Biomaterials 52 (2015) 240–250

Contents lists available at ScienceDirect

Biomaterials

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

Hierarchical targeted hepatocyte mitochondrial multifunctional chitosan nanoparticles for anticancer drug delivery

Zhipeng Chen [a, d, *, 1], Liujie Zhang [b, 1], Yang Song [c], Jiayu He [a], Li Wu [a], Can Zhao [a], Yanyu Xiao [d], Wei Li [a], Baochang Cai [a], Haibo Cheng [a], Weidong Li [a, *]

[a] Department of Pharmacy, Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Nanjing University of Traditional Chinese Medicine, Nanjing 210046, PR China
[b] Department of Pharmacy, Henan University, Kaifeng 475001, PR China
[c] Department of Hospital Pharmacy, The 302nd Hospital of PLA, Beijing 100039, PR China
[d] State Key Laboratory of Natural Medicines (China Pharmaceutical University), Nanjing 210009, PR China

ARTICLE INFO

Article history:
Received 20 July 2014
Received in revised form
27 January 2015
Accepted 1 February 2015
Available online

Keywords:
Hierarchical
Mitochondrial-targeted
Multifunctional nanoparticles
pH-response
Drug delivery
Cancer therapy

ABSTRACT

The overwhelming majority of drugs exert their pharmacological effects after reaching their target sites of action, however, these target sites are mainly located in the cytosol or intracellular organelles. Consequently, delivering drugs to the specific organelle is the key to achieve maximum therapeutic effects and minimum side-effects. In the work reported here, we designed, synthesized, and evaluated a novel mitochondrial-targeted multifunctional nanoparticles (MNPs) based on chitosan derivatives according to the physiological environment of the tumor and the requirement of mitochondrial targeting drug delivery. The intelligent chitosan nanoparticles possess various functions such as stealth, hepatocyte targeting, multistage pH-response, lysosomal escape and mitochondrial targeting, which lead to targeted drug release after the progressively shedding of functional groups, thus realize the efficient intracellular delivery and mitochondrial localization, inhibit the growth of tumor, elevate the antitumor efficacy, and reduce the toxicity of anticancer drugs. It provides a safe and efficient nanocarrier platform for mitochondria targeting anticancer drug delivery.

1. Introduction

The mitochondrion is a membrane-bound organelle found in most eukaryotic cells. This organelle is vital to the cell's energy metabolism (as the cell's “power house”) and for the regulation of programmed cell death (as the cell's “arsenal”). Accordingly, the mitochondrion is a prime target for pharmacological intervention, and recent researches have revealed that mitochondria exert both vital and lethal functions in physiological and pathological scenarios [1–3]. The mitochondrion controls the activation of apoptotic effector mechanisms by regulating the translocation of pro-apoptotic proteins from the mitochondrial inter-membrane space to the cytosol. Conversely, mitochondria play a major role in multiple forms of non-apoptotic cell death and, in particular, regulated necrosis [4,5]. Recently, increasing evidence has suggested that mitochondrial dysfunction is responsible for a variety of human disorders including neurodegenerative diseases, diabetes, obesity and cancer [6–8]. Due to the key regulatory functions of mitochondria that are often altered in neoplasia, mitochondrially targeted compounds represent a promising approach for eradicating cancer cells [9–11].

Mitochondrial targeting is based on the negative potential on the matrix face of the inner mitochondrial membrane. Because the potential may be 20–60 mV (in negative values) higher in the mitochondria of cancer cells compared with non-malignant cells, this mitochondrial feature can be used for drug delivery in cancer therapy [12–14]. Mitochondrial targeting provides a disciplined approach for cancer cell destruction, however, difficulties remain in accomplishing this goal. Most of the drug carriers may be degraded or destroyed before these drugs gain access to their mitochondrial targets owing to the delivery of these nanocarriers from the injection site to the final target sites of action consists of

* Corresponding authors. Department of Pharmacy, Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Nanjing University of Traditional Chinese Medicine, Nanjing 210046, PR China. Tel./fax: +86 25 85811050.
E-mail addresses: czpcpu2000@hotmail.com (Z. Chen), liweidong0801@163.com (W. Li).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biomaterials.2015.02.001
0142-9612/

various transport steps with multiple physiological and biological barriers. Up to now, there are only a few drug delivery systems that can deliver drugs to the mitochondria of cancer cells [15–17].

