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Physico-chemical and toxicological characterization of iron-containing albumin nanoparticles as platforms for medical imaging

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ABSTRACT

Iron oxide-containing magnetic nanoparticles (MNPs) have certain advantages over currently used contrast agents for tumor imaging by magnetic resonance imaging (MRI) as they offer the possibility of functionalization with ligands and tracers. Functionalized MNPs also may be used for targeted tumor therapy. In the current study nanoparticles (NPs) consisting of recombinant human serum albumin (rHSA) with incorporated hydrophilic (NH_4) $2Ce(IV)(NO_3)_6$-γ-Fe_2O_3 particles (CAN maghemite particles) for medical imaging were produced and characterized. For this purpose CAN maghemite particles were incorporated into an rHSA matrix to yield rHSA-NPs. The resulting NPs were analyzed by transmission electron microscopy, photon correlation spectroscopy, and atomic absorption. The sizes of the manufactured NP were 170 ± 10 nm, and the zeta-potential was −50 ± 3 mV. The NPs remained stable when stored after lyophilization with sucrose 3% [w/v] as a cryoprotector. They showed pro-inflammatory properties without cell and animal toxicity *in vivo* and were highly contrasting in MRI. In conclusion, this report introduces novel rHSA NP with favorable properties containing iron oxide for detection by MRI.

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Introduction

In 2008 7.6 million cancer-related deaths were registered by the World Health Organization [1]. Diagnosis of the tumors at earlier stages would enable a higher percentage of patients to be treated with potentially curative therapies and would, therefore, be highly beneficial. During the last decades, magnetic resonance imaging (MRI) and also positron emission tomography (PET) became essential medical imaging techniques for cancer diagnosis, especially for brain and abdominal tumors. Considerable progress came from continuous improvement of the MRI scanners and contrast agents. At present two major types of MRI contrast agents are available: T1-shortening contrast agents such as gadolinium or manganese chelates, producing signal increase, and T2-shortening contrast agents such as iron compounds reproducing the underlying MRI signal.

Currently, small molecule gadolinium chelates are almost exclusively used in clinical MRI practice. To overcome their inherent limitations, large efforts are currently under way to develop NP-based MRI contrast agents. Their properties can be tailored to achieve a desirable biodistribution and to cross biological barriers such as for instance the blood–brain barrier [2,3]. Moreover, they can be functionalized for active targeting. Nevertheless, iron oxide nanoparticles are the only NPs that have been introduced into routine clinical imaging. Thus, superparamagnetic iron oxide (SPIO) NPs have been used in MRI of unclear liver lesions [4]. These contrast agents are rapidly taken up by liver macrophages, the density of which is lower in malignant liver tumors as compared to normal liver tissue, thus contrasting malignant tumors in the liver. A new approach was the development of hydrophilic water-compatible maghemite (γ-Fe_2O_3) NPs (CAN stabilized maghemite NP). They show a low aggregation tendency and are very well suited for the preparation of biocompatible magnetic nanoparticles (MNP) [5].

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At present, major challenges in the development of NP-based contrast agents encompass the development of appropriate matrices that can be readily functionalized with ligands for efficient targeting, and to obtain appropriately targeted NPs. One of the main approaches is the entrapment of magnetic iron NPs into polymers [6]. The selected polymer has to fulfill the following requirements: it has to be biodegradable, non-toxic and it has to offer functional groups to modify the surface for the active targeting processes.

Different NP matrices, such as serum-derived and recombinant human serum albumin (rHSA) and synthetic polymers, including poly(lactide-co-glycolide) (PLGA), or poly-(n-butyl cyanoacrylate) (PBCA) have been used for NPs [7–10]. Recently non-toxic HSA nanoparticles conjugated with gadolinium for hepatic imaging that are non-toxic and stable in serum were developed [11]. HSA is a well-known and widely used excipient in pharmaceutical formulations such as vaccines and biologicals. It is obtained by fractionation of human plasma, which has the potential of viral or prion contamination. To avoid these contaminations, rHSA, which has been expressed in *Pichia pastoris* [12] with a consistent high yield and quality, was used in the present study and represents an alternative to HSA from human sources. Using this material, highly contrasting magnetic NPs for MRI were developed. The preparation process was reproducible and robust. *In vitro* and *in vivo* studies were performed to provide information about safety and biological effects. MRI measurements showed the contrasting capacity of the obtained NPs.

