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Long term in vivo biotransformation of iron oxide nanoparticles

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ABSTRACT

The long term outcome of nanoparticles in the organism is one of the most important concerns raised by the development of nanotechnology and nanomedicine. Little is known on the way taken by cells to process and degrade nanoparticles over time. In this context, iron oxide superparamagnetic nanoparticles benefit from a privileged status, because they show a very good tolerance profile, allowing their clinical use for MRI diagnosis. It is generally assumed that the specialized metabolism which regulates iron in the organism can also handle iron oxide nanoparticles. However the biotransformation of iron oxide nanoparticles is still not elucidated. Here we propose a multiscale approach to study the fate of nanomagnets in the organism. Ferromagnetic resonance and SQUID magnetization measurements are used to quantify iron oxide nanoparticles and follow the evolution of their magnetic properties. A nanoscale structural analysis by electron microscopy complements the magnetic follow-up of nanoparticles injected to mice. We evidence the biotransformation of superparamagnetic maghemite nanoparticles into poorly-magnetic iron species probably stored into ferritin proteins over a period of three months. A putative mechanism is proposed for the biotransformation of iron-oxide nanoparticles.
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1. Introduction

The long term outcome of nanoparticles (NPs) in the body is a major concern, which has been raised by the recent development of nanotechnology and nanomedicine. How nanomaterials interact with living constituents and are processed by the organism are justifiably the most-asked questions whenever an application of nanomaterials is proposed [1]. Safety issues are becoming prominent in the development of new nanodevices, especially in the biomedical field. Inorganic nanoparticles that exhibit unique optical or magnetic properties are of special interest for diagnosis and therapeutic applications exploiting their response to physical, remotely activated, stimuli [2,3]. However, while tremendous advances have been made in the tuning of the chemical and physical hallmarks of NPs, their fate once injected in the organism is still not controlled. In particular, the transformations induced by cellular activity on inorganic nanoparticles are largely unknown yet.

Most studies carry out in vitro assessments of NPs toxicity using cellular models [1,4–6]. They point out some critical parameters governing the biological response to nanomaterials, such as their size, composition, surface reactivity, architecture, stability with respect to the biological medium and intracellular distribution. However, such studies always require validation in vivo, where most of these parameters are no longer controlled. Hence reliable methods are eminently desirable to monitor the fate of inorganic nanoparticles directly in the organism.

One approach to assess the in vivo behaviour of inorganic nanoparticles is to rely on their most salient properties, their physical properties, which precisely make their interest for applications. For example, optical properties of plasmonic or luminescent nanoparticles can be exploited to study their biodistribution [7]. Similarly, magnetic properties of iron oxide NPs enable their follow-up by MRI, a particularly attractive non-invasive imaging technique, paving the

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way of many diagnostic applications [8]. However a quantitative relationship between the physical signal and the amount of nanoparticles is often compromised, because the physical properties may be impacted by the biological environment or by the peculiar organization adopted by nanomaterials when immerged in the blood flow or processed by cells [9]. It is particularly true for MRI which relies on the indirect effect of the magnetic field produced by nanoparticles on the dynamics of surrounding protons [10]. Therefore a reliable monitoring of nanoparticles should be based on their specific physical properties, independently of their environment.

Here we propose to refer on the superparamagnetic properties of nanomagnets to monitor their fate in vivo. We perform complementary techniques of nanomagnetism in order to specifically quantify iron oxide NPs based on their magnetic moment and to assess their degradation and biotransformation over time. The progressive modification and loss of their characteristic magnetic properties are interpreted as the signatures of the transformations operated by cells on nanoparticles. Because the degradation obviously occurs at a nanometer scale, the magnetic follow-up is here complemented by a nanoscale structural characterization of the intracellular outcome of nanoparticles. Our multiscale approach, to evaluate biologically-induced changes of the nanoparticle state, applies here to iron oxide NPs used as MRI contrast agent. Although iron-based NPs have never shown acute or sub-acute toxicities, the way they are handled by the iron metabolism is still not elucidated [11–14]. The present results are thus directly relevant to current safety considerations in clinical diagnostic and therapeutic uses of nanomagnets.

