2, 3-Dimercaptosuccinic Acid-Modified Iron Oxide Clusters for Magnetic Resonance Imaging

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ABSTRACT: Over the last decade, various magnetic nanomaterials have been developed as magnetic resonance imaging (MRI) contrast agents; the greatest challenges encountered for clinical application have been insufficient stability. In this paper, a lyophilization method for 2, 3-dimercaptosuccinic acid-modified iron oxide (γ-Fe_2O_3@DMSA) nanoparticles was developed to simultaneously overcome two disadvantages; these include insufficient stability and low-magnetic response. After lyophilization, the clusters of γ-Fe_2O_3@DMSA with the size of 156.7 ± 15.3 nm were formed, and the stability of the lyophilized powder (γ-Fe_2O_3@DMSA-LP) increased up to over 3 years. It was also found that rehydrated γ-Fe_2O_3@DMSA-LP could be ingested by RAW264.7 cells in very large quantities. Results of pharmacokinetics and biodistribution studies *in vivo* indicated that γ-Fe_2O_3@DMSA-LP is a promising liver-targeted material. Furthermore, it also exhibited higher MRI efficiency and longer imaging time in the liver than the well-known product Feridex®. Moreover, results of vascular irritation and long-term toxicity experiments demonstrated γ-Fe_2O_3@DMSA-LP could be a nontoxic, biocompatible contrast agent *in vivo*. Therefore, the proposed γ-Fe_2O_3@DMSA-LP can be used as a potential MRI contrast agent in clinic for hepatic diseases. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:4030–4037, 2014

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INTRODUCTION

For a long time, drugs were considered as chemical substance used in treatment for diseases; therefore, diagnostic drugs in research of pharmaceutical sciences have been ignored. For safe and effective use of diagnostic drugs in patients, a suitable dosage is required as with most therapeutic drugs. On the contrary, hepatic diseases, including hepatocellular carcinoma, hepatic cyst, and hepatic hemangioma,[1] do not always cause obvious and wide morphologic abnormalities. This means that the diagnosis of qualitative imaging plays an important role in the treatment of liver diseases.

Magnetic resonance imaging (MRI) is an imaging technique used primarily in clinic to produce high-quality images of the inside of human body based on the different relaxation times of hydrogen atoms.[2] Currently, the most used contrast agent for MRI in clinic is a gadolinium (Gd) chelate, for example, Magnevist® (gadopentetate dimeglumine), but the *in vivo* distribution is not specific, and the elimination occurs too quickly, and what is worse is that parts of patients appeared adverse reaction of nephrogenic systemic fibrosis. Therefore, high-sensitivity MRI contrast agent is in urgent need to provide additional contrast for liver from other tissues.

Currently, there are only two liver-targeting MRI contrast agents used in clinic, dextran or carboxydextran-coated magnetite colloidal particles (Feridex® and Resovist®). On the nanoscale, magnetic particles possess a strong tendency to agglomeration because of its high-specific surface area and interaction induced by magnetism. Increasing particle size and/or increasing saturation magnetization directly increases aggregation and sedimentation of magnetic nanoparticles dispersion. This aggregation is amplified during the long-term storage in aqueous solution. The physical and chemical instability is the greatest challenge of nanoparticule systems involving liposomes, solid nanoparticles, and nanoemulsion, which will lead to precipitation and degradation. Surface modification and reduction in the size distribution are typical methods for obtaining stable nanoparticule systems. Both aforementioned contrast agents can be stabilized by using macromolecular coatings (polymers, such as dextran and carboxydextran) and by maintaining a monodisperse size to resist aggregation. However, this kind of nanoparticular colloid is generally complex, cost-consuming, and requires several separation steps to obtain pure macromolecules, resulting in very low yield.

Lyophilization, also known as freeze-drying, is a wise industrial process, which removes water from a frozen sample by sublimation and desorption under vacuum. Compared with other drying methods, lyophilization has the advantage of not damaging to temperature- or oxidative-sensitive materials and can limit bacterial growth. Above all, once the free water was removed completely, nanoparticles can be stored for long periods of time and can be transported conveniently. There are lots of lyophilized liposomal drugs used in the clinic, such as AmBisome® (NeXstar Pharmaceuticals Inc., Boulder, USA) and Myocet™ (Elan Pharmaceuticals Inc., Princeton, NJ, USA).[3] In this paper, we selected small molecular

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2, 3-dimercaptosuccinic acid-modified maghemite nanoparticles (γ-Fe_2O_3@DMSA) as liver-targeted contrast agent, which was not easily oxidized as compared with magnetite and can be produced in large quantities by coprecipitation. Lyophilization technology of γ-Fe_2O_3@DMSA developed here could simultaneously produce clusters of γ-Fe_2O_3@DMSA and turn the colloid into powder. It is reported that the iron oxide clusters showed higher magnetic response and a considerable increase in MRI relaxivity than that of individual ones because of the interaction between the aggregated nanocrystals.[4–7] Therefore, the lyophilized powder (γ-Fe_2O_3@DMSA-LP) was expected not only to improve the stability, but also enhance the MRI contrast efficiency.

In this study, the cellular uptake *in vitro*, pharmacokinetics, and biodistribution *in vivo* were sequentially investigated to explore the mechanism of liver targeting. The MRI and toxicity experiments were conducted and revealed that the proposed γ-Fe_2O_3@DMSA-LP could be used as a potential biocompatible MRI contrast agent in clinic for hepatic diseases.

