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Original Article

Multifunctional near-infrared dye-magnetic nanoparticles for bioimaging and cancer therapy

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ABSTRACT

Theranostics based on nanoparticles have developed rapidly in the past decade and have been widely used in the diagnosis and treatment of liver cancer, breast cancer, and other tumors. However, for skin cancers, there are limited studies. In the present study, we successfully synthesized a theranostic nanoparticle by grating IR820 onto the surface of chitosan-coated magnetic iron oxide, IR820-CS-Fe_3O_4, showing an excellent magnetic resonance imaging (MRI) capability and cytotoxic effects against melanoma under irradiation with a near-infrared (NIR) laser (808 nm) *in vitro*. Furthermore, good stability for up to 8 days and negligible cytotoxicity were observed. These characteristics are important for biomedical applications of nanoparticles. In conclusion, we provide a novel and potential theranostic platform for melanoma treatment and detection.

Introduction

Cancer still remains one of the major causes of death worldwide [1]. To the best of our knowledge, effective tumor treatment depends on accurate detection and real-time monitoring [2]. To achieve these goals, the integration of diagnosis and treatment is inevitable. With the rapid development of nanotechnology, there have been many considerable advances in the diagnosis and treatment of cancer. Theranostics based on nanomaterials is a technology that combines therapeutic agents (e.g., chemotherapeutic drugs, light-thermal conversion agents, and photosensitizers) and imaging agents (e.g., X-ray contrast agents, magnetic resonance imaging (MRI) contrast agents, and fluorescent dyes) in nanoparticles to improve the cancer therapy [3,4]. In the past several years, some theranostic nanoparticles have been used in the study of breast cancer, liver cancer, and other tumors [5–8]; however, this technique has not been reported for skin tumors. With the incidence of melanoma (one of the major causes of death) growing rapidly in recent years and an increasing tendency in the younger population [9], there is an urgent need to explore the therapeutic efficacy of theranostic nanoparticles for melanoma.

Phototherapy based on nanomaterials is a minimally invasive tumor treatment technology that has developed rapidly in recent years. It includes photothermal treatment (PTT) and photodynamics therapy (PDT). Both of these methods use near-infrared (NIR) light with a strong penetration ability as the light source, through irradiating nanomaterials with a light-heat conversion ability, photosensitizers to generate heat, or singlet oxygen to kill tumor cells [10,11]. Compared with traditional radiotherapy and chemotherapy, this strategy is less limited by side effects; thus, it can be performed many times. Moreover, it can greatly enhance the effect of radiotherapy and chemotherapy as well as reverse drug resistance to chemotherapy [12–15]. Nanomaterials used in phototherapy are divided into two major categories. Inorganic materials such as gold as well as carbon nanomaterials as therapeutic agents show good antitumor effects [16,17]. But their shortcomings, such as difficult excretion, poor degradation, and potential toxicity, undeniably limit their application. Organic nanomaterials have emerged as a novel type of phototherapeutic agent because of their good biocompatibility, easy degradation, and high light-thermal conversion efficiency. Indocyanine green (ICG), approved by the Federal Drug Administration (FDA), can be used for NIR imaging

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and plays an important role in clinical disease diagnosis [18]. ICG is also a potential PTT agent [14]. Tang et al. have reported that ICG used in thermotherapy has a synergistic effect with chemotherapeutic drugs in the treatment of tumors and can reduce the occurrence of side effects [19]. However, the stability of ICG is poor, and it tends to adsorb proteins nonspecifically in plasma. These disadvantages limit its use in PTT. Similar to ICG, IR820 is a new indocyanine green dye with a strong absorption in the NIR region; in addition, it is more stable and easily modified. Thus, it can also be used for PTT. It has been shown that IR820 can produce heat under NIR laser irradiation; therefore, it affects the metabolism of tumor cells, leading to cell death [20]. IR820 can also increase the generation of singlet oxygen in cells so as to affect PDT [21]. Hence, IR820 is a promising NIR dye for the diagnosis and treatment of cancer.

