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The biocompatibility evaluation of iron oxide nanoparticles synthesized by one pot process for intravenous iron supply

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This paper reports a new synthesis method to control the size of iron oxide nanoparticles (IONs) by adding sodium citrate during fabrication to obtain sodium citrate-modified iron oxide nanoparticles (SCIONs). The method was simpler and more effective than the synthetic process of ferumoxytol, a commercial nano-sized iron agent. Compared with other iron agents which were prepared using branched polymers to form a carbohydrate shell, our SCIONs were tightly bound within a nonionic carbohydrate matrix. The physicochemical properties of SCIONs were characterized, and the results showed that these nanoparticles could be stably stored in water for six months without sediment. The cytotoxicity evaluation of SCIONs indicated that they were biocompatible to cells. The effect of iron supply by SCIONs was assessed by measuring the retention of iron ions in the serum, and the results demonstrated that the synthesized SCIONs are very promising for intravenous iron supply.

Introduction

Parenteral iron therapy is effective in a variety of diseases which are caused by iron deficiency. The most used parenteral iron agents are divided into two categories: i) intramuscular injection agents; ii) intravenous injection agents (Fig. S1). Existing strategies have their limitations. For example, the intramuscular injection agents work slowly and associate with many adverse reactions. In addition, they would color the muscle at the injection site. The intravenous iron agents have been widely used in clinical practice due to their fast absorption and rare side effect.\textsuperscript{1-5} Intravenous iron agents include iron-chelated agents and nanometer iron agents (NMIA). Compared with iron-chelated agents, NMIA has higher iron concentrations and good curative effect for anemia.\textsuperscript{6, 7}

NMIA generally consist of an iron core and a modified shell, such as dextran, glucose and polysaccharide.\textsuperscript{8} The iron cores of NMIA were usually composed of hydroxyl-bonded Fe\textsuperscript{3+} or iron trioxide. The iron agent produced by Luipold Pharmaceuticals Inc. was made up of iron-hydroxyl and glucose. The sizes of glucose-coated NMIA and the iron core were approximately 35 nm and 7 nm, respectively.\textsuperscript{9} The ferumoxytol prepared by AMAG Inc. Ltd. is another kind of NMIA, which was composed of Fe\textsubscript{3}O\textsubscript{4} nanoparticles. The size of ferumoxytol is 17-31 nm. Currently, the efficiency of iron supplement was better compared with other iron supplements, which can also be used in magnetic resonance imaging.\textsuperscript{10, 11}

Iron oxide nanoparticles (IONs) serve as the iron core of NMIA.\textsuperscript{6, 8} At present, the main synthetic methods of IONs are chemical co-precipitation,\textsuperscript{12-17} thermal decomposition,\textsuperscript{18, 19} hydrothermal method,\textsuperscript{20, 21} and sol-gel method.\textsuperscript{22} Compared with other methods, chemical co-precipitation is simple, low temperature and relatively rapid. However, the modification procedure of IONs obtained from chemical co-precipitation is complex. For example, in the preparation of ferumoxytol, carboxymethyl-dextran was coated onto the IONs as carbohydrate shell.\textsuperscript{11} Therefore, a straightforward and environmental friendly method for synthesizing IONs coated with modified shell need to be developed.

As a stabilizer and acidity regulator in food industry, sodium citrate (SC) can also be used as anticoagulant in the pharmaceutical industry.\textsuperscript{23} In the process of IONs modification, SC is employed as stabilizer in the after-treatment of IONs obtained by hydrothermal method, thermal decomposition method and co-precipitation method.\textsuperscript{24, 25} Polyethyleneimine (PEI) coated IONs were synthesized through co-precipitation and modified by SC and PEI, which was used in the absorption for hexavalent chromium.\textsuperscript{26}

This work provided a simple and one pot method for synthesizing IONs with different sizes by adding different concentrations of SC. The generated IONs were termed as SCIONs. The stability of SCIONs was tested in different salinity levels. The efficiency of iron supply in serum and in vivo was investigated by injecting SCIONs into serum and the body of mice. The biocompatibility of SCIONs in vivo was studied by measuring the
toxicity in cells.

Experimental

Materials

Ferric chloride (FeCl₃·6H₂O, F102739-500g), ferrous chloride (FeCl₂·4H₂O, 110778-500g), ammonium hydroxide (NH₄OH, 25-28%, A112081-500ml), sodium citrate (SC, S116312-500g), nitric acid (HNO₃, N116240-500ml), and potassium thiocyanate (KSCN, P112195-500g) were purchased from Aladdin. Dulbecco modified eagle medium (DMEM, C11995500BT), roswell park memorial institute medium (RPMI 1640, 118755008T) and fetal bovine serum (FBS, 10099-141) were obtained from Thermo Fisher Scientific. Cell counting kit-8 (CCK-8, CK040-4) was purchased from Dojindo Laboratories. Reactive oxygen species (ROS) assay kit (C13000) of 2', 7'-dichlorofluorescein diacetate (DCFH-DA) was prepared by Applygen Technologies Inc.. Mouse fibroblast cell line (3T3) and Mouse macrophagecyte cell line (Raw 264.7) were supplied by Shanghai Ge Fan Biological Technology, Inc. Balb/c mice (male, 18-22 g) were supplied by Vital River Laboratory (VRL).

