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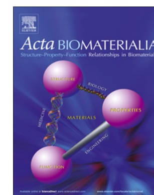

Full length article

Time-course assessment of the aggregation and metabolization of magnetic nanoparticles

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ABSTRACT

To successfully develop biomedical applications for magnetic nanoparticles, it is imperative that these nanoreagents maintain their magnetic properties *in vivo* and that their by-products are safely metabolized. When placed in biological milieu or internalized into cells, nanoparticle aggregation degree can increase which could affect magnetic properties and metabolization. To evaluate these aggregation effects, we synthesized citric acid-coated iron oxide nanoparticles whose magnetic susceptibility can be modified by aggregation in agar dilutions and dextran-layered counterparts that maintain their magnetic properties unchanged. Macrophage models were used for *in vitro* uptake and metabolization studies, as these cells control iron homeostasis in the organism. Electron microscopy and magnetic susceptibility studies revealed a cellular mechanism of nanoparticle degradation, in which a small fraction of the particles is rapidly degraded while the remaining ones maintain their size. Both nanoparticle types produced similar iron metabolic profiles but these profiles differed in each macrophage model. Thus, nanoparticles induced iron responses that depended on macrophage programming. *In vivo* studies showed that nanoparticles susceptible to changes in magnetic properties through aggregation effects had different behavior in lungs, liver and spleen. Liver ferritin levels increased in these animals showing that nanoparticles are degraded and their by-products incorporated into normal metabolic routes. These data show that nanoparticle iron metabolization depends on cell type and highlight the necessity to assess nanoparticle aggregation in complex biological systems to develop effective *in vivo* biomedical applications.

Statement of Significance

Magnetic iron oxide nanoparticles have great potential for biomedical applications. It is however imperative that these nanoreagents preserve their magnetic properties once inoculated, and that their degradation products can be eliminated. When placed in a biological milieu nanoparticles can aggregate and this can affect their magnetic properties and their degradation. In this work, we showed that iron oxide nanoparticles trigger the iron metabolism in macrophages, the main cell type involved in iron homeostasis in the organism. We also show that aggregation can affect nanoparticle magnetic properties when inoculated in animal models. This work confirms iron oxide nanoparticle biocompatibility and highlights the necessity to assess *in vivo* nanoparticle aggregation to successfully develop biomedical applications.

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1. Introduction

Magnetic nanoparticles (NPs) appeared around two decades ago as promising tools for biomedical applications [1,2]. The possibility to modify their size, shape and surface chemistry [3] for drug

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delivery, to specifically target tumors using external magnetic fields, and to employ their magnetic properties for magnetic-fluid hyperthermia raised hope for improved cancer treatment [4–6]. However, in spite of these early promises only few of these compounds have reached the clinical practice [7].

An obstacle that magnetic nanoparticles encounter for their eventual use in the clinic is the difference in behavior that these materials can present in aqueous suspension and in *in vitro* and *in vivo* settings [8]. This is due to nanoparticle aggregation in biological fluids that, among other parameters such as the particle size and shape, can strongly influence magnetic properties [9]. Several research groups are evaluating particle aggregation through the formation of a protein corona in serum-containing media [10–12]. These results, although extremely relevant for particle *in vivo* hydrodynamic size alterations, are still far from reproducing a complex biological setting. Not all cells within the same organ should necessarily accumulate NPs in the same aggregation status and this could have strong effects on their magnetic properties. Yet, little attention is being paid to aggregation rates within living systems, a critical parameter to develop effective applications [13–17].

Another essential aspect for magnetic nanoparticle safe implementation in biomedical applications and their approval by regulatory agencies lies in their biotransformation. The fate of these materials once they performed their purpose needs to be studied. Since iron is part of several vital processes [18] and organisms have mechanisms that transport and store iron in non-toxic forms [18], iron oxide magnetic nanoparticles are predicted to be safely eliminated in biological systems. There is growing evidence that iron oxide nanoparticles trigger iron-coping mechanisms in cells and that the degradation products of these materials are incorporated into normal iron metabolic routes [19–25]. There are nonetheless gaps in knowledge in the field; and for instance how aggregation state affects magnetic nanoparticle metabolization has not been explored. One of the main difficulties to fill this knowledge gap lies in the detection of magnetic nanoparticles at very low concentrations in biological matrices. Alternating current (AC) magnetic susceptibility measurements can identify, quantify and follow the transformations of magnetic nanoparticles in biological samples with almost no need for sample processing. Its only limitation is the volume of material that can be fitted inside the gelatin capsules used to perform the magnetic measurements (typically approximately 100 mg of dried sample [26]). Importantly, these measurements can distinguish between nanoparticles and endogenous iron [27,28]. This technique is therefore ideally suited to analyse the fate and transformation of particles within cells or tissues [29].