A therapeutically feasible mitochondrial-targeted carrier should be able to accomplish these four sequential tasks: a) tightly encapsulate an anticancer drug into a nanoparticle to avoid degradation during blood circulation and after endocytosis; b) selectively adsorb onto cancer cells; c) rupture the endosomes to facilitate endosomal escape of the nanoparticle; and d) transform into a positively charged nanoparticle so as to bind to mitochondria. Despite extraordinary advances, the majority of previous reports have only investigated one or two issues such as endosomal escape [18,19] or charge conversion [20–22]. Hence, an easily prepared nanocarrier system that can overcome successive physiological and biological barriers ranging from blood circulation to mitochondria for anticancer drug delivery is highly desirable.

A nano-delivery system may be a reasonable choice for achieving the aforementioned four functions. Chitosan (CTS), a natural polymer derived from crustacean shells, has attracted particular attention as a drug carrier. Their simple and mild preparation methods, low toxicity, good stability, controlled drug release and ability to overcome biological barriers have made chitosan nanoparticles (CTS-NPs) popular in drug and gene delivery applications [23–25]. Biodegradable nanoparticles formulated from chitosan have been extensively investigated for delivery of different agents including peptides [26–28], plasmid DNA [29,30] and chemotherapy drugs [31–33]. Currently, efforts have focused on the preparation of chitosan nanoparticles for applications in organelle targeting drug delivery systems. Herein, we reported two multifunctional, structurally simple, chitosan derivatives N-glycyrrhetinic acid-polyethylene glycol (PEG)-chitosan (NGPC) and N-quaternary ammonium-chitosan (NQC) – as basic construction unit of the mitochondrial-targeted nanocarrier system that can accomplish the above-mentioned four tasks. NQC carries quaternary amine groups and NGPC contains PEG (pH-cleavable Schiff's base) as a hydrophilic block, with two chitosan chains as a hydrophobic block, which assemble into the multifunctional nanocomposite particles (MNPs) by crosslinking of tripolyphosphate. Brucine, a hydrophobic drug, was chosen as a typical "natural anticancer drug" acting on the mitochondria to trigger the apoptosis of tumor cells [34]. It was encapsulated into the CTS-NPs as mentioned above through a hydrophobic interaction, denoted as brucine/NGPC-NPs, brucine/NQC-NPs and brucine/MNPs. As depicted in Fig. 1, the primary characteristics of MNPs include long circulation, liver parenchyma cell targeting and pH responsivity to the mildly acidic tumor microenvironment and the acidic intracellular compartment, successively. Smart MNPs, after hydrolysis in the lysosome, achieve endosomal escape and mitochondrial targeting. Typically, hydrophilic chain PEG and target molecule glycyrrhetinic acid (GA) are present on the surface of MNPs during blood circulation. As MNPs arrive at the tumor site by means of the GA, followed by endocytosis into the endosomes and lysosomes, the pH-cleavable Schiff's base generates a stronger positive surface charge of the MNPs. The imidogen on the surface of the MNPs facilitates proton influx (proton sponge effect) to endolysosomes, leading to endolysosomal bursting and creating favorable conditions for MNPs to escape from endolysosomes to the cytoplasm (pH 7.2–8.0). Meanwhile, MNPs with a highly positive charge have the ability to aggregate at the mitochondria by electrostatic interactions due to the higher negative potential of cancer cell mitochondria. Therefore, intelligent MNPs provide a safe and efficient carrier system for successive mitochondrial hydrophobic drug delivery.

2. Materials and methods

2.1. Materials, cell culture and animals

4-Formylbenzoic acid, N, N-Dicyclohexylcarbodiimide (DCCI) and Tripolyphosphate (TPP) were purchased from Sinopharm Chemical Reagent Co., LTD and used as received. 1-Hydroxybenzotriazole (HOBt) and 3-chloro-2-hydroxypropyltrimethyl ammonium chloride were purchased from Shanghai DiBo chemical technology co., LTD. Glycyrrhetinic acid(GA, Xian fu jie pharmaceutical co., LTD), PEG_{2000}(Guangdong guanghua chemical plants co., LTD) and Chitosan (CTS, Nantong green god biological engineering co., LTD) were used as purchased. Brucine was obtained from Tokyo Chemical Industry Co, Ltd (Tokyo, Japan). Coumarin 6 (C6), chlorpromazine, sucrose, amiloride, nystatin and sodium azide were purchased from Sigma–Aldrich Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle Medium (high glucose) cell culture medium (DMEM, Hyclone®) were purchased from Sunshine BioTechnology Co., Ltd. (Nanjing, China). Fetal bovine serum (FBS, Gibco®), penicillin-streptomycin solution (Gibco®), trypsin (Gibco®), phosphate buffered saline (PBS, Gibco®) were provided by Pufei Biotechnology Co., Ltd. (Shanghai, China). All other chemicals were used as purchased without further purification.

