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Carboxylic mannan-coated iron oxide nanoparticles targeted to immune cells for lymph node-specific MRI *in vivo*

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ABSTRACT

Carboxylic mannan (CM)-coated super paramagnetic iron oxide nanoparticles (CM-SPIONs) were prepared to target antigen-presenting cells (APCs), including macrophages, by the specific interaction between the mannose ligand tethered on CM-SPION and mannose receptors on APCs. Carboxylic mannan was synthesized by introducing the aldehyde group to mannan by oxidation, followed by the conversion of aldehyde groups to carboxyl groups. CM-SPION exhibited uniform-sized nanoparticles with a highly negative surface charge appropriate for longer blood circulation. It was demonstrated that CM-SPION could target macrophages bearing mannose receptors more specifically than polyvinyl alcohol (PVA) or dextran-coated SPION. The *in vitro* and *in vivo* toxicities of CM-SPION were evaluated, and the results showed that the LD_{50} of CM-SPION was much higher than that of mannan-SPION (80 mg Fe/kg *vs.* 44 mg Fe/kg in mice, respectively). The uptake of CM-SPION by peritoneal macrophages was also confirmed with Prussian blue staining and magnetic resonance (MR) phantom tube imaging. In the *in vitro* uptake study visualized by MR phantom tube imaging, the intracellular uptake of CM-SPION was much faster than those of dextran-coated SPION (Dex-SPION) and PVA-coated SPION (PVA-SPION) at the initial hours of incubation, and increased drastically up to 24 h post-incubation. The *in vivo* uptake of CM-SPION in lymph nodes (LNs) was tracked by MR imaging (MRI) after subcutaneous injection in a rat model. It was found that the injected CM-SPION predominantly accumulated in the popliteal LN, and the *in vivo* accumulation rate with CM-SPION in the LN was comparable to that of Dex-SPION, the positive control, as measured by a signal drop in MR intensity. Histological analysis with Prussian blue staining also confirmed the accumulation of SPION in the LN.

1. Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) have been used as a contrast agent in magnetic resonance imaging (MRI) or as a carrier platform in the applications of drug (Butoescu, Seemayer, et al., 2009; Butoescu, Jordon, et al., 2009; Munnier et al., 2008) and gene delivery (Lee, Kim, Kim, & Kim, 2002; Ran et al., 2010). SPIONs can be synthesized by different methods, including co-precipitation, reaction in constrained environments, thermal decomposition, sol–gel reaction, polyol methods, aerosol/vapor methods, and sonolysis (Laurent et al., 2008). To increase its stability, SPION has been coated by many kinds of hydrophilic materials such as dextran, polyethylene glycol (PEG), poly-vinyl alcohol (PVA), chitosan, and others (Chastellain, Petri, & Hofmann, 2004; Lee et al., 2002; Liu et al., 2011; Lu, Yin, Mayers, & Xia, 2002; Petri-Fink, Chastellain, Juillerat-Jeanneret, Ferrari, & Hofmann, 2005; Schöpf et al., 2005; Shen, Weissleder, Papisov, Bogdanov, & Brady, 1993). However, SPION medical applications are limited due to several problems such as aggregation, toxicity, and lack of tissue specificity during systemic circulation (Gupta & Gupta, 2005; Lin, Lee, & Chiu, 2005; Neenu, Gareth, Romisa, & Shareen, 2010).

The properties of nanoparticles can be significantly altered by surface modification. The typical approach to tailoring the surface

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properties of nanoparticles is mediated by surface coating or encapsulation with biocompatible materials (Caruso & Antonietti, 2001). Besides improving its durability and suspensibility in biological environments, and its biocompatibility, the functional coating on the surface of nanoparticles can also be used to immobilize or tailor the bio-distribution of foreign molecules (Bucak, Jones, Laibinis, & Hatton, 2003). The surface coating of SPION has helped to resolve its drawbacks encountered in *in vivo* with unmodified SPION. The carboxyl group has been introduced as a surface coating for SPION, and this approach has been shown to have many advantages (Yu & Chow, 2004). Negatively charged SPION usually has the free carboxyl group on its surface, and this can be utilized for covalent attachment with proteins and other therapeutic molecules. The carboxyl group can also improve the dispersion of iron oxide nanoparticles in a biological system by improving the hydrophilicity (Yu & Chow, 2004). Negatively charged nanoparticles have less chance of interacting with the negatively charged plasma membrane of the cells and the negatively charged proteins encountering during circulation before reaching the target tissue; this results in increased systemic circulation (He, Hu, Yin, Tang, & Yin, 2010).

Targeting to specific cells or tissue is an important issue for the medical application of nanoparticles. For instance, SPION coated with polyvinylbenzyl-*O*-β-D-galactopyranosyl-D-gluconamide (PVLA)-galactose moieties can be targeted to hepatocytes *via* asialoglycoprotein receptors, which allows for the enhanced MR contrast imaging of the rat liver after intravenous administration (Yoo et al., 2007). Moreover, dextran-coated SPION (Dex-SPION) has been approved in clinical trials as an MRI contrast agent with commercial names such as Lumirem, Endorem, and Sinerem. Dex-SPION could be targeted to macrophages through scavenger receptors, which have been already used for the diagnosis of metastatic liver and metastatic LN in MRI (Bellin et al., 1994; Corot, Robert, Idée, & Port, 2006; Harisinghani et al., 2003; Raynal et al., 2004). Carboxy dextran SPION (SH U 555 A, RESOVIST) has also been approved in clinical trial settings as a faster contrast agent for liver MRI (Reimer & Balzer, 2003). The compound can be regarded as safe and well tolerated. Even bolus injections cause no cardiovascular side effects, lumbar back pain, or clinically relevant laboratory changes. It also prevents nanoparticle aggregation and makes magnetic nanoparticles highly hydrophilic (Reimer & Tombach, 1998). It is also reported that dextran SPION accumulates in the LN after local injection. Once it reaches the LN, it is trapped by the immune cells in the LN (Mehvar, 2000).