Preparation of SC modified iron oxide nanoparticles (SCIONS)

The SCIONS were synthesized by chemical co-precipitation of ferric chloride and ferrous chloride in the presence of sodium citrate (SC). The procedures were described as follows: ferric chloride (0.6 g), ferrous chloride (0.3 g) and SC (0.02, 0.10, 0.20, 0.40, 0.60, 1.0 g) were dissolved in 20 ml distilled water, and the molar ratio of ferric chloride and ferrous chloride was 2:1. We controlled the additive amount of SC to obtain IONs of different sizes, and the additive amounts of SC were controlled from 0 to 5 wt%, respectively. After filtered by 0.22 μm membrane (Millipore), the mixture was heated to 80 °C coupled with stirring. Then, ammonium hydroxide was added to this mixture and the black solution of IONs was obtained after reacting for an hour. Finally, the nanoparticles were dialyzed by using distilled water in a beaker and the end products were called SCIONS.

Characterization of SCIONS

Fourier transform infrared spectra were collected from SCIONS in KBr from 4000 cm⁻¹ to 450 cm⁻¹ by Fourier transforms infrared spectrometer (FT-IR) (Spectrum One, Perkin Elmer Instruments Co. Led). Zeta potential and size distribution of SCIONS were measured by laser particle size analyzer (Zetasizer Nano ZS, Malvern). The efficiency of iron supplement in vitro and in vivo was evaluated by adding SCIONS in solutions with different amounts of SC. After washed by PBS for three times, these cells were dyed by DCFH-DA probe for 30 min and the mixture was stirred for 24 h. The ROS level was determined based on the reaction between ROS, such as the hydroxyl radical, and DCFH-DA. Mice macrophagecyte cells (raw 264.7) were chose in this work. These cells were allowed to grow for 12 h in a roswell park memorial institute medium (RPMI 1640) plus 10% fetal bovine serum (FBS) at 37°C under 5% CO₂. The adherent cells were dyed by DCFH-DA probe for 30 min and washed by PBS for three times. Then the stimulants were added into these dyed cells and these cells were incubated for 2 h. Finally, fluorescence intensity of treated cells was measured by microplate reader.

Cytotoxicity of SCIONS

A normal cell line, mouse embryo fibroblasts (3T3) were used in this study. The cells were cultured in dulbecco modified eagle medium (DMEM) plus 10% fetal bovine serum (FBS) at 37°C under 5% CO₂. The cells were plated at a dose of 10⁴ in 96-hole cell culture plate with three parallel and were allowed to grow for 12 hours. For preventing the evaporation of medium, phosphate buffer saline (PBS) was injected into the holes of 96-well plate. Then the SCIONS were added into the medium and the medium containing no agent was counted as control. The cells were incubated for 24 h with different amounts of SCIONS. After washed by PBS for three times, the toxicities of SCIONS were examined by CCK8 kit at 450 nm by multimode reader.

Results and discussion

As shown in Fig. 1, we employed SC as surfactant to disperse ferric chloride and ferrous chloride in distilled water. Carboxyl groups of SC have good adsorption ability to form chelates with metal atomic, and the chelates can stably exist in solution. Complexes formed by precursors and SC would control the reaction process and prevent the aggregation of nanoparticles. The nanoparticles obtained were named as SCIONS.
The presence of IONs in SCIONs was characterized by FTIR (Fig. 2). The absorption peak at 580 cm$^{-1}$ confirms the presence of Fe-O group, which was consistent with standard graph of IONs. The results showed that the small absorption peaks of Fe-O were found with the increased concentration of SC.

Size distribution and zeta-potential of SCIONs were measured by laser granularity analyzer (Fig. 3). The sizes of SCIONs were varied from 220 nm to 3 nm. The average sizes of SCIONs decreased along with the increase of SC amount. The results proved that the addition of SC was effective for size controlling of SCIONs in preparation process. When the additive amount of SC was 5 wt%, the average size of SCIONs was 3.11 nm. The variation of zeta potential of SCIONs was also exhibited in Fig. 3, in which the zeta potential decreased as the concentration of SC increased. Due to the addition of SC in synthetic procedure, the zeta potential of SCIONs was in a trend from a positive value to a negative one because of more carboxyl groups in solution. A comparative study of the physicochemical properties of intravenous iron agents was conducted, showing that the zeta potential of iron agents was ranged from -43.2 mV to +3.7 mV at pH value close to physiological pH. The dynamic light scattering was also applied to confirm the average sizes of these iron agents, and the size of them was within 50 nm. As a consequence, the size of SCIONs should be controlled within 50 nm to meet the requirement as a kind of iron agents.

TEM image of SCIONs were displayed in Fig. 4. As the existence of 5 wt% SC in the synthetic procedure, we can see the particles were homodispersed in water, and the average size measured by Nano Measurer 1.2 was 2.18 nm, which was smaller than that measured by DLS.