Methods

Preparation of magnetic iron particles

A solution of $FeCl_3$•$6H_2O$ (240.0 mg, 0.9 mmol) dissolved in degassed Milli-Q (Milli-Q Direct-Q UV, EMD Millipore, Darmstadt, Germany) water using argon-gas for degassing (4.5 mL) was mixed with an aqueous solution of $FeCl_2$•$4H_2O$ (97.5 g, 0.45 mmol, 4.5 mL H_2O). This solution was kept under N_2 and ultrasonicated (Bransonic® ultrasonic cleaner bath, 2510E MTH model, 42 kHz at full power) for 5–10 min at room temperature. Then, a concentrated 24% [w/v] aqueous NH_4OH (0.75 mL) solution was introduced in one shot, resulting in an immediate black precipitation of magnetite (Fe_3O_4) particles. The sonication was continued for additional 10 min. The resulting Fe_3O_4 particles were transferred into a glass bottle, magnetically decanted (using an external magnet), and washed with degassed doubly distilled H_2O (ddH_2O) (3 × 40 mL) until neutrality. Black free flowing magnetite particles were stored as a 30 mL suspension in ddH_2O before any further processing, followed by an aging process for a minimum storage time of at least 2 hours at room temperature. In the next step, CAN-stabilized maghemite particles (CAN, $(NH_4)2Ce(IV)(NO_3)_6$-γ-Fe_2O_3 particles) were prepared. Firstly, the former aqueous magnetite suspension (30 mL) was magnetically decanted to separate the magnetite particles from its aqueous storage phase. CAN (300.0 mg, 0.547 mmol) dissolved in 12.0 mL MeCOMe was introduced onto decanted magnetite particles, followed by the addition of 12 mL degassed Milli-Q water. The corresponding mixture was ultrasonicated using a high-power sonicator (Sonics®, Vibra cell, 750 W, power modulator set to 25%) equipped with a titanium horn (1 hour, 0 °C) under an inert argon atmosphere. At this stage optionally, the resulting highly stabilized hydrophilic CAN-γ-Fe_2O_3 particles were purified by washing with ddH_2O (3 × 10 mL) using Amicon® Ultra-15 centrifugal filter devices (100 K) processed at 4000 rpm during 5–6 min (room temperature, 18 °C) and redispersed in ddH_2O (15 mL).

CAN maghemite-containing rHSA NP preparation

rHSA NPs were prepared using a desolvation technique developed by Langer et al. [10]. Firstly, the rHSA had to be dialyzed using a Slide-A-Lyzer Dialysis Cassette MWCO 3500 kDa (Pierce, Rockford, IL). Dialysis took place in two cycles of 2 hours, respectively, and one cycle of 15 hours using Milli-Q water as dialysis medium. Subsequently, the dialyzed protein was freeze-dried using a Christ Epsilon 1-4 (Christ Epsilon 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). For rHSA NP preparation, rHSA was dissolved to 100 mg/mL in a 10 mM NaCl solution, adjusted to pH 8.5, and filtered through a sterile filter (FP 30/0 2CA, Schleicher & Schuell, Dassel, Germany). Afterwards, desolvation took place by addition of ethanol into the protein solution under constant stirring using a pump rate of 1 mL/min (ISMATEC IPN, Glattbrugg, Switzerland). This procedure enabled a defined NP formation process. The 61 freely available amino groups on the surface of the desolved protein were inactivated by the twofold excess of the bifunctional crosslinker glutaraldehyde added to the suspension (calculated on the basis of 61 free amino groups per molecule of rHSA). The NP suspension then was stirred at room temperature for 24 hours to ensure a quantitative crosslinking process.

Excess glutaraldehyde was removed from the NP suspension by three cycles of centrifugation (20 min × 20,100*g*) and resuspension of the centrifugation pellet in Milli-Q water. The resuspension steps were performed in an ultrasonic bath (Bandelin, Sonorex, Berlin, Germany). Finally, the amount of NP in suspension was determined gravimetrically.

The CAN maghemite-containing rHSA nanoparticles (MNPs) were prepared by a procedure identical to the one described for rHSA NP. In brief, to 0.5 mL dialyzed rHSA (200 mg/mL in 10 mM NaCl solution) either 1000 μg or 2000 μg of iron in terms of CAN maghemite particles was added and incubated for 1 hour at 20 °C (Eppendorf thermomixer, 300 rpm, Hamburg, Germany) to obtain 1000-MNP or 2000-MNP, respectively.

Characterization of CAN maghemite-containing rHSA NP

NPs were characterized with respect to their particle size, polydispersity, and zeta potential by dynamic light scattering (DLS) using a Zetasizer Malvern Nano ZS (Malvern Instruments Ltd., Malvern, UK). For this purpose the samples were diluted 1:100 with filtered Milli-Q water (FP 30/0 2CA, Schleicher & Schuell).

Iron determination

The iron content of the magnetic nanocore system was determined by atomic absorption spectroscopy (AAS) using flame ionization detection (Thermo Scientific iCE 3000 instrument, Waltham, MA). Standards of 0, 1.0, 2.5, 5.0, and 7.5 ppm of iron were prepared in a mixture of ethanol 96% [w/v] and water 30%/70% [v/v]. The supernatants obtained during all three purification steps were analyzed without dilution. An air-acetylene flame at 1.0 L/min was used, and the absorbance was determined at 248.3 nm. Four seconds of chamber pre-conditioning and three determinations, each one of 4 seconds, were performed for each sample. The final volume necessary for every analysis was 5 mL. A deuterium lamp was used for the background correction.

Transmission electron microscopy

For transmission electron microscopy the diluted samples were collected on pioloform-coated copper grids and fixed with diluted methanol overnight. A Philips C12 transmission electron microscope (Eindhoven, NL) was used for sample analysis.

Preparation of NP samples for freeze-drying study and long term storage

In the first set of experiments, the optimal freeze-drying excipient (sucrose, mannitol, trehalose, respectively) was determined. For this purpose, 5 mg of magnetic NPs was mixed with different amounts of freeze-drying excipient (1%, 3%, and 5% [w/v]), and Milli-Q water was added to a final volume of 1 mL. The samples were transferred into 5 mL freeze-drying vials (Wheaton, Capitol Scientific, USA), and the stoppers (Wheaton, Capitol Scientific, USA) were adjusted for only

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