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Cellular uptake, antitumor response and tumor penetration of cisplatin-loaded milk protein nanoparticles

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ABSTRACT

The casein nanoparticles cross-linked by transglutaminase were prepared, and cisplatin (CDDP), as a model antitumor drug, was loaded into the casein nanoparticles. These nanoparticles were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), and zeta potential. The uptake and penetration of nanoparticles in 2- and 3-dimensional SH-SY5Y cells were examined at 37 °C and 4 °C. The in vivo biodistribution of the nanoparticles was investigated using near-infrared fluorescence (NIRF) imaging and ion-coupled plasma mass spectrometry (ICP-MS). The antitumor effect of CDDP-loaded nanoparticles was evaluated on hepatic H22 tumor-bearing mice model via intravenous administration. It is found that the obtained nanoparticles showed spherical shape with the size of 257 nm, and drug loading content of 10%. These CDDP-loaded casein nanoparticles have the extraordinary capabilities to penetrate cell membrane barriers, target tumor and inhibit tumor growth. The tumor growth inhibition of CDDP-loaded nanoparticles is 1.5-fold higher than that of free CDDP. Further, the penetration examination of the CDDP-loaded casein nanoparticles in the tumor tissue demonstrated that the nanoparticles had the ability to penetrate the tumor and deliver CDDP to the tumor more deeply and affect the cells far from the vasculature.

1. Introduction

Recently, the nanoparticles based on the proteins such as albumin, gelatin and silk protein have been widely used as the drug delivery systems [1–3] due to their good biocompatibility, biodegradation into natural products, lack of toxicity, and non-antigenicity. Moreover, protein-based nanoparticles have active targeting ability except passive targeting based on the enhanced permeation and retention effect (EPR effect). For example, albumin nanoparticles can utilize albumin receptor (gp60)-mediated transcytosis through microvessel endothelial cells in angiogenic tumor vasculature and target the albumin-binding protein SPARC (Secreted Protein, Acidic and Rich in Cysteine), which is overexpressed in a majority of tumors [4]. Although two albumin-based particulate formulations have been approved for clinical use [5,6], the utilization of native proteins in terms of particulate drug delivery systems is still limited. In particular, the inherent bioactivities of protein-based nanoparticles have not been fully explored yet. For example, some proteins including homeodomain transcription factors [7], the herpes simplex virus-1 protein [8] and the HIV-1 transactivator Tat protein [9,10] are capable of penetrating cellular membrane, leading to increase drug retention in tumors and overcome drug resistance by increased intracellular drug concentrations in tumor. However, up to now, the membrane penetration of therapeutic cargos is mainly dependent on a series of short peptides called cell-penetrating peptides (CPPs), not cell-penetrating proteins and nanoparticles themselves. The strategy is often based on the conjugation of CPPs with cargos, thereby holding the risk to alter the biological activity of the cargoes. Moreover, it is generally accepted that CPPs mostly use energy-dependent endocytosis pathways across the membrane bilayer [11,12], resulting in endosomal sequestration and decreased bioavailability in the cytoplasm or nucleus [13]. In addition, as small molecular peptides, these CPPs are non-selective between tumor and normal tissues. Thus, the development of the protein-based nanoparticles drug delivery systems in which the particles themselves have drug-carrying and non-endocytotic cell-penetrating abilities is highly desirable.

Casein is a main ingredient of milk proteins, which is comprised of αs_1-, αs_2-, β-, and κ-casein (molar ratio 4:1:4:1, respectively) [14]. The caseins are proline-rich proteins and have distinct hydrophobic and hydrophilic domains. As a natural food product, casein is inexpensive, readily available, non-toxic and biodegradable [15]. In recently years, some casein-based oral drug delivery systems have

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been developed [16,17]. In our previous work [18], we prepared casein hollow spheres by molecular self-assembly in aqueous solution, and found that these casein hollow spheres have extraordinary capability to penetrate cell barriers. Unlike most of CPPs which cross cellular membranes in an endocytosis fashion, the casein hollow spheres can penetrate cell membrane in a non-endocytosis-dominant uptake mechanism, irrespective of cancer cell types and temperatures [19]. However, due to their relatively larger hydrodynamic diameter (about 379 nm at pH = 3.2 and 500 nm at pH = 7.4), these casein hollow spheres were not considered to be an ideal injectable drug delivery system.

