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Relaxation of ferromagnetic nanoparticles in macrophages: In vitro and in vivo studies

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Abstract

The relaxation characteristics of magnetic nanoparticles ($CoFe_2O_4$) were investigated in J774A.1 macrophages and after voluntary inhalation. In dry form 25% of the particles showed Néel relaxation. Relaxation in macrophages occurred within minutes and could be inhibited by fixation, showing Brownian relaxation and intracellular transport processes. Relaxation in the lung happened similarly, but was dependent on the time after deposition. The particles were cleared from the lung within 2 weeks.

Keywords: CoFe; Cobalt ferrite nanoparticles; Macrophage; Ingestion; Relaxation; Inhalation; Lung; Clearance

1. Introduction

It has been shown that ingested ferromagnetic particles in the micron size range are novel tracers for the study of cytoskeleton-dependent cell functions, such as phagocytosis, intracellular transport mechanisms and cytoskeletal mechanical integrity [1–3]. Alveolar macrophages (AM) are defence cells in the lung and are therefore very potent for engulfing foreign materials, such as particles or bacteria. Ingested particles are surrounded by a membrane and form phagosomes, which fuse with lysosomes due to intracellular transport. Such ferromagnetic particles can also be inhaled and used to measure AM functions in vivo in the human lung.

Epidemiological studies have attributed a specific health hazard of environmental particles [4] and the fraction of ultrafine particles (diameter-<100 nm) seems to play a specific role [5]. The fraction of ultrafine particles contributes less to the mass, but much to the number and specific surface

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area of environmental particles, and many studies have shown specific toxicity of ultrafine particles in vitro and in vivo [6–8].

The fate of inhaled ultrafine particles (diameter-<100 nm) in living cells and in the lung is still unclear. These particles are not only phagocytized by macrophages, but they can enter epithelial cells or may pass the epithelial barrier and enter the blood circulation, where they can be transported to other organs or tissues (heart, brain) and cause inflammatory reactions [9,10]. The relaxation characteristics of magnetic nanoparticles can give information on the specific location within cells or within the lungs, such as in liquid, on the epithelium, or within cells. The purpose of this study is the investigation of relaxation and intracellular transport processes in macrophages using ferromagnetic nanoparticles.

2. Methods

Preparation of magnetic nanoparticles: Ferrimagnetic $CoFe_2O_4$-nanoparticles were prepared by co-precipitation of mixed Co^{2+} and Fe^{3+} solutions in the molar ratios with solved KOH at 95 °C [11,12]. The precipitate was repeatedly washed in deionized water and then stabilized with citric acid. The primary particle size was 13 ± 3.5 nm (mean $\pm$ standard deviation). In water solution, the particles form a colloid, which is stable over months. The particles are single domain particles and most of the particles have a remanent magnetization. Attractive magnetic forces between the particles are superimposed by electrical repulsive surface charges.

Relaxation of magnetic nanoparticles: After the application of a magnetic field H the particles of a ferrofluid return to equilibrium by two different mechanisms [13]:

Brownian relaxation is accomplished by bulk rotational diffusion of the particle within the carrier liquid according to [14]

$$\frac{B(t)}{B_0}=\exp(-t/\tau_B),\quad \tau_B=\frac{1}{2D}=\frac{\kappa V\eta}{2kT}, \tag{1}$$

with a relaxation time constant τ_B, where κ is the shape factor ($\kappa=6$ for spheres), V is the hydrodynamic volume of the particle including the coat of surfactant, η is the dynamic viscosity of the carrier liquid, and kT is the thermal energy.

Néel relaxation is the rotation of the internal magnetization vector inside the particle, with a time constant of [15]

$$\tau_N=\tau_0\exp(\Delta E/kT), \tag{2}$$

where τ_0 is usually approximated as 10^{-9} s, and

$$\Delta E=KV(1-h)^2 \tag{3}$$

is the energy barrier assuming uniaxial non-interacting particles, $h=H/H_k$ is the reduced field and H_k is the internal field due to anisotropy. In real ferrofluids there is always a distribution of energy barriers due to a distribution of the magnetic anisotropy constants K (crystalline and predominantly shape anisotropy) as well as of the volumes of the magnetic particle cores V. Particles with relaxation times faster than the measurement time (a common value is 100 s) are called superparamagnetic. Particles that do not relax by the Néel mechanism within the measurement period are called blocked particles and do not contribute to the magnetization change within the considered time window.