MATERIALS AND METHODS

Materials

All the chemicals were purchased from Sinopharm Chemical Reagent Company, Ltd. (SCRC, Shanghai, China) unless otherwise specified. $(CH_3)_4NOH$ and DMSA were purchased from Shanghai Zhuorui Chemical Reagent Company, Ltd. Maltose was purchased from Shanghai Huixing Biochemical Reagent Company, Ltd. Sucrose, polyethylene glycol (PEG 400), dextran, glycine, and leucine were purchased from Sinopharm Chemical Reagent Company, Ltd. Poly vinyl pyrrolidone (PVP K30) was purchased from BASF (BASF Corporation, Mount Olive, N.Y.). RAW264.7 cells were purchased from Shanghai Cellular Institute of China Scientific Academy. RPMI 1640 medium (containing 10% fetal calf serum, 100 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin), glucose-free RPMI 1640 medium, and fetal calf serum were purchased from Shanghai BoooCle Biopharm Company, Ltd. All the chemicals were of analytical reagent grade.

Male white rabbits (1.6–2.2 kg) and male SD mice (18–22 g) were obtained from Animal Experiment Center of Southeast University. Animal experiments were performed via a protocol approved by the Institutional Animal Care and Use Committee of Southeast University.

Methods

Preparation of γ-Fe_2O_3@DMSA-LP

γ-Fe_2O_3 were synthesized by chemical coprecipitation and subsequently coated with DMSA as previously described.[8] Briefly, a mixed solution of $FeCl_3 \cdot 6H_2O$ (0.01 M) and $FeSO_4 \cdot 7H_2O$ (0.006 M) was adjusted to pH 9.0 by aqueous ammonia solution (1.5 M) under a N_2 environment. After washing with water and ethanol, the obtained magnetite nanoparticles were dispersed in water and the pH was adjusted to 3.0 using 0.1 M HCl. Then, these magnetite nanoparticles were oxidized into reddish-brown γ-Fe_2O_3 nanoparticles at about 95°C–100°C for 1 h. For DMSA coating, 0.01 M DMSA was added to the γ-Fe_2O_3 nanoparticles solution. Finally, the products were washed repeatedly with water.[9,10]

The preparation of γ-Fe_2O_3@DMSA-LP was performed with a vacuum freeze dryer (FreeZone.6L, Labconco Corp., Kansas City, MO). The γ-Fe_2O_3@DMSA colloidal suspension was mixed with different skeleton materials and stabilizers, followed by being sterilized and prefrozen for 12 h, and finally dried under reduced pressure to remove moisture.

Characterization

Scanning electron microscopy (SEM) was utilized to identify the microstructure of γ-Fe_2O_3@DMSA-LP with a S4800 microscope (Hitachi High Technologies Japan Inc., Tokyo, Japan). X-ray diffraction (XRD) was used to determine the crystal structure of the sample on a *D*/max-rA diffractometer with CuKα radiation (Rigaku Corporation, Tokyo, Japan). The morphology of rehydrated γ-Fe_2O_3@DMSA-LP was observed by transmission electron microscopy (TEM) using a JEM-2100 (JEOL, Tokyo, Japan). Magnetization measurements were carried out with a 7407 vibrating sample magnetometer (Lake Shore, Westerville, OH, USA). The size and zeta potential of the rehydrated γ-Fe_2O_3@DMSA-LP were determined at 25°C by photon correlation spectroscopy instrument (Malvern Zetasizer 3000; Malvern Instruments Company, Worcestershire, UK).

Stability

At 0.5, 1, 2, and 3 years after preparation, γ-Fe_2O_3@DMSA-LP was rehydrated with sterile water. To determine the stability in clinical use, the size, zeta potential, and iron concentration at 0, 4, and 12 h after rehydration were measured.

Intracellular Uptake of Iron

RAW 264.7 cells were seeded in 48-well plates at density of 1.5×10^4 cells per well and left overnight. After exposure to the rehydrated γ-Fe_2O_3@DMSA-LP at concentrations of 50, 100, 200 μg Fe per milliliter in medium at 37°C for 1 h, the cells were washed, stained with 2% potassium ferrocyanide (Perl's reagent), and nuclear fast red. Stained cells were observed with a light microscope (Axioplan Imaging II; Zeiss, Oberkochen, Germany) and photographed.

Pharmacokinetics and Biodistribution

Mice ($n = 3$) were injected via the tail vein with the rehydrated γ-Fe_2O_3@DMSA-LP at a dose of 1.5 mg Fe per kilogram. At several time intervals after injection blood, liver, kidneys, and spleen were collected and digested with HNO_3 and iron concentrations were determined by the *O*-phenanthroline colorimetric method.[10] The determination of iron concentration before injection was for elimination of endogenous iron.

MRI *In Vivo*

Male SD mice ($n = 4$) were injected intravenously through the tail vein with the rehydrated γ-Fe_2O_3@DMSA-LP (5.05 mg Fe kilogram). All mice were examined by MRI at several time intervals after injection. MRI images were measured on an Avanto-1.5T Magnetic Resonance Imaging Equipment (Siemens Healthcare, Erlangen, Germany). Experimental parameters for the spin-echo images were TR 5500 ms, TE 101 ms, 2 NEX, and FOV 14 × 14 cm^2.

Safety Assay

Vascular irritation test were performed as previously described.[11] Rehydrated γ-Fe_2O_3@DMSA-LP (1.85 mg Fe per