It was previously reported that mannan-coated SPION (mannan-SPION) could be specifically targeted to macrophages by the interaction with mannose receptors on antigen-presenting cells (APCs) (Yoo et al., 2008). Mannan is a cell wall component of microorganisms, consisting of D-mannose residues expanded by a-(1,6)-, a-(1,3)-, and a-(1,2)-linkages. Mannan is also recognized by the mannose receptors of APCs and reticuloendothelial cells, which mainly reside in the normal LN. In our previous study, it was also demonstrated that mannan-SPION was specific to immune cells in LN due to mannose receptor-mediated endocytosis, facilitating preferential uptake in APCs and achieving faster acquisition and enhanced contrast of MR imaging in target tissue as compared with Dex-SPION and PVA-coated SPION (PVA-SPION) (Vu-Quang et al., 2011). It was also previously reported by our group that SPION coated with β-glucan could target APCs, since glucan was reported to elicit immune responses through the activation of macrophages with an immune cell-specific (1,3)-β-D-glucan receptor or dectin-1 receptor. β-Glucan-coated SPION was internalized by the immune cells residing in the metastatic liver, which aided in discrimination between metastasized tumor regions and normal hepatic parenchymal tissue. β-Glucan also induces the immune system, which aids in anti-tumor activity (Vu-Quang et al., 2012). However, the direct exposure of immune cells to a higher molecular yeast mannan component coated on mannan-SPION might cause severe immune responses after administration (Ataoglu, Dogan, Mustafa, & Akarsu, 2000). It has been hypothesized that the carboxylation of mannan coated on the surface of iron oxide nanoparticles might reduce this toxic response. Thus, carboxylic mannan-coated SPION (CM-SPION) was synthesized by introducing the carboxyl group to mannan with periodate/sodium chlorite reactions. Following this, the carboxylic mannan was coated on SPION in order to alleviate the systemic toxicity caused by the direct exposure to higher molecular mannan. We investigated the physicochemical properties, the *in vitro* and *in vivo* uptakes of CM-SPION using MRI and assessment of systemic toxicity. This uptake was also confirmed with Prussian blue staining.

2. Materials and methods

2.1. Materials

Mannan from *Saccharomyces cerevisiae* was purchased from Sigma–Aldrich Chemical Co. (MO, USA). Hydroxyl-amine hydrochloride, 2,4-dihydroxy-benzaldehyde, and sodium chlorite were also purchased from Sigma–Aldrich. Sodium periodate, ethylene glycol, and hydrogen peroxide solution were purchased from Samchun Chemicals (South Korea). The dialysis membrane (MWCO 1000 Da) was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA).

2.2. Carboxylation of mannan

To introduce aldehyde groups onto mannan, mannan was oxidized following the standard methods with minor modifications (Massia & Stark, 2001; Mislovičová, Masárová, & Gemeiner, 2001). Mannan (400 mg, 2.3 mmol mannose) was dissolved in 16 mL of distilled water (DW); then, sodium periodate solution (0.1 M, 0.45 mmol or 0.90 mmol) was slowly dropped into the solution under continuous stirring at 4 °C in the dark for 1 h. The oxidation reaction was stopped by adding ethylene glycol (0.9 mmol or 1.8 mmol) and stirred for another 30 min. The oxidized mannan was purified by dialysis using a dialysis membrane (MWCO 1000 Da) against DW at 4 °C for 1 day. The purified oxidized mannan was then lyophilized to obtain a white solid and kept at 4 °C.

The number of aldehyde groups of mannan was quantitatively analyzed using the standard protocol with a slight modification (Zhao & Heindel, 1991). Aldehyde-mannan (11 mg) was dissolved in hydroxyl-amine hydrochloride solution (0.25 M, 5 mL) with a pH of 2.14. The solution was sonicated for 2 h and stored overnight at room temperature. Degree of aldehyde groups of oxidized mannan was determined by titration of the HCl, which was generated by treating the aldehyde functionality with a measured amount of hydroxylamine hydrochloride. The titration was performed using NaOH (0.1 M) solution until the end point was reached at pH 2.14. Finally, the amount of aldehyde groups in the mannan was calculated by comparing the titer value of NaOH against the amount in 2,4-dihydroxy-benzaldehyde, a standard aldehyde reagent for this titration method. The degree of aldehyde functionalization on the mannose ring was estimated to be 19% or 29%.

To convert the aldehyde groups to carboxylic acid groups, aldehyde mannan was oxidized using sodium chlorite (Haaksman, Besemer, Jetten, Timmermans, & Slaghek, 2006; Raach & Reiser, 2000). Aldehyde mannan (340 mg, 19% ring-opened mannose or 370 mg, 29% ring-opened mannose) was dissolved in 20 mL DW and hydrogen peroxide (1.2 equiv. to mannose ring) was added in an ice bath. Then, sodium chlorite solution (0.5 M, 1.2 equiv. to mannose ring) was slowly dropped into the solution and the pH was adjusted to 5. After removal from the ice bath, the mixture was stirred and