The human hepatocellular liver carcinoma cell line HepG2 was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in DMEM with 10% (v:v) FBS and 1% penicillin/streptomycin in an incubator (Thermo Scientific, USA) at 37 °C under an atmosphere of 5% CO_2 and 95% relative humidity. The cells were subcultured approximately every 2 days at 80% confluence at a split ratio of 1:3.

The animal experiments were carried out with the approval from the Ethics Committee of Nanjing University of Traditional Chinese Medicine and in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Wistar rats (180–220 g), Kunming (KM) mice (18–22 g) and Male ICR mice (SPF grade) (18–22 g) were bought from College of Veterinary Medicine Yangzhou University (Jiangsu, China), and maintained in a light-controlled room kept at a temperature of 25 ± 2 °C and a relative humidity of 55 ± 5% with free access to standard food and water. The animals were fasted for at least 24 h prior to experiment but given water freely.

2.2. Synthesis of NGPC and NQC

2.2.1. Synthesis of NGPC

The synthesis of NGPC involved three steps of chemical modification on GA by esterification. Briefly, GA (471 mg, 1 mmol), *N, N*-Dicyclohexylcarbodiimide (248 mg, 1.2 mmol) and 1-Hydroxybenzotriazole (162 mg, 1.2 mmol) were dissolved in dichloromethane (20 mL), and stirred for 1 h at room temperature. The temperature of solution was lowered to below 10 °C, PEG_{2000} was added to the solution, and stirred overnight. This reaction mixture was denoted as solution 1.4-Formylbenzoic acid (150 mg, 1 mmol), *N, N*-Dicyclohexylcarbodiimide (248 mg, 1.2 mmol) and 1-Hydroxybenzotriazole (162 mg, 1.2 mmol) were dissolved in dichloromethane (20 mL), were dissolved in dichloromethane (20 mL), and stirred for 1 h at room temperature. The temperature of solution was lowered to below 10 °C, and the solution 1 was added and stirred overnight, followed by stirring for 1 h after 20 ml water was added. The reaction mixture was filtered, the filtrate was washed with saturated sodium bicarbonate solution (10 mL × 3) and saturated salt water (100 mL × 1) successively, and then dehydrated overnight with hydrous sodium sulfate. The dichloromethane solution was evaporated with vacuum distillation to obtain grease products. Then the grease products and chitosan (161 mg, 13.4 μmol) were dispersed in the 30 mL DMSO and stirred for 48 h at room temperature. The product was filtered, washed with DMSO, and the ice water was added. The mixture solution was filtered and washed with distilled water, followed by freeze-drying.

2.2.2. Synthesis of NQC

The synthesis of NQC is relatively simple. Specifically, the chitosan (4.0 g) and 40% sodium hydroxide solution (6 mL) were added to 50 mL isopropanol and stirred for 4 h at 55 °C, and then 50% 3-chloro-2-hydroxypropyltrimethyl ammonium chloride solution (32 mL) was added and stirred for 6 h at 65 °C. The reaction solution was adjusted to pH 7.0 with 10% hydrochloric acid solution and filtered, washed with 85% methanol solution (60 mL × 3), and absolute ethyl alcohol (50 mL × 3) successively, and then dried overnight under vacuum at 80 °C.

2.3. Preparation and characterization of brucine/CTS-NPs

2.3.1. Preparation of CTS-NPs

Brucine/NGPC-NPs were prepared by the modified method previously reported [35,36]. Briefly, 100 mg NGPC was dissolved in 10 ml of 50% ethanol solution and extruded through polycarbonate membrane filters with a pore size of 0.45 μm. And then 10 mg brucine was added to the above solution and stirred for 1 h at room temperature. About 1.25 ml of tripolyphosphate (TPP) (10 mg/mL, pH7~8) was dropwisely added to the NGPC solution under magnetic stirring (100 r/min). The NPs solution was centrifuged at 20,000 rpm for 30 min and then washed three times to remove unencapsulated drug. Finally, the dispersed solution was lyophilized 24 h for further use.

Brucine/NQC-NPs and Brucine/MNPs were prepared as above, except NQC and the same quality of NQC and NGPC were used instead of NGPC. At the same time, C6-