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Super-paramagnetic loaded nanoparticles based on biological macromolecules for *in vivo* targeted MR imaging

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ABSTRACT

Target-specific MRI contrast agent based on super-paramagnetic iron oxide–chitosan-folic acid (SPIONP-CS-FA) nanoparticles was fabricated by using an ionotropic gelation method, which involved the loading of SPIONPs at various concentrations into CS-FA nanoparticles by electrostatic interaction. The SPIONP-CS-FA nanoparticles were characterized by ATR-FTIR, XRD, TEM, and VSM techniques. This study revealed that the advantages of this system would be green fabrication, low cytotoxicity at iron concentrations ranging from 0.52 mg/L to 4.16 mg/L, and high water stability (pH 6) at 4 °C over long periods. Average particle size and positive zeta-potential of the SPIONP-CS-FA nanoparticles was found to be 130 nm with narrow size distribution and 42 mV, respectively. In comparison to SPIONP-0.5-CS nanoparticles, SPIONP-0.5-CS-FA nanoparticles showed higher and specific cellular uptake levels into human cervical adenocarcinoma cells due to the presence of folate receptors, while *in vivo* results (Wistar rat) indicated that only liver tissue showed significant decreases in MR image intensity on T_2 weighted images and T_2^* weighted images after post-injection, in comparison with other organs. Our results demonstrated that SPIONP-CS-FA nanoparticles can be applied as an either tumor or organ specific MRI contrast agents.

1. Introduction

Over the past decade, magnetic nanoparticles (MNPs) have evolved from being academic curiosities through to addressing breakthrough applications in medical diagnostics, and the treatment of diseases such as cancer, cardiovascular disease, and neurological disease [1–4]. Super-paramagnetic iron oxide nanoparticles (SPIONPs), including magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3), were the first to be developed and are now widely used MNPs-based contrast agents due to their chemical stability, low toxicity, and biodegradability [5,6]. Generally, clinical magnetic resonance imaging (MRI) contrast agents can be classified into two major classes based on their magnetic properties; T_1 or positive contrast agents, and T_2 or negative contrast agents. Paramagnetic agents shorten the longitudinal relaxation time (T_1) and create bright contrast, resulting in greater signal intensity with positive enhancement (hyper-intensity) in T_1 weighted images. On the other hand, super-paramagnetic agents also shorten the transverse relaxation time (T_2) and provide darkness in images, resulting in decreases in signal intensity with negative enhancement (hypo-intensity) in T_2 weighted images, and T_2^* weighted images.

Recent advances have seen target-specific contrast agents based on MNPs being developed for improving the potential sensitivity, specificity, and detectability of current imaging techniques [5–8]. Several targeting agents have been proposed as cancer markers to be used in both preclinical and clinical settings, for specific interaction with the following receptors and growth factors: somatostatin, transferrin, $\alpha_v\beta_3$ integrin, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), carcinoembryonic antigen (CEA), prostate stimulating membrane antigen (PSMA), and folate [9–12]. Since folate receptor (FOLR) is significantly over-expressed on the surface membranes of many cancer types including brain, breast, lung, thyroid, cervix, pancreas, colon, renal, ovary, uterus, prostate, and hematopoietic cells of myelogenous origin [12–20], targeting FOLR is potentially an attractive strategy for treatment of these cancer types. However, an issue with using SPIONPs for this purpose is their propensity to undergo aggregation in water or tissue fluid, which limits *in vitro* and *in vivo* magnetic-based

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isolation and detection strategies, and therefore their usefulness in clinical MRI applications [21,22]. Coating SPIONPs with natural polymers such as chitosan (CS), a natural cationic polysaccharide derived from chitin which has been widely used in medical applications due to its non-toxic nature, low immunogenicity, excellent biocompatibility and biodegradability, and thus preventing their aggregation, would potentially alleviate these problems and enable SPIONPs to be used as effective contrast agents [23,24]. We recently reported the loading of SPIONPs within CS nanoparticles using an ionotropic gelation method, with pentasodium triphosphate (TPP) as a cross-linking agent [25]. Our results demonstrated that SPIONPs loaded within CS/TPP nanoparticles enhanced their ability to be dispersed in aqueous solution, and all particles were lower than 130 nm in size. Additionally, particles retained their superparamagnetic properties at room temperature with an r_2/r_1 value of 6.81, suggestive of being suitable as T_2 contrast agents for MR imaging.

However, no attention has been paid thus far to the development of SPIONPs loaded within CS-folic acid (FA) (SPIONP-CS-FA) nanoparticles by using an ionotropic gelation method. This method is very simple, which involves dropping aqueous solution of TPP into CS-FA solution at room temperature. Our system is not harmful to human health and the environment because of no toxic wastes and no utilization of organic solvents. Therefore, incorporation of FA onto the SPIONP-CS surface may impart greater sensitivity of the SPIONP-CS to FOLR, and subsequent targeting of these agents to cancer cells and animal cancer models. This could be due to targeting cell membranes and enhancing endocytosis of SPIONP-CS-FA nanoparticles via the FOLR. Prior to further study in animal cancer models, the focus of this report is to fabricate and determine physicochemical properties of SPIONP-CS-FA nanoparticles by ATR-FTIR, DLS, TEM, XRD, and VSM techniques. The effects of SPIONP-CS-FA nanoparticles, which used as MR contrast agent, on T_2 weighted (T_2W) and T_2^* weighted ($T_2{*}W$) images as well as *in vitro* targeted cellular uptake in human cervical adenocarcinoma cells (HeLa cells) were investigated. Furthermore, targeted *in vivo* biodistribution in healthy animals (Wistar rat) was also investigated.

