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Construction of doxorubicin-loading magnetic nanocarriers for assaying apoptosis of glioblastoma cells

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ABSTRACT

Magnetic nanoparticles (MNPs), in comparison with traditional drug solutions or suspensions, represent a promising vehicle to achieve the controlled drug delivery to targeted cell/tissue regions in cancer treatment. In this study, the biodegradable chitosan-modified magnetite (Fe_3O_4) NPs (CS-MNPs) are firstly synthesized using as nanocarriers, and then encapsulated with anti-tumor drug doxorubicin (DOX) to construct DOX-loaded CS-MNPs (DOX-CS-MNPs), which are further applied to assay apoptosis of glioblastoma multiforme U251 cells. The properties of the DOX-CS-MNPs including particle size, shape and magnetization, are characterized. The stability, drug release, magnetic response and redispersion of the DOX-CS-MNPs within an external magnetic field are evaluated. Furthermore, the biological effects of the DOX-CS-MNPs on U251 glioblastoma cells, particularly cytotoxicity, cell viability, actin cytoskeleton and apoptosis rate, are subsequently investigated. The data show that the prepared DOX-CS-MNPs are spherical in shape with average diameter of 60 nm approximately. The fabricated DOX-CS-MNPs also exhibit specific properties including low aggregation, high saturation magnetization, satisfactory magnetic-responsive aggregation, and redispersion in water, etc. The biological assays show that the DOX-CS-MNPs can efficiently enter the cells, reduce cell viability, and inhibit cell proliferation in a dose-dependent manner, and a high rate of cell apoptosis is induced in U251 glioblastoma cells after DOX-CS-MNPs treatment. Therefore, the present results indicate that the constructed DOX-CS-MNPs may be a promising vehicle for efficiently inhibiting proliferation of human U251 glioblastoma cells.

1. Introduction

Malignant brain tumors are a leading cause of death worldwide [1,2]. To this day, malignant glioma therapy remains a great challenge. Traditional brain tumor chemotherapy has long been used to improve the prognosis of patients with malignant gliomas, especially those with distant metastasis [3]. However, traditional chemotherapy is mainly limited by the low selectivity of anti-tumor drugs, which causes severe side effects and inadvertent damage to healthy cells [4,5]. To treat malignant brain tumors effectively, glioma cells must be selectively killed to protect normal cells. Hence, the development of efficient tumor cell-targeted therapies for treating brain tumors has become a crucial and urgent need [6].

Magnetic nanoparticles (MNPs), especially superparamagnetic iron oxide (SPIO) NPs, such as magnetite (Fe_3O_4) and/or maghemite (γ-Fe_2O_3), have been exploited as anti-tumor drug delivery carriers in the tumor cell-targeted treatment because of their nano-sized diameters (between 1 and 100 nm) and intrinsic superparamagnetic properties when added to an external magnetic field [7,8]. However, the raw SPIO NPs without surface coatings or modifications behave erratically and can readily aggregate and precipitate in aqueous solutions and blood plasma, which seriously hinders their applications *in vitro* and *in vivo* [9]. Therefore, the naked SPIO NPs must be functionalized to improve their stability and promote their biocompatibility upon modification for further attachment of biomolecules [10,11].

To achieve SPIO NPs with satisfactory water-dispersibility and stability, and biocompatibility, researchers have fabricated NPs with coating layers, such as polymers [12,13], dendrimers [14,15], polypeptides [16,17], and albumin [18,19]. Numerous studies show that the coated and/or surface-modified SPIO NPs composed of iron oxide cores and polymeric shells are generally characterized with excellent dispersibility and high stability in water, moderate drug-loading capacity, and specific targeting of cells or tissues, and these NPs are thus particularly powerful tools for targeted drug delivery and therapy clinically [20–22].

Chitosan (CS), as a natural polymer, is widely used as a coating layer for SPIO NPs because of its membrane-penetration, biodegradability, and biocompatibility [23,24]. Structurally, CS has many

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amino groups that are potentially ionized easily in weakly acidic environments, thereby allowing the polymer to readily bind to negatively charged surfaces such as cell membranes. Moreover, the outer membrane surface of tumor cells usually possesses more negative charges than normal cells [25]. Thus, CS-MNPs, when used as anti-tumor drug carriers, can increase drug concentrations in the specific tumor tissues or cells, improve drug delivery efficiency, prolong action times, and decrease unwanted side effects [26–30]. The utilization of CS-MNPs as drug carriers can overcome the shortcomings of traditional anti-cancer drug solutions or suspensions in drug delivery and cancer treatment. Moreover, the high magnetic susceptibility of the iron oxide core of the CS-MNPs enables non-invasive manipulation of MNPs by a magnetic field. Therefore, the application of functionalized SPIO NPs that integrate therapy and diagnosis could also facilitate the monitoring of therapeutic efficacy during treatment [31,32].

