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The preliminary study of immune superparamagnetic iron oxide nanoparticles for the detection of lung cancer in magnetic resonance imaging

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ABSTRACT

To improve the sensitive and specific detection of metastasis of lung cancer, this study fabricated immune superparamagnetic iron oxide nanoparticles (SPIONs) used in magnetic resonance (MR) immumoimaging. These SPIONs were coated with oleic acid and carboxymethyl dextran, and then conjugated to mouse anti-CD44v6 monoclonal antibody. The physicochemical properties of magnetic nanoparticles without monoclonal antibody were characterized by X-ray powder diffraction (XRD), thermogravimetric analysis (TGA), and Fourier transform infrared spectroscopy (FTIR). The sizes of the nanoparticles were determined by dynamic light scattering measurements (DLS) and transmission electron microscope (TEM). Coated nanoparticles could well disperse in water with low dosage of CMD as the Fe/CMD ratio is 1/1 and 2/1 (w/w). Importantly, these SPIONs have relatively high saturation magnetization, as measured by vibrating sample magnetometer (VSM). They could efficiently become the transversal relaxation times (T2) contrast agent to improve detection limit through measured *in vitro* magnetic resonance imaging (MRI) and actively target human lung adenocarcinoma (A549) cells *in vitro* cell culture. Thus, these immune SPIONs are potentially useful for lung tumor-targeting diagnosis.

1. Introduction

Lung cancer is the most common cancer worldwide and the leading cause of cancer related deaths in many countries. Tumor metastasis is the leading cause of death in the patients with lung cancer.[1–4] Around 30% of lung cancer was diagnosed with synchronous distant metastasis, and 50% to 60% of distant metastases occurred in the course of treatment, and 80% to 90% lung cancer patients died of metastases.[5] Therefore, sensitive and specific detection of metastasis is the key to improving the treatment efficacy and the prognosis of lung cancer patients. However, traditional imaging methods have low sensitivity and specificity due to they mainly depend on morphologic characteristics to judge metastases.[6] The problem on how to sensitively and specifically diagnose lung cancer metastasis is to be solved urgently.

In the metastasis of lung cancer, a wide variety of tumor specific proteins are differentially expressed.[7] By detecting the differential expressions of these proteins, the metastasis may be sensitively and specifically detected. In available imaging methods, magnetic resonance imaging (MRI) technology is by far the most promising to detect the development of lung cancer at molecular level.[8] Gadolinium-based agents are commonly used contrast agents for MRI. However, these agents can't realize the specific imaging of cancers, as they have rapid exertion, poor target-specific biodistribution and undesired side-effect.[9–11] Currently, superparamagnetic iron oxide nanoparticles (SPIONs), which have an increasing potential application in drug delivery[12–14] and hyperthermia treatment,[15] are attracting much attention for magnetic resonance imaging contrast agent. This is because SPIONs can enhance the proton transverse relaxation of water tissues,[16–19] consequently providing MR T2 shortening effects.[20] However, these naked SPIONs have a large surface area/volume ratio that leads to nonspecific adsorption of plasma proteins onto the particles' surfaces and agglomeration; as a result, they are quickly eliminated by the reticular endothelial system (RES) such as macrophages.[21,22]

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Therefore, for clinical application, surface modification of SPIONs is necessary. Recently, several synthetic and natural polymers such as polyethylene glycol (PEG),[23] dextran,[24] and heparin,[25] all of which have good biocompatibility, have been employed to encapsulate SPIONs, accordingly to enhance their water dispersibility and extend blood circulation times. However, most of these surface modified SPIONs exhibited non-specific binding to tumor cells that correlated with low efficiency and low specificity of nanoparticles to tumor cells.

Although numerous scientists have devoted their vigor to *in vivo* cancer imaging studies,[25–27] the sensitivity in specific tissue hardly meet the clinical application needs. Fortunately, active targeting drug delivery systems provide a novel approach to overcome this dilemma.[12] Active targeting moieties, such as ligands,[28] antigens,[29] or monoclonal antibodies,[30] conjugated to surface modified SPIONs can achieve an enhanced effect of tumor-targeting specificity and efficacy using clinical MRI, which bind specifically to a receptor that is overexpressed in tumor cells, but not in normal cells. Modified by carboxymethyl dextran (CMD), SPIONs have no acute adverse effect on cells even at a high dosage.[31] And they are stable in aqueous solution and can effectively conjugate with monoclonal antibodies.[30]

As a transmembrane glycoprotein receptor and a variant isoform of CD44, CD44v6 has been identified as biological (protein) marker for metastatic behavior in epithelium-derived cancer.[32,33] Transfection of spliced variant CD44v6 was capable of conferring metastatic potential on cells of a nonmetastatic tumor cell line, thus regulating tumor invasion, progression, and metastasis of carcinoma in rat experimental models.[34] D. Situ et al. have proven that the high expression of CD44v6 could be detected in the primary lesions of stage I non-small cell lung cancer,[35] indicating that binding with the receptor CD44v6 can be applied to detect and prevent lung cancer metastasis. SPIONs modified with hyaluronic acid (HA) have been used in the diagnosis and treatment of CD44-overexpressing cancer.[14,36,37] Moreover, anti-CD44v6 single chain antibody conjugated nanoparticles have been used as efficient probes for targeted MR imaging of cancer cells overexpressing CD44v6 receptors *in vitro* and xenografted tumor models *in vivo*.[38] Other studies on iron oxide nanoparticles preparation and surface functionalization showed that the SPIONs targeted to CD44 have a great potential application in MR imaging.[39–41]

Herein, to prepare water dispersible SPIONs that can actively target lung cancer cells, these nanoparticles were first prepared by the method of chemical coprecipitation.[23] The CMD containing carboxyl groups (COOH) in their repetitive unit, in combination with oleic acid, were used as steric stabilizers to encapsulate these SPIONs. Afterwards, mouse anti-CD44v6 monoclonal antibodies were conjugated with CMD to achieve early and specific diagnosis. We investigated the physicochemical properties, magnetic property, *in vitro* MRI and *in vitro* targeting property of the SPIONs.

