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Synthesis of tumor-targeted folate conjugated fluorescent magnetic albumin nanoparticles for enhanced intracellular dual-modal imaging into human brain tumor cells

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ABSTRACT

Superparamagnetic iron oxide nanoparticles (SPIO NPs), utilized as carriers are attractive materials widely applied in biomedical fields, but target-specific SPIO NPs with lower toxicity and excellent biocompatibility are still lacking for intracellular visualization in human brain tumor diagnosis and therapy. Herein, bovine serum albumin (BSA) coated superparamagnetic iron oxide, i.e. γ-Fe_2O_3 nanoparticles (BSA-SPIO NPs), are synthesized. Tumor-specific ligand folic acid (FA) is then conjugated onto BSA-SPIO NPs to fabricate tumor-targeted NPs, FA-BSA-SPIO NPs as a contrast agent for MRI imaging. The FA-BSA-SPIO NPs are also labeled with fluorescein isothiocyanate (FITC) for intracellular visualization after cellular uptake and internalization by glioma U251 cells. The biological effects of the FA-BSA-SPIO NPs are investigated in human brain tumor U251 cells in detail. These results show that the prepared FA-BSA-SPIO NPs display undetectable cytotoxicity, excellent biocompatibility, and potent cellular uptake. Moreover, the study shows that the made FA-BSA-SPIO NPs are effectively internalized for MRI imaging and intracellular visualization after FITC labeling in the targeted U251 cells. Therefore, the present study demonstrates that the fabricated FITC-FA-BSA-SPIO NPs hold promising perspectives by providing a dual-modal imaging as non-toxic and target-specific vehicles in human brain tumor treatment in future.

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Introduction

Superparamagnetic iron oxide (SPIO) nanoparticles, mostly magnetite (Fe_3O_4) and/or maghemite (γ-Fe_2O_3), have employed as promising tools for biomedical applications, including targeted cell labeling and separation [1–3], diagnostic magnetic resonance imaging (MRI) [4,5], immunoassays [6,7], gene/drug targeted delivery and cancer targeted therapy [8–10], due to their nanosized diameter and outstanding superparamagnetic properties. However, the "bare" SPIO nanoparticles without surface coating or modification are erratic and can readily aggregate and precipitate in aqueous solutions and blood plasma, which seriously hinders their applications either *in vitro* or *in vivo* [11,12]. Extensive efforts have thus been made to design and construct sophisticated SPIO nanoparticles with desired properties including stable water dispersion and excellent biocompatibility so far. For instance, different coating layers were used to decorate nanoparticles, including silica [13], polymers [14–16], heparin [17,18], and polypeptides [19–21], allowing nanoparticles to be suspended in water, saline solution, and culture medium, and making them suitable for *in vitro* and *in vivo* experiments. But these fabricated nanoparticles usually displayed cytotoxicity at a differing extent [22]. Fortunately, bovine serum albumin, as an ideal coating protein has shown versatile properties including non-toxicity, non-immunogenicity, good biocompatibility and ligand-binding [23,24]. Owing to its high binding capacity and less side-effects, albumin-coating magnetic nanoparticles have gained considerable attention in tumor targeted cell labeling and intracellular imaging analysis [25,26].

Recently, targeted delivery is highly desirable for reducing unintended side effects and toxicity of magnetic nanoparticles [27,28]. Numerous targeting ligands, such as monoclonal antibodies

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[29,30], cationic transfection agent [31,32], and cell membrane receptors are often used to functionalize magnetic nanoparticles to improve affinity and specificity in tumor treatments [33,34], by providing more binding sites for cell, gene/drug and other conjugates. Among these, folic acid (FA) ligand, has a high affinity, and enables selectively binding to folate receptor anchored cell surface and up-regulated in many human tumors [35–37]. Therefore, the FA ligand is preferentially considered as a useful molecule to develop an efficient receptor-mediated tumor specific delivery for magnetic nanoparticles.

In the recent years, accredited to advantageous combinations of fluorescence imaging and magnetic response, the development of multifunctional fluorescent magnetic nanocarries has attracted increasing attention [38,39]. These fluorescent magnetic nanoparticles generally possess such attractive features as fluorescence and superparamagnetism, which allow for a controlled and monitored movement of nanoparticles in cells, and help to probe specific bioactive molecules in the localized domains or compartments but without disturbing other parts in the live cells.

In this study, superparamagnetic iron oxide, i.e. γ-Fe_2O_3 nanoparticles (SPIO NPs) were first synthesized and surface-coated with non-immunogenic BSA, and then conjugated with tumor-specific ligand FA to prepare a targeting FA-BSA-SPIO NPs. The tumor U251 cells, as a most common and lethal type in human brain malignant tumor [40], were used as a model to incubate with the prepared FA-BSA-SPIO NPs at varying concentrations including 25, 50, 100, 250 and 500 μg/mL, and the biological effects of the prepared FA-BSA-SPIO NPs on the treated U251 cells were investigated in detail. Furthermore, the prepared FA-BSA-SPIO NPs were decorated with fluorescent dye FITC for intracellular internalization and visualization. Our study demonstrated that the finalized FITC-FA-BSA-SPIO NPs could provide a dual-modal imaging as non-toxic and target-specific vehicles in human brain tumor treatment in future.