Gadolinium, a common contrast agent used in MRI, is restricted due to its fast metabolism and potential toxicity. The FDA has announced a public health warning that gadolinium might lead to nephrogenic fibrosis or nephrogenic and fibrotic dermopathy [22]. Compared with gadolinium, magnetic iron oxide nanoparticles have some advantages, such as a good biocompatibility, high detection sensitivity, and targeting effects [23,24]. Therefore, it is a good choice to select iron oxide nanoparticles in theranostics [25]. However, the instability of iron oxide nanoparticles in a physiological environment hinders their application. Thus, it is imperative to determine how to improve their stability.

In this study, by chemical connecting IR820 onto the surface of chitosan-coated magnetic iron oxide (CS-Fe_3O_4), we successfully constructed IR820-CS-Fe_3O_4 nanoparticles. The nanoparticles showed good stability for up to 8 days and negligible cytotoxicity. When IR820 was introduced to the nanoparticles, the amount of singlet oxygen produced by IR820 under NIR laser irradiation was significantly increased. Moreover, an excellent MRI capability and phototherapeutic effect of IR820-CS-Fe_3O_4 nanoparticles could be seen *in vitro*.

Materials and methods

Materials

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and ferric dichloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) were purchased from Xilong Chemical Co. Ltd. (Guangdong, China). Low molecular weight chitosan was purchased from Sigma–Aldrich. Other chemicals and reagents used in this study were as described previously [26].

Methods

Nanoparticles

CS-Fe_3O_4 was synthesized by the coprecipitation method. First, CS (0.25 g, low molecular weight) was dissolved Milli-Q water (50 mL, a little acetic acid was added to help dissolve CS) and purged with nitrogen for 30 min to remove the oxygen in the reaction system. Meanwhile, the solution was heated up to reflux. Then, $FeCl_3 \cdot 6H_2O$ (0.7295 g, 2.7 mmol) and $FeCl_2 \cdot 4H_2O$ (0.3575 g, 1.8 mmol) were dissolved in diluted HCl solution (2 mL, 1 M). After that, the mixture of iron precursors was quickly injected into the hot polymer solution, following the addition of the concentrated ammonia wate to adjust the pH value to 6.9. After refluxing for 40 min, the reaction system was cooled to room temperature, transferred to a dialysis membrane (MWCO: 12,000–14,000), and dialyzed against Milli-Q water for 65 h. Then, the CS-Fe_3O_4 colloid was filtered and stored at 4 °C for the following experiments, and the iron concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES).

For the synthesis of IR820-CS-Fe_3O_4, IR820 (15 mg, 0.0177 mmol) was first reacted with 6-aminohexanoic acid (16.25 mg, 0.1239 mmol) in 1 mL of anhydrous N′,N′-dimethylformamide at 85 °C to generate the IR820-linker. The reaction was carried out under a nitrogen atmosphere. After the activation, 3 mL of CS-Fe_3O_4 colloid ([CS-Fe_3O_4] = 6 mg/mL) was added to the solution to react overnight, and then it was dialyzed against deionized water for 48 h. The IR820-CS-Fe_3O_4 nanoparticles were collected for further experiments. In order to study the cellular uptake and intracellular localization, CS-Fe_3O_4 was labeled with fluorescein isothiocyanate (FITC).

Characterization

A 200-kV transmission electron microscope (FEI Tecnai G2) and dynamic light scattering (Malvern Instruments Ltd., Malvern, UK) were used to characterize the morphology, size, and zeta potential of the nanoparticles. ICP-OES (PE8000, PerkinElmer, USA) was used to determine the absolute concentrations of Fe in each sample. A 7.0-T small-animal MRI instrument (BioSpec70/20USR, Bruker) was used to measure the MRI capability of IR820-CS-Fe_3O_4. Ultraviolet (UV)–visible absorption spectra of the nanoparticles were analyzed by UV–visible spectrophotometry (Perkin Elmer Lambda 850).

Cell culture

The human melanoma cell line (A375) was purchased from Shanghai Cell Collection (Shanghai, China). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM glutamine, 100 U/mL penicillin, and 1 μg/mL streptomycin (Invitrogen).

