from ZhongshiDuqing Biotech Co., Ltd. (Shandong, China). The isoelectric point of SPI is about 4.5. Compound enzymes were obtained from Doing Higher biotech Co., Ltd. (Nanning, China). Acryloyl chloride and 3-aminophenyboronic acid were provided by Aladdin Industrial Corporation. 4,4′-Azobis(4-cyanovaleric acid) (ACVA), *N*-hydroxysuccinimide (NHS), and *N*-(3-dimethylaminopropy)-*N*′-ethylcarbodiimide hydrochloride (EDC) were obtained from Mackin Biochemical Co., Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC) was purchased from TCI Development Co., Ltd. (Shanghai, China). Doxorubicin hydrochloride (DOX) was purchased from Meilun Biological Technology (Dalian, China). Folic acid hydrate (FA) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were obtained from Sigma Chemical Co., Ltd. Male ICR mice (18–22 g, and 6–8 weeks old) were purchased from Anhui Medical University (Anhui, China).

2.2. Preparation of SP and FA-SP

SPI was dissolved in 100 mL deionized water, and stirred for 15 min at 90 °C by a magnetic stirrer. Compound enzymes (0.5%) were added to the solution, after the temperature and pH of mixture solutions was then adjusted to be 50 °C and 7.5, respectively. The reaction was carried out for 9 h, and maintained the temperature and pH stable. Finally, the temperature was increased to be 90 °C for 10 min to stop the reaction. The above solution was cooled to room temperature to give mixed soy protein, which was then centrifuged at 4500 rpm for 20 min. The supernatant was collected, and dialyzed by a dialysis bag with a cut-off molecular weight of 8 KDa, and finally low molecular weight soy protein (SP) was obtained by vacuum freeze drying. The molecular weight of original SPI, mixed soy protein, and SP were measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

FA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL, EDC and NHS were added at a molar ratio of FA:EDC:NHS = 1:1.2:1.2. The mixture was stirred at 37 °C for 1 h to give activated FA. The activated FA was added dropwise into SP solution at pH 8.0 with the molar ratio of FA to SP to be 1:5. FA was conjugated to SP by the interaction between the carboxylic group of FA and the amino group of SP. After 24 h, the mixture solution was dialyzed against deionized water for 48 h, and dried by lyophilization to give FA-conjugated SP (FA-SP). The chemical structure of FA-SP was analyzed using a Fourier transform infrared (FT-IR) spectrophotometer. And then FA, FA-SP, and SP were dissolved in a $NaHCO_3$ buffer, and detected using UV absorption by a microplate reader. The grafting degree was determined by comparing amino group content of primary SP and FA-SP using TNBS method [28].

2.3. Preparation of SP and FA-SP NPs

N-3-Acrylamidophenylboronic acid (APBA) was synthesized as reported previously [29]. SP (20 mg) and APBA (20 mg) was dispersed in 10 mL of distilled water, and a certain amount of initiator ACVA was added to the solution. The temperature of system was raised to 90 °C under a nitrogen atmosphere. The opalescence appeared in the reaction system, which was a signature of the formation of SP NPs. The resulting suspension was cooled at room temperature, filtered, and dialyzed against distilled water for 24 h using a cut-off molecular weight of 14 kDa to remove any aggregation and residual monomers. FA-SP NPs was prepared as the same method as that of SP NPs.

2.4. Characterization of SP and FA-SP NPs

The average hydrodynamic diameter and size distribution of NPs were evaluated by dynamic light scattering (DLS) using a Zeta Siser Nano Series (Malvern, United Kingdom). The stability of SP and FA-SP NPs was observed in various conditions. Particle size and count rates in different pH values and physiological conditions such as saline, PBS, FBS, and cell culture medium (RPMI 1640) were measured by DLS at room temperature. Zeta potential of NPs was obtained with Zetaplus. All samples were diluted to proper concentration before measurement, and the measurements were repeated three times.

The morphology of NPs was investigated by transmission electron microscopy (TEM) and scansion electron microscopy (SEM). For TEM observation, SP and FA-SP NPs were diluted to applicable concentration, and added onto a copper grill covered with nitrocellulose. Samples were allowed air-dried at room temperature, and then examined using a TEM (JEM-2100, Japan). For SEM observation, one drop of SP and FA-SP NPs solution was placed on the surface of a silicon wafer, and air-dried at room temperature. Then the wafer was coated with a thin layer of gold prior to observation.

2.5. Preparation of DOX-loaded SP and FA-SP NPs

DOX-loaded nanoparticles (DOX/SP and DOX/FA-SP NPs) were prepared by adding a certain amount of DOX into SP or FA-SP NPs at pH 8.0. The mixture was slowly stirred at room temperature for 8 h in the dark. Then DOX-loaded NPs were separated by centrifuging at 10,000 rpm for 30 min. The amount of un-loaded DOX in supernatant was quantified by a microplate reader (Molecule Devices, USA) at the excitation wavelength of 480 nm. The drug loading content (LC) and loading efficiency (LE) were evaluated as Eqs. 1 and 2:

$$\text{Drug loading content}\% = \frac{\text{Weight of the DOX in NPs}}{\text{Weignt of the DOX-loaded NPs}} \times 100\% \quad (1)$$

$$\text{Drug loading efficiency}\% = \frac{\text{Weigtht of the DOX in NPs}}{\text{Weight of the feeding Dox}} \times 100\% \quad (2)$$

2.6. In vitro drug release

DOX release profiles of DOX/SP and DOX/FA-SP NPs was measured as follows. 1 mL of DOX/SP or DOX/FA-SP NPs suspension was placed into a dialysis bag (MWCO 14 kDa), and dialyzed against 5 mL of 0.01 M phosphate buffered saline (PBS) at 37 °C. The pH value of system was adjusted to 4.0, 5.0, and 7.4, respectively. After a predetermined period, 5 mL of release medium was withdrawn, and 5 mL of fresh PBS was added. DOX concentration in release medium was measured by a microplate system (Molecule Devices, USA) at an excitation wavelength of 480 nm and an emission wavelength of 590 nm. The cumulative amount of DOX released from NPs was plotted against time.