Experimental

Reagents and materials

Superparamagnetic iron oxide, i.e. γ-Fe_2O_3 nanoparticles (SPIO NPs) used in this study were prepared from magnetite (Fe_3O_4) according to the methods as previously proposed elsewhere [41,42]. The human U251 cell line was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell culture medium and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (CA, USA). Folic acid (FA), bovine serum albumin (BSA), *N*-hydroxysuccinimide (NHS), 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), 3-(4, 5-dimethylthiazol-2-diphenyl-tetrazolium) bromide (MTT), potassium ferrocyanide (Perls reagent), fluorescein diacetate (FDA), propidium iodide (PI), dimethyl sulfoxide (DMSO), and Triton X-100 solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neutral red were obtained from Beyotime Biotech (Jiangsu, China). Fluorescent dye FITC and 4, 6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Other reagents and chemicals were purchased from local commercial suppliers and were of analytical reagent grade, unless otherwise stated. Deionized (DI) water (Milli-Q, Millipore, Bedford, MA) was utilized to prepare aqueous solutions.

Synthesis of FA conjugated BSA-SPIO NPs

In the present study, the SPIO NPs were synthesized as a carrier core through the chemical coprecipitation method [41,42]. The SPIO NPs were then conjugated with BSA. In brief, 2 mL of prepared SPIO NPs (10 mg/mL) were dispersed into 40 mL of aqueous BSA solution (1.0 mg/mL), and the mixture was sonicated for 5 min, and then stirred at 150 rpm for 2 h at room temperature. The dispersion was washed with deionized (DI) water to obtain BSA-SPIO NPs by removal of unconjugated BSA under magnetic field. The obtained BSA-SPIO NPs were finally suspended in DI water and stored at 4 °C until use.

The prepared BSA-SPIO NPs were then decorated with FA via a chemical method as previously reported with minor modifications [43]. Briefly, the activated FA with amine reactive succinimidyl ester was prepared by reaction between 10 mL of FA (0.1 M) and 1-Ethyl-3-[3-dimethylaminopropyl] carbodimide hydrochloride (EDC, 0.2 M) in MES buffer (pH 5.5), stirred at 150 rpm for 15 min at room temperature in dark. 0.5 M *N*-hydroxysulfosuccinimide (Sulfo-NHS) in MES buffer (pH 5.5) was then added into the above EDC-FA solution, and the reaction was stopped after 6 h. Finally, the prepared amine reactive FA-NHS ester was incubated with BSA-SPIO NPs for 2 h in dark to obtain the FA-BSA-SPIO NPs. The obtained FA-BSA-SPIO NPs were washed several times with phosphate buffered saline (PBS, pH 7.4) and re-dispersed in PBS (pH 7.4) stored at 4 °C.

Preparation of fluorescent FA-BSA-SPIO NPs

The prepared FA-BSA-SPIO NPs were modified with fluorescein isothiocyanate (FITC) through chemical reaction between FA-BSA-SPIO NPs and FITC. Firstly, 50 mg of FA-BSA-SPIO NPs were resuspended in 5 mL of borate buffered saline (pH 8.4), and then mixed with 100 μg/mL of FITC solution, and incubated for 2 h stirring at 60 rpm at room temperature in dark. The prepared FITC-FA-BSA-SPIO NPs suspension was washed with PBS (pH 7.4) for three times under magnetic field, and then observed with an inverted fluorescence microscope (Eclipse TE 2000-U, Nikon, Kyoto, Japan) equipped with a high-resolution CCD camera (CV-S3200, JAI Co., Japan). Finally the prepared FITC-FA-BSA-SPIO NPs were suspended in RPMI-1640 medium and stored at 4 °C. Subsequently, the FITC-FA-BSA-SPIO NPs were up taken and internalized into U251 cells for intracellular imaging (Scheme 1).

In vitro MRI assessment of FA-BSA-SPIO NPs

The prepared FA-BSA-SPIO NPs were dispersed in DI water with iron concentrations in the range of 0–25 μg Fe/mL and imaged on a MesoMR23-60-I MRI scanner (Niumag, Shanghai, China) at a magnetic field of 1.5 T at room temperature. The T_2-weighted images were acquired at the following parameters: K matrix = 192 × 256, slice width = 5.0 mm, multiple echo time (TE) = 180 ms, repetition time (TR) = 3000 ms.

Cellular uptake of U251 cells with FA-BSA-SPIO NPs

The U251 cells were routinely cultured as previously indicated elsewhere [9]. The U251 cells were regularly monitored using an inverted light microscope, and the culture medium was replaced every two days. The U251 cells were normally passages in 1:3 ratio every three days to maintain exponential growth stage. At the exponential growth phase, the U251 cells were treated with 0.25% trypsin for 3 min at 37 °C to harvest cells in Ca^+ and Mg^+ free PBS (pH 7.4). The U251 cells were then seeded at a density of 2×10^4 cells/well and then incubated in a 24-well plate for 12 h. The varying concentrations of non-targeting BSA-SPIO NPs and targeting FA-BSA-SPIO NPs were then added to each well respectively, and incubated for 48 h, followed with fixation with 4% paraformaldehyde. After that, Prussian blue (PB) staining was used to determine the cellular uptake of the treated U251 cells by incubation with 2% potassium ferrocyanide (Perls reagent) and 2%

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