To study iron oxide nanoparticle degradation and its cellular effects, it is critical to use biologically pertinent models. In this aspect, macrophages are highly relevant to nanoparticle metabolization studies as they can capture and probably degrade inoculated iron oxide nanoparticles [30–32], and this in turn could alter their activation [23,33,34]. Macrophages are tissue resident cells of the mononuclear phagocytic system that are activated by environmental cues and modify their function accordingly [35]. Macrophage stimulation results in a continuum of activation profiles [36]. At one end of this spectrum, classically activated macrophages (also denominated M1 macrophages) promote inflammatory responses, while at the other end alternatively activated macrophages (also denominated M2 macrophages) antagonize inflammatory responses [37,38]. Macrophage activation affects the way they process iron [39], and conversely iron content in the milieu can alter macrophage responses [40]. M1 macrophages sequester iron to deprive bacteria from this essential nutrient during inflammation [39,41], whereas M2 macrophages favor iron release to promote tissue repair [39]. The iron response of tissue resident macrophages is also likely to depend on their intrinsic specialization, with for instance spleen red pulp macrophages and liver Kupffer cells involved in iron homeostasis [35,42]. It is thus crucial to study how aggregation could alter NPs transformation and affect iron metabolism in different macrophage populations.

To evaluate the effects of iron oxide nanoparticle aggregation on their magnetic properties and metabolization, we synthesized iron oxide nanoparticles with different behavior when aggregated in agar dilutions. We evaluated their uptake in three *in vitro* macrophage models and how their degradation affected iron metabolism. Finally we studied the *in vivo* effects of iron oxide nanoparticle aggregation on their magnetic properties and metabolization after intravenous injection.

2. Materials and methods

2.1. Magnetic nanoparticle synthesis

Maghemite particles coated with citric acid (NPs-CIT) or with dextran covalently bound to the citric acid layer (NPs-DEXT) were prepared for this work. Maghemite nanoparticles (NPs) were prepared by co-precipitation. A NH_4OH solution (75 mL, 25%, from Fluka – Riedel de Haën, Germany) was added to a $FeCl_2$ (0.175 mol L^{-1}, from Sigma Aldrich, Germany, ≥99.0%) and $FeCl_3$ (0.334 mol L^{-1}, from VWR International, France, 27% Aqueous solution) solution at room temperature under vigorous stirring for 5 min, washed three times with distilled water by magnetic decantation, and acid treated to fully oxidize the magnetic iron oxide to maghemite [43]. Maghemite particles were then coated with citric acid (from Sigma Aldrich, Austria, ≥99.5%) (NPs-CIT) as described [44,45]. Briefly, a solution of citric acid (40 mL, 0.1 mol L^{-1}) was added dropwise to the nanoparticles (50 mL, [Fe] ≈ 20 mg mL^{-1}, pH 3). The mixture was then heated at 80 °C for 30 min under vigorous mechanical stirring. Dextran-coated maghemite nanoparticles (NPs-DEXT) were obtained by binding covalently amino-functionalized dextran to the citric acid *via* 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, from Sigma Aldrich, Germany) activation of the carboxylic acids. Briefly, carboxymethyl-dextran sodium salt (8 g, from Sigma Aldrich, Germany) was modified with a 1 mol L^{-1} ethylenediamine solution (pH 4.75) (80 mL), pH adjusted to 4.75, EDC (160 mg) added and pH readjusted to 4.75. The solution was stirred for 12 h and purified by dialysis using a membrane with a cut off of 12,000–14,000 Da. This amino-modified carboxymethyldextran solution (90 mL of an estimated concentration of 40 mg mL^{-1} of dextran, pH 7.0) was slowly added to a 10 mL NPs-CIT suspension ([Fe] = 7 mg mL^{-1}) at pH 7.0 under sonication. EDC (383 mg) was then added, pH readjusted to 7.0, and the suspension was stirred for 12 h. The final NPs-DEXT suspension was purified by dialysis for several days using a 50,000 Da cut off membrane [46].

2.2. Magnetic nanoparticles characterization

Iron concentration was measured by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) using OPTIME 2100DV apparatus from Perkin Elmer (USA) after acid digestion. Maghemite core size was determined from Transmission Electron Microscopy (TEM) micrographs using a JEOL JEM1010 (100 kV) microscope (Japan). TEM grids were prepared by drying at room temperature a drop of the particles suspended in water onto a carbon coated copper grid. Mean particle size and distribution were evaluated by manually measuring the largest internal dimension of at least 170 particles (Straight line tool, ImageJ software, US National Institutes of Health). Infrared spectra (IR) of the samples diluted in 2% KBr were recorded between 4000 and 250 cm^{-1} in a Bruker IFS 66V-S spectrometer (USA) and a Nicolet FT-IR 20SXC

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