2. Materials and methods

2.1. Design of the study

In this study, we aimed to assess the biodistribution and outcome of P904 nanoparticles (Guerbet SA) administrated intravenously to mice. P904 nanoparticles consist in 8 nm superparamagnetic cores of maghemite coated by hydrophilic derivatives of glucose. These nanoparticles are currently developed by Guerbet SA, as a contrast agent for non-invasive imaging of inflammation by MRI. The diagnosis and therapeutic follow-up of various pathologies involving inflammatory processes, such as atherosclerosis or obesity, may benefit from the development of P904 iron oxide nanoparticles. P904 NPs are expected to be uptaken by cells of the reticulo-endothelial system, especially the macrophages which are present or recruited in inflammatory areas, allowing their non-invasive detection [15–17].

In our study, we tested two doses to evaluate the long term fate of nanoparticles in mice: 1000 μmol of iron/kg, the dose previously proposed for the detection of inflammation in atherosclerotic plaques [16] and 50 μmol of iron/kg, the potentially-used dose in human clinic. P904 nanoparticles were followed during three months after injection at low dose in normal healthy mice (C57-B6 mice) and during 44 days after injection at high dose in obese mice (ObOb leptin-deficient mice). We choose obese mice to assess if inflammatory macrophages located within adipose tissue can uptake P904 nanoparticles and thus allow quantification of obesity-related inflammation.

Besides their imaging applications, P904 nanoparticles are representative of a class of sugar-coated iron oxide ferrimagnetic nanoparticles, with a typical size around 8 nm, which have found applications for magnetic drug targeting (when associated with a drug carrier) [18], cell targeting (when internalized in cells) [19,20], magnetic hyperthermia [21] or magnetically-assisted drug delivery [22]. In all these applications, a precise knowledge of the long term in vivo outcome of NPs is mandatory.

2.2. Magnetic nanoparticles

Superparamagnetic P904 NPs were provided by Guerbet (Roissy, France). They are synthesized by coprecipitation of ferric and ferrous ions in alkaline medium following a classical procedure [23]. They consist in a ferrimagnetic core of maghemite coated by a hydrophilic derivative of glucose. The magnetic size distributions, deduced from the field-dependent magnetization curve, fit a log-normal distribution with characteristic diameter $d_0 = 7.2$ nm and polydispersity index $\sigma = 0.24$.

2.3. Animal model

All animal experiments were conducted in keeping with French Agriculture Ministry guidelines. A total of 62 mice were used in the study: 30 of them were male 8-weeks-old C57/Bl6 mice (Janvier, France) (mean weight 25 ± 3 g), the other 32 mice were 8-weeks old leptin-deficient "ob/ob" mice (B6.V-Lepob, Janvier, France) showing obesity (weight range 47 ± 5 g). 25 C57/Bl6 mice and 27 obob mice were injected with P904 nanoparticles respectively at a dose of 50 μmol/kg (*low dose*) and 1000 μmol/kg (*high dose*) via a retro-orbitary route. 5 C57/Bl6 mice and 5 obob mice served as control. Five injected C57/Bl6 mice were sacrificed at each different time-points after P904 injection (Days, D1, D7, D30, D60 and D90). Six injected obob mice were sacrificed at D3, D10, D31 and D44 and only 2 mice at D24. Liver, spleen and inflammatory adipose tissue (only for obob mice) were excised and shared for ICP-OES, FMR, SQUID and TEM characterizations.