2. Experimental section

2.1. Materials

All chemical reagents and solvents obtained from commercial suppliers were used as received. Ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$ >99%), ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$ >99%), oleic acid (OA, ≥97%), sodium hydroxide (≥98%), aqueous ammonium hydroxide (25–28%, w/w), bromoacetic acid (≥9 8%) were purchased from Chengdu Kelong Chemical Co., Ltd., China. Dextran T-70 was purchased from Aladdin; 4-(2-hydroxyethyl)-1-piperazineethane- sulfonicaci (HEPES, ≥99.0%) was purchased from Amersco. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, ≥99.0%) was purchased from J&K Chemical Ltd; N-hydroxysulfosuccinimide (NHS, ≥ 98.0%) was purchased from Alfa Aesar. Fluorescein isothicynate (FITC, 90%) was purchased from Acros Organics. Mouse anti-CD44v6 was purchased from Invitrogen and DAPI was purchased from Sigma; human lung adenocarcinoma cell (A549) was obtained from Huaxi Hospital, Sichuan University, China.

2.2. Synthesis of carboxymethyl dextran (CMD)

Five grams of dextran T-70 was added into 25 mL, 2 mol/L NaOH solution, which contains 0.1 mol/L bromoacetic acid. The mixture was stirred for 24 h at room temperature. Then the solution was dialyzed against dilute hydrochloric acid and deionized water for 24 h, respectively. White spumescence blocks were obtained after freeze dehydration and the yield is 71%.

2.3. Synthesis of Fe_3O_4 nanoparticles modified by oleic acid (OA) in combination with carboxymethyl dextran (CMD)

The preparation of Fe_3O_4 nanoparticles is based on an existing method that belongs to chemical coprecipitation.[23,42] The details are described as follow: $FeCl_3{\cdot}6H_2O$ (5.41 g, 0.02 mol) and $FeCl_2{\cdot}4H_2O$ (1.99 g, 0.01 mol) were added into a 250 mL three-necked flask with 100 mL deoxygenated deionized water under nitrogen flow quickly; and the solution was heated to 80 °C in half an hour, then the stirring speed was modulated to 800 r/min; 25 mL aqueous ammonium hydroxide was dropwise added at the rate of 3 drops per second. The solution's color changed gradually from brown to black, indicative of the formation of iron oxide particles. Finally, 1 mL oleic acid was added into the black solution, and the dispersion was continuously stirred for another 1 h under a nitrogen flow to complete the reaction. The solutions were naturally cooled to room temperature and dialyzed against deionized water for two days to remove the ammonia and impurities. The resulting OA coated SPION (SPION-OA) stock solution with Fe_3O_4 concentration of 18.56 mg/mL, which was determined by the theory calculation, was stored at 4 °C before use. Additionally, the naked SPIONs could be obtained with the same process without oleic acid.

Desired amount of carboxymethyl dextran (CMD) (variable from 0.1 mg to 100 mg) with the concentration 1.856 mg/mL in water blended with 5 mL SPION-OA solution, which stem from the stock solution diluted tenfold to make up mixture solution with different mass ratio of Fe/CMD (10/1, 8/1, 6/1, 4/1, 2/1, 1/1, 1/2, 1/4, 1/5, 1/6, 1/7, 1/10). The mixture was sonicated at 120 W for 2 h. To remove the remaining free polymer in solution, an external magnetic field was applied to the solution using a magnet. Within minutes, all the black particles sank toward the magnet, and the supernatant was discarded. The black precipitate was washed twice and sonicated at 200 W using an ultrasonic processor (KQ5200 DE, Kunshan Ultrasonic Instrument Co., Ltd., China) in equal volume of distilled water to redisperse it. The resulted Fe_3O_4 nanoparticles modified by oleic acid (OA) in combination with carboxymethyl dextran (SPION-OA-CMD) were stored at 4 °C before use.

2.4. Conjugation antibody to SPION-OA-CMD

Coupling of mouse anti-CD44v6 to the SPION-OA-CMD was performed by utilizing the carboxyl groups on the particle surface.[43] Here we should point out that the SPION-CMD and SPION-OA are not stable during the process of conjugation antibody to carboxyl. Three milliliters of SPION-OA-CMD was diluted with 0.3 mL HEPES solution (1 mol/L). Then 0.5 mg 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 0.75 mg N-hydroxysulfosuccinimide (NHS) were added into the solution and incubated for two hours with mechanical shaking at the pH = 5.37. Then the pH value was adjusted to 7.4 by 0.5 M NaOH, followed by addition of 20 μL antibody into 1 mL of the reaction complex and incubated over night with mechanical shaking. The resulting