XRD spectrum of SCIONs was illustrated in Fig. 5. The peaks with 2θ values of 30.09, 35.44, 43.07, 53.43, 56.96, 62.55 and 74.00 are corresponded to the crystal planes (220), (311), (400), (422), (511), (440) and (533) of crystalline magnetite (JCPDS File no. 01-085-1436), respectively. The spinel crystal structure of magnetite is very similar to that of maghemite. The iron oxide nanoparticles are often a mixture of magnetite and maghemite.

The stability of SCIONs in saline water was studied by adding them into solution with different concentrations of sodium chloride, ranging from 24.00 to 0.19 wt%. It can be seen that the nanoparticles were stable in solution of 0.75 wt% sodium chloride, and the same result was obtained in 0.90 wt% sodium chloride solution, indicating that the nanoparticles are suitable for normal physiological condition. When the iron oxides in saline water at higher NaCl concentrations than 0.90 wt%, such as 24.00 wt%, the particles would deposit in 4 hours, and then would happen even in 3 wt% saline water.
SCIONs in preparation procedure were 5.0 wt%.

IONs in serum would combine hemoglobin and iron ions in nanoparticle would release into the serum within 24 hours. Iron supply efficiency of SCIONs was conducted by adding SCIONs into serum and DI-water for 24 hours. The efficiency of SCIONs for iron supply in serum was provided in Fig. 6 and that in DI-water of the control group was shown in Fig. S4. In Fig. 6, iron concentrations increased as the dose of SCIONs increased, higher than that in control group. The results confirmed that SCIONs would associate with hemoglobin in serum and dissolve in serum. Moreover, the efficiency of iron supplement was dose-dependent. The iron concentrations in serum after administration were also calculated, and the iron concentration in serum was dose dependent. The maximum concentration of iron was 5 μg/ml at a 500 mg dose in 45 minutes, and the iron percent was 1.5%. The iron concentration in serum was 15 μg/ml at the concentration of 500 μg/ml for 24 hours, and the dissolved percent of SCIONs was 3.04% (Fig. S5). Compared with the literature, the supply of iron was more effective with lower level of iron agents.

It was reported that IONs which was injected into mice would exist in blood and circulate for 24 hours ending up in organization. Results in serum also illustrated that IONs would associate with protein in serum and resolve in serum. It was reported that the effect on haemoglobin of parenteral iron and oral iron source was different, and the haematocrit of patients increased 5.5±1.9% and 9.2±3.4% on day 7 and 17, respectively. The iron concentrations in serum after receiving parenteral iron were calculated by some researchers, and the iron level increased from 345±273 to 359±140 μg/L. On the contrary, iron level of the control group decreased from 458±206 to 131±121 μg/L. The results above indicated that iron agents were efficiency to anemia. The iron supply efficiency of SCIONs in mice was demonstrated in Fig. 7. However, as the program went on, the body weight of mice decreased because continuous blood drawing. Compared with the control group, iron concentrations decreased more smoothly after continuous medication in experimental groups. The reduction rate of iron in experimental group was 27.92±4.39% on day 6, and that in control group was 38.70±1.95%, which showed that SCIONs can be used in iron supply for normal mouse.

Fe₃O₄ would produce reactive oxygen species (ROS) in metabolic process, and the induced oxidative stress may cause tissue damage. In addition, ROS has been reported to be involved in signal transduction. Hence, high ROS levels can damage cell structure and function, and induce apoptosis or necrosis, which may ultimately result in pathological changes and lead to organ dysfunction or cancers. As a consequence, the amounts of ROS in treated cells must be detected. Fluorescence intensity of cells (the content of ROS) was collected at 525 nm via excitation at 485 nm, which was displayed in Fig. 9. After incubation with SCIONs, fluorescence intensity of cells was measured at 2, 5, 12, 24 and 48 hours by a microplate reader. ROS concentration in experimental group (SCIONs and 5.0 wt% sodium citrate) was less than that in control group because the citrates on SCIONs would
react with ROS produced by cells. The above results indicated that SCIONs would not produce ROS in the procedure of cell metabolism, and these particles were safe to cells.

![Cell viability of 3T3 cell line after treated by SCIONs.](image1)

![ROS concentrations of Raw 264.7 cells treated by sodium citrate, SCIONs and PBS.](image2)

**Fig. 8** Cell viability of 3T3 cell line after treated by SCIONs.

**Fig. 9** ROS concentrations of Raw 264.7 cells treated by sodium citrate, SCIONs and PBS.

### Conclusions

In summary, we have synthesized a series of SCIONs with different particle sizes from 220 nm to 3 nm. It was found that the size distribution and zeta potential of SCIONs decreased with an increasing concentration of SC. SCIONs can be stored stably in saline solution for long time when the concentrations of sodium chloride ranged from 0.75 to 0.90 wt%. SCIONs were non-toxic for 3T3 cells and the concentrations of ROS in SCIONs-treated cells were no more than that in control group. The excellent iron supplemental efficiency of SCIONs in serum and mice showed that these SCIONs are promising for NMIA.

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### Notes and references