2.4. Iron quantification by FMR and ICP-OES

Ferromagnetic Resonance was performed with a Varian E102 EPR (Electron Paramagnetic Resonance) spectrometer operating at Q band (9.26 GHz). The first derivative of the power absorption dW(B)/dB was recorded as a function of the applied field B in the range 0–6000 Gauss. The modulation field had a frequency of 100 kHz and an amplitude of 10 G. The area of the FMR absorption curve (calculated by a double integration of the spectrum dW(B)/dB) was proportional to the amount of P904 nanoparticles in the sample. An absolute calibration was performed using suspensions of P904 nanoparticles (2 μl sample) at different concentrations quantified by ICP-OES [24]. Before FMR measurements, the excised pieces of organs were first weighed, rinsed, finely sliced and let dry for 3 days in an oven at 80 °C. The dried organ samples were weighed again and introduced into a quartz tube suitable for FMR measurements. For adipose tissue, the dehydrated tissue was sucked in a PTFE microtube and then pushed away in the bottom of the quartz tube. The net mass of dehydrated organs in the quartz tube was then weighed and FMR spectra were recorded.

Total iron content in organs was quantified by inductively coupled plasma - optical emission spectrometry (ICP-OES). After excision, samples were conserved at −80 °C until preparation for ICP-OES measurements. The organs were mineralized in 3.5 ml of nitric acid and 1.5 ml of chloridric acid for 4 h at 100 °C and then treated with 1.5 ml of H_2O_2 for 1 h at 95 °C on a heat block. The volume was adjusted to 10 ml with distilled water and samples were analyzed for iron content by ICP-OES (Ultima, HORIBA Jobin Yvon, Edison, NJ).

2.5. SQUID magnetization measurements

Magnetization measurements were carried out on a Quantum Design MPMS-5S SQUID magnetometer. Field-dependent magnetization curves were measured at 300 K as a function of the external magnetic field in the range $0–3\times10^4$ Gauss. Temperature-dependent magnetization was measured at low field (50 Gauss) for temperatures increasing from 5 K to 300 K for field cooled (FC) and zero-field cooled (ZFC) samples. Magnetic field during cooling was 50 Gauss. Dried organs were prepared as for FMR quantification and introduced in an SQUID gelatin capsule for measurements. For spleen and adipose tissue, the samples stemming from the different mice sacrificed at each time point ($n = 5$) were pooled. For liver samples, SQUID measurements were performed for different individual mouse to demonstrate reproducibility among animals. In all cases, the (temperature-independent) diamagnetic signal coming from the capsule and tissue was subtracted from the measured magnetization.

2.6. Transmission electron microscopy (TEM)

For electron microscopic analysis, organs were cut into 1 mm^3 pieces after excision and fixed with 2% glutaraldehyde in 0.1 M Na pH 7.2 cacodylate buffer for 4 h at room temperature and then postfixed with 1% osmium tetroxide containing 1.5% potassium cyanoferrate, contrasted with uranyl acetate 2% in water, gradually dehydrated in ethanol (30%–100%) and embedded in Epon. For a previsualization (specially to locate macrophages in adipose tissue), 1 micron sections were collected onto glass slides, conterstained with methylen blue-Azur II and imaged on an epi-fluorescent microscope (DMRB – Leica - France) with a 63x plan apochromat oil immersion lens. Thin sections (70 nm) of selected zones were collected onto 200 mesh cooper grids, and conterstained with lead citrate before examination with Zeiss EM902 electron microscope operated at 80 kV– (MIMA2- UR1196 Génomique et Physiologie de la Lactation, INRA, Plateau de Microscopie Electronique 78352 Jouy-en-Josas, France).

High resolution micrographs were acquired with a charge-coupled device camera MegaView III CCD camera and analysed with ITEM software (Eloïse – SARL – Roissy CDG – France). High magnification TEM, electron diffraction and X-ray energy-dispersive spectroscopy (XEDS) experiments were carried out on a Technai G2F20 field-emission electron microscope operating at 200 kV and equipped with an energy-dispersive X-ray analyzer. XEDS spectrum imaging combines the imaging and analytical capabilities of electron microscopy. A dark field image in scanning mode (STEM mode) of the area of interest is first collected and this image is divided into an array of pixels with a user defined resolution (here 100 by 100). The nanometric STEM probe is then scanned over the same area, but stopped for a pre-determined time interval (here 500 ms), at each pixel. During this time interval, an entire X-ray spectrum from 0 to 20 keV is acquired. The resulting array of spectra is known as a spectrum image data cube. Chemical maps were reconstructed from the data cubes with

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