Cytotoxicity assay

A Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) was used to test the cytotoxicity of A375 cells after treatment with the nanoparticles. A375 cells were seeded in 96-well plates at a density of 6×10^3 cells per well and incubated overnight. The following day, the medium was replaced with fresh medium containing CS-Fe_3O_4 and IR820-CS-Fe_3O_4 at different concentrations. After exposure for 24 h, 100 μL of medium containing CCK-8 (10%) was added to the cells, and the plate was incubated at 37 °C for 2 h. Finally, the absorbance was detected at 450 nm, with a reference at 600 nm, using a multifunctional microplate reader (Infinite 200, TECAN).

Cellular uptake and intracellular localization in cells

Flow cytometry was applied to measure the cellular uptake of FITC-CS-Fe_3O_4 nanoparticles. Briefly, A375 cells were seeded in 6-well plates at a density of 2×10^5 cells per well and grown until 80% confluent. After incubation for 3 h with 10, 20, or 40 μg/mL FITC-CS-Fe_3O_4, respectively, the cells were washed with phosphate-buffered saline (PBS) and centrifuged (1000 rpm, 5 min) after digestion to collect the cells. Next, the cells were washed with PBS and analyzed by flow cytometry (BD, USA).

A375 cells were exposed to 20 μg/mL FITC-CS-Fe_3O_4 for 1 h and then rinsed with PBS. Next, the cells were incubated in serum-free medium containing Lyso Tracker Red (50 nM) for 1 h and Hoechst 33,258 (5 μg/mL) for 15 min at 37 °C. Photographs were taken under a laser scanning confocal microscope (Leica, Germany) (Lyso Tracker Red: excitation of 577 nm, emission of 590 nm; Hoechst 33,258: excitation of 350 nm, emission of 460 nm; FITC: excitation of 495 nm, emission of 519 nm).

Heat and singlet oxygen generation measurement

Aqueous IR820-CS-Fe_3O_4 and IR820 (0, 8, 16, and 32 μg/mL) were irradiated under the NIR laser (808 nm, 8 W/cm^2) for 5 min. The temperatures at different time points (0, 15, 30, 45, 60, 90, 150, 210, and 270 s) were recorded by a thermal imager (FLIR, USA). Next, singlet oxygen sensor green diluent (5 μM) was mixed with IR820-CS-Fe_3O_4 or IR820 (5 μM) at the same volume before the NIR laser irradiation (808 nm, 8 W/cm^2) for 0, 0.5, 1, 2, 3, 4, and 5 min, respectively. The signal intensity was measured by a microplate reader (excitation of 507 nm and emission of 531 nm).

Live/dead cell viability assay

A375 cells were exposed to different concentrations (0, 4, 8, and 16 μg/mL) of IR820-CS-Fe_3O_4 nanoparticles and IR820 for 3 h. After NIR laser irradiation (808 nm, 8 W/cm^2) for 5 min, calcein acetoxymethyl ester (calcein AM) (2 μg/mL) and propidium iodide (PI) (2 μg/mL) were diluted in serum-free medium to stain live and dead cells at 37 °C for 35 min. The images were taken by an inverted fluorescence microscope (Olympus IX71, Japan).

Statistical analysis

All results were expressed as the mean ± standard deviation (SD). Differences between groups were evaluated using one-way analysis of variance, and multiple comparison between groups was performed by the Student–Newman–Keuls method with SPSS 16.0 software. $P < 0.05$ was considered statistically significant.

Results

CS-Fe_3O_4 nanoparticles were synthesized by the coprecipitation method. Energy dispersive X-ray spectroscopy confirmed that CS-Fe_3O_4 was synthesized and contained the elements of Fe and O (Fig. 1A). The transmission electron microscopy image showed that the mean size of the CS-Fe_3O_4 nanoparticles was approximately 8.2 nm and that their shape was spherical with good uniformity (Fig. 1B). These results verified the successful synthesis of the CS-Fe_3O_4 nanoparticles. The ICP-OES results indicated that the percentage of iron in the nanoparticles was 37.1%. The UV–visible spectrum showed that IR820 has a peak absorption at 688 nm. The concentration of IR820 was detected by the light absorbance at