In order to fully explore and utilize the bioactivity of casein, at present work, we further design and optimize the preparation strategy of casein particles. In particular, we use a natural cross-linker, transglutaminase (TGase) to replace glutaraldehyde which commonly used. These modifications significantly decrease the size of casein particles and improve the particle stability in various pH media. Further, we investigate the penetration of the casein nanoparticles in two-dimensional monolayer cells and three-dimensional multicellular tumor spheroids (MCTS), and the distribution in tumor-bearing mice based on near-infrared fluorescence (NIRF) imaging. When these casein nanoparticles are used as the carriers to delivering cisplatin (CDDP) in cancer treatment, the tumor suppression is evaluated. Finally, the tumor penetration of CDDP-loaded casein nanoparticles in H22 tumor-bearing mouse is assessed by immunohistochemical analysis.

2. Materials and methods

2.1. Materials

Casein from bovine milk which is dephosphorylation and made of αs_1-, αs_2-, β-, and κ-casein was purchased from Sigma–Aldrich. Acrylic acid (AA, Nanjing Chemical Reagent Co., Ltd.) was distilled under reduced pressure in nitrogen atmosphere. Propionic acid was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. Potassium peroxydisulfate ($K_2S_2O_8$, Nanjing Chemical Reagent Co., Ltd.) was recrystallized from deionized water before use. Rhodamine B isothiocyanate (RBITC) was purchased from SunShineBio Co., Ltd (Nanjing, China). Cisplatin (CDDP) was provided by shandongboyuan Co., Ltd. (Jinan, China). Rat anti-mouse CD31 (BD Pharmingen™), Alex-488 streptavidin, donkey anti-rabbit Alex-488, donkey anti-rat Alex-594 were obtained from Invitrogen. Cleaved caspase-3 antibody was purchased from Cell Signaling Technology Company. All other reagents were of analytical grade and used without further purification. Murine hepatic H22 cell line was obtained from Shanghai Institute of Cell Biology (Shanghai, China). Male ICR mice (6–8 weeks old and weighing 18–22 g) were purchased from Animal Center of Drum-Tower Hospital (Nanjing, China).

2.2. Preparation of casein nanoparticles

Casein (80 mg) was dispersed in 40 mL of acrylic acid (133 μL) and propionic acid (35 μL) aqueous solution under magnetic stirring and the temperature was raised to 90 °C. When the solution became clear, a predetermined amount of $K_2S_2O_8$ was added into the reaction system under a nitrogen atmosphere to initiate the polymerization of AA monomers. As the opalescence appeared in the reaction system, which was a signature of the formation of casein-poly(acrylic acid) (casein-PAA) nanoparticles, the reaction was allowed to proceed for another 10 min at 90 °C. The suspension was filtered with filter paper to remove any aggregation. The resultant suspension was dialyzed against water (pH = 3.0) for 24 h using a dialysis bag with a cutoff molecular weight of 14 kDa to remove residual monomers and other small molecules.

To improve the stability of casein-PAA nanoparticles, the pH of the suspension was adjusted to around 5.5, and then a predetermined amount of TGase was added to the suspension to cross-link casein moiety of casein-PAA nanoparticles. The cross-linking reaction was allowed for 72 h at 37 °C under magnetic stirring. Finally, the cross-linked product was again dialyzed against distilled water for 24 h to remove residual TGase, PAA in the particles and the other small molecules.

2.3. Preparation of CDDP-loaded casein nanoparticles

CDDP was dissolved in the suspension of casein nanoparticles (1 mg/mL), which was allowed to shake at 37 °C for 48 h to obtain CDDP-loaded casein nanoparticles. And then, this suspension was treated with the method developed by Kataoka's group to remove free CDDP [20]. Briefly, the unbound CDDP was removed by dialysis against distilled water using a dialysis bag with a molecular weight cutoff 14 kDa for 48 h.