In the present work, the magnetic Fe_3O_4 NPs are used as potential carrier cores to prepare the CS-MNPs by surface coating with chitosan as shell modifiers. The synthesized CS-MNPs are then loaded with a commonly used anti-tumor drug DOX to construct the DOX-loaded CS-MNPs (DOX-CS-MNPs), which are used to specifically inhibit proliferation of human U251 glioblastoma multiforme cells. The properties of the constructed DOX-CS-MNPs including particle size and shape, magnetization, stability in water, magnetic response and redispersion to an external magnetic field, drug releasing, etc. are analyzed. Furthermore, the biological effects of the constructed DOX-CS-MNPs on U251 glioblastoma cells, including cytotoxicity, cell viability, and apoptosis rate, are also investigated. The results show that the constructed DOX-CS-MNPs can effectively prohibit cell proliferation, and induce a high rate of cell apoptosis in U251 glioblastoma cells, which indicates the prepared DOX-CS-MNPs may be a promising anti-tumor drug delivery model for efficiently inhibiting proliferation of human U251 glioblastoma cells.

2. Materials and methods

2.1. Reagents and materials

Superparamagnetic magnetite (Fe_3O_4) NPs utilized in this study were prepared according to the methods described elsewhere [33,34]. Chitosan (deacetylation degree $\geqslant$ 90%, MW = 60 kDa) was obtained from Guoan Biological Technology Co., Ltd. (Xian, China). The human U251 glioblastoma cell line was obtained from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell culture medium and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (CA, USA). Fluorescein isothiocyanate (FITC), DOX, 3-(4,5- dimethylthiazol-2-diphenyl-tetrazolium) bromide (MTT), fluorescein diacetate (FDA), propidium iodide (PI), Hoechst H33258, dimethyl sulfoxide (DMSO), Triton X-100 solution, and paraformaldehyde were purchased from Sigma–Aldrich (St. Louis, MO, USA). The fluorescent dye, 4, 6-diamidino-2-phenylindole (DAPI), was purchased from Molecular Probes Inc. (Eugene, OR, USA). Rhodamine phalloidin was obtained from Cytoskeleton Inc. (Denver, CO, USA). Apoptotic DNA ladder detection kit was purchased from Keygen Biotech (Nanjing, China). Other reagents and chemicals used were purchased from local commercial suppliers and of analytical reagent grade unless otherwise stated. De-ionized (DI) water (Milli-Q, Millipore, Bedford, MA, USA) was used to prepare aqueous solutions.

2.2. Principle of DOX-CS-MNPs fabrication for assaying cell apoptosis

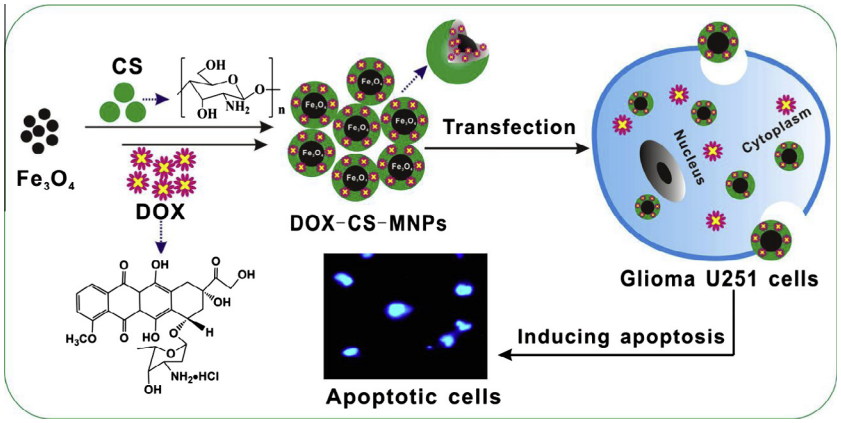
The general principle of DOX-CS-MNPs fabrication for assaying apoptosis of human U251 glioblastoma multiforme cells starts with the synthesis of magnetic Fe_3O_4 NPs followed with chitosan modification, and the DOX encapsulation of the CS-modified MNPs is then performed through a reverse microemulsion method. Subsequently, the constructed DOX-CS-MNPs are delivered to human U251 glioblastoma multiforme cells, supposed to specifically inhibit cell proliferation and induce apoptosis (Scheme 1).

2.3. Synthesis of superparamagnetic Fe_3O_4 NPs

As the magnetic nanocarriers used in the present study, the Fe_3O_4 NPs were prepared according to the previous methods proposed elsewhere [33,34]. First, 4.5 mL $FeCl_3$ (2 mol/L dissolved in 2 mol/L HCl) was added to 15.5 mL of ultra-purified water, and then 3 mL Na_2SO_3 (1 mol/L) was subsequently added dropwise into the mixture within 1 min while stirring. When the solution color changed from red to light yellow, the solution was added into 120 mL NH_4OH (0.85 mol/L) while vigorously stirring. A black precipitate quickly formed and was completely crystallized for another 40 min. The black precipitate was washed with deoxygenated water by magnetic decantation and then dried to form Fe_3O_4 powder.

2.4. Construction of DOX-CS-MNPs

The reverse microemulsion method was used to prepare DOX-CS-MNPs with some modifications [35]. Specifically, 40 mg chitosan powder was dissolved in 2 mL of (2% w/v) acetic acid solution, and 36 mg Fe_3O_4 NPs was then added into this mixture to form a



Scheme 1. Schematic representation of the DOX-CS-MNPs fabrication for assaying cell apoptosis of U251 glioblastoma cells. Generally, magnetic Fe_3O_4 NPs are firstly synthesized and then surface-modified by chitosan, and DOX loading through a reverse microemulsion method to construct DOX-CS-MNPs. The fabricated DOX-CS-MNPs are finally delivered to glioblastoma multiforme U251 cells to inhibit cell proliferation and induce apoptosis.