2. Materials and methods

2.1. Materials

Chitosan (CS) having an average M_W of 10 kDa was purchased from OilZac Technologies Co., Ltd. (Bangkok, Thailand). The degree of deacetylation (DDA = 96%) was determined by ^{1}H NMR spectroscopy. Anhydrous ferric chloride ($FeCl_3 \geq 98\%$) was purchased from Fluka-Chemika (Deisenhofen, Germany). Ferrous sulfate ($FeSO_4{\cdot}7H_2O$, 99.5%), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO) and glacial acetic acid (CH_3COOH, 99.9%) were purchased from Carlo Erba Reagents (Italy). *N*-hydroxysuccinimide (NHS, 98+%), pentasodium triphosphate (TPP) and folic acid (FA) were purchased from Acros Organics, Geel (Belgium). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma–Aldrich (USA). Dialysis tubing with a molecular weight (3500 MW) cut-off was purchased from Cellu Sep T1 (Membrane Filtration Products, Inc., Segiun, TX, USA). All chemicals and reagents were used without further purification. MilliQ Plus (18.2 MΩ, Millipore, Schwalbach, Germany) purified water was used to make all aqueous solutions.

2.2. Synthesis of SPIONPs

The SPIONPs were synthesized through a modified co-precipitation method from that reported by Wei and coworkers [26]. Briefly, 1.62 g of $FeCl_3$ and 1.39 g of $FeSO_4{\cdot}7H_2O$ (2:1 molar ratio of Fe(III): Fe(II)) was dissolved in 10 mL of 12 M HCl. This solution was added dropwise with stirring to a three-necked round bottom flask containing 50 mL of 1.5 M NaOH solution at room temperature under nitrogen atmosphere, followed by stirring for 30 min. The reaction mixture was then heated at 70 °C for 2 h. Following this, the suspension was cooled down with an ice water bath (1–5 °C) and the black precipitate was isolated by centrifugation at 3000 rpm for 10 min. After washing with DI water four times the precipitate was suspended in 100 mL of DI water and sonicated using an ultrasonic bath for 30 min. The colloidal suspension thus obtained (pH 6) was stored at 4 °C in the dark prior to further use.

2.3. Preparation of SPIONP loaded CS-FA nanoparticles

The CS-FA was synthesized by covalent cross-linking technique [27]. Briefly, CS (100 mg) was added to 10 mL of 1% (v/v) aqueous acetic acid solution and stirred at room temperature until a clear solution resulted. At the same time, 2.73 mg of FA (0.01 eq/NH_2 of GlcN) was dissolved in 3 mL of DMSO, and the solution was stirred at room temperature in the dark. The carboxyl group of FA was activated by adding 1.42 mg of EDC, and 1.42 mg of NHS (molar ratio of FA: EDC: NHS at 1: 1.2: 2) to the FA solution, followed by stirring for 1 h. Following this, the activated FA solution was added dropwise into the CS solution, and the mixture was stirred at room temperature for 24 h in the dark. Subsequently, the CS-FA solution was dialyzed in DI water for 3 days to remove DMSO and unreacted FA. The dialysed product was then lyophilized in the dark to afford CS-FA for the loading experiments. The above solution was freezed in a liquid nitrogen to form ice state. The samples were then vacuum dried in freeze-dry (CHRIST, GAMMA 2–16 LSC) at −80 °C and 0.1 mbar for 24 h. The SPIONPs loaded CS-FA triphosphate (SPIONPs-CS-FA) nanoparticles were prepared by using an ionotropic gelation method, according to our previously reported protocol [25]. The SPIONP-CS-FA nanoparticles, made from different volumes of the SPIONP colloidal solutions (0.1 mL, 0.3 mL or 0.5 mL) are abbreviated herein as SPIONP-0.1-CS-FA, SPIONP-0.3-CS-FA, and SPIONP-0.5-CS-FA, respectively. The total Fe concentrations in all samples were determined by using ICP-OES (Table 1).

2.4. Characterizations

2.4.1. ATR-FTIR analysis

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were recorded on a Nicolet 6700 spectrometer (Thermo Company, USA) using the single bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE) at 25 °C.

2.4.2. XRD analysis

X-ray diffraction (XRD) patterns were recorded on a Bruker D8 ADVANCE diffractometer (Bruker, Switzerland) with CuKα radiation (λ = 1.5406 Å).

2.4.3. TEM analysis

Transmission electron microscope (TEM) images were obtained on a JEM-100CX II TEM instrument operating at 120 kV. TEM samples were prepared by placing an aqueous suspension of the nanoparticles onto carbon-coated copper grids and then air drying before examination.

2.4.4. VSM analysis

Vibrating sample magnetometry (VSM) was performed on a Lakeshore Model 740H VSM Head Drive instrument (Cryotronics, Inc., Ohio, USA). The hysteresis of magnetization was obtained by