2.4. Characterization of the nanoparticles

The mean hydrodynamic diameter and size distribution of nanoparticles were evaluated by dynamic light scattering (DLS) using a Brookheaven BI9000AT system (Brookheaven Instruments Corporation, USA). Zeta potentials of the samples were measured by Zetaplus (Brookheaven Instruments Corporation, USA). All DLS measurements were done with a laser wavelength of 660.0 nm at 25 °C, and each batch was analyzed in triplicate. The obtained dried samples were mixed with KBr powder and pressed to a plate for FT-IR measurement. FT-IR spectra were recorded on a vacuum FT-IR Spectrometer (Bruker VERTEX80V, Germany). The morphology of casein nanoparticles was observed by transmission electron microscopy (TEM, JEOLTEM-100, Japan). One drop of the nanoparticle suspension was placed on a 200-mesh nitrocellulose-covered copper grid. The grid was allowed to dry at room temperature without staining, and was examined with the TEM.

2.5. Drug loading content and encapsulation efficiency

The Pt content in the casein nanoparticles was measured by ion-coupled plasma mass spectrometry (ICP-MS, Perkin–Elmer Corporation, USA). Briefly, the CDDP-loaded casein nanoparticles were decomposed in hot nitric acid. After being evaporated to dryness, they were dissolved in 2 N hydrochloric acid solution. Then, the Pt concentration in the solution was measured by ICP-MS. The following equations were used to evaluate the drug loading content and encapsulation efficiency.

$$\text{Drug loading content } \% = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the feeding drug}} \times 100\%$$

$$\text{Encapsulation } \% = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the feeding drug}} \times 100\%$$

2.6. In vitro CDDP release from the nanoparticles

The release of CDDP from casein nanoparticles in phosphate buffered saline (PBS, 0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl) at 37 °C was evaluated by the dialysis method as reported previously [18]. Briefly, the CDDP-loaded nanoparticle solution of known platinum drug concentration was placed inside a dialysis bag (MWCO, 14 kDa). The dialysis bag was placed in PBS buffer and gently shaken at 37 °C in a water bath at 80 rpm. The released Pt outside of the dialysis bag was sampled at a predetermined time interval, and measured by ion-coupled plasma mass spectrometry (ICP-MS, Hewlett–Packard 4500).

2.7. Stability of CDDP-loaded nanoparticles

The stability of CDDP-loaded nanoparticles was observed by measuring the particle size as a function of time. The samples were dissolved in PBS with pH of 7.4 at 37 °C and evaluated by DLS with a Brookheaven BI9000AT system. At various time points, the hydrodynamic diameters and the scattering light intensity were measured by DLS at 25 °C.

2.8. In vitro cytotoxicity and cellular uptake

Cytotoxicity of CDDP-loaded casein nanoparticles against human derived neuroblastoma cell line (SH-SY5Y cells) was assessed by MTT assay. SH-SY5Y cells were cultured in DMEM (Dulbecco's modified Eagle essential medium), supplemented with 10% (v/v) inactivated FBS (fetal bovine serum), and antibiotics (10 U mL^{-1} penicillin and 10 $\mu g mL^{-1}$ streptomycin). Cells were cultured in a humidified 5% CO_2 incubator at 37 °C. SH-SY5Y cells were seeded on 96-well plates with a density around 5000 cells/well and allowed to adhere for 24 h prior to the assay. The cells were co-incubated with a series of doses of free CDDP, empty casein nanoparticles and CDDP-loaded nanoparticles at 37 °C for 24 h. Then, 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) indicator dye (5 mg/mL in PBS, pH = 7.4) was added to each well, and the cells were incubated for another 2 h at 37 °C in the dark. The medium was withdrawn and 200 μL acidified isopropanol (0.33 v/v HCl in isopropanol) was added in each well and agitated thoroughly to dissolve the formazan crystals. The solution was transferred to 96-well plates and immediately monitored on a microplate reader (Bio-Rad, Hercules, CA, USA.). Absorption was measured at a wavelength of 490 nm and 620 nm as a reference wavelength. The values obtained were expressed as a percentage of the control cells to which no drugs were added.

To trace the cellular uptake of nanoparticles, the casein nanoparticles were labeled with rhodamine B isothiocyanate (RBITC). The RBITC-labeled nanoparticles were prepared as follows: 1 mL of anhydrous DMSO containing 1 mg RBITC was added into 4 mL casein nanoparticles solution, and the mixture was stirred for 24 h at room temperature in the dark. Then, the RBITC-labeled casein nanoparticles were separated by centrifugation and unreacted RBITC was removed. Finally, the obtained RBITC-labeled nanoparticles were dispersed in aqueous solution for in vitro cellular uptake.