Since in general, both mechanisms contribute to the magnetization, an effective relaxation time of

$$\tau_{eff}=\frac{\tau_B\tau_N}{\tau_B+\tau_N} \tag{4}$$

applies for every single particle fraction. If Brownian rotation is inhibited (e.g. by freezing or drying the sample) τ_{eff} equals τ_N. The time-dependent magnetization of a fraction of identical particles follows a simple exponential law:

$$M(t)=M(t_0)\exp(-t/\tau_{eff}). \tag{5}$$

The distribution of energy barriers results in a distribution of effective relaxation times. Assuming a constant distribution of energy barriers, integration over the distribution of τ leads to [16,17]

$$M(t)=C+S\ln(t), \tag{6}$$

where C is a constant and S is called coefficient of magnetic viscosity.

Relaxation of magnetic particles in macrophages: Ingested micron-sized magnetic particles are

transported by intracellular forces along the cytoskeleton and these transport processes cause a decay of aligned particles, which is also described as Brownian rotational motion, where the energy kT is replaced by intracellular randomization energy E_r [18,19]. This energy is about 3 magnitudes of orders higher than thermal energy kT and it was shown that the transport needs an intact cytoskeleton and depends on the ATP status of the cell [20]. Assuming a Newtonian viscosity of the cytoskeleton, relaxation follows an exponential decay:

$$\frac{B(t)}{B_0} = \exp(-t/\tau_B), \quad \tau_B = \frac{1}{2D} = \frac{\kappa V \eta}{2E_r}. \tag{7}$$

Relaxation and twisting of micron-sized magnetic particles in macrophages revealed a cytoplasmic viscosity of $\eta \approx 100\,\mathrm{Pa\,s}$ (viscosity of water $\approx 1\,\mathrm{mPa\,s}$) and an intracellular transport energy of $E_r \approx 5 \times 10^{-18}\,\mathrm{J}$ ($kT = 4.3 \times 10^{-21}\,\mathrm{J}$ at 37 °C).

Measurement of Néel relaxation: Relaxation was measured with particles in dry form, where the physical rotation of the particle was inhibited. A droplet of the particle suspension was put on to chromatography paper and the water was allowed to evaporate. A decay of the magnetization under this condition is due to the intrinsic Néel relaxation. Relaxation was measured after the particles were aligned in a strong magnetic field pulse ($\approx 100\,\mathrm{mT}$, some milliseconds). The loss of alignment (relaxation) was continuously detected for 5 min using a fluxgate sensor [21].

Detection of magnetic nanoparticles in J774A.1 macrophages: J774A.1 macrophages were incubated together with magnetic nanoparticles for up to 24 h. J774A.1 cell line was obtained from European Collection of Animal Cell Cultures. J774A.1 is provenient from a tumour of a female BALB/c/NIH mouse [22]. This mouse monocyte macrophage cell line grows at 37 °C in RPMI 1640 medium containing 5% fetal calf serum (FCS) in 5% CO_2 with a doubling time of ≈ 2 days. Magnetic particles binding assay: magnetic nanoparticles were incubated with cells in medium with or without serum (w or w/o FCS). Two million cells were suspended in 2 ml medium and incubated in plastic Petri dishes (30 mm diameter, Nunc Inc.) for 1 h at 37 °C. After removal of the medium half of the probes were set to serum-free medium for 1 h. The suspension of magnetic nanoparticles (in medium w or w/o FCS) was added in final concentration of 2 and 20 μg/ml/million cells and incubated for 3 and 24 h, respectively. Before relaxation measurements the medium was exchanged and non-adherent cells and free particles were removed. Relaxation was measured after the particles were aligned in a strong magnetic field pulse ($\approx 100\,\mathrm{mT}$, some milliseconds). The loss of alignment (relaxation) was detected using fluxgate sensors [21].

After the relaxation measurements the viability was tested by incubation with probidium iodide (PI). PI positive cells (PI+) were analysed in a fluorescence microscope after counting at least 100 cells in the visible area. In addition, relaxation measurements were repeated after cell fixation with 2.5% glutaraldehyde in phosphate buffer (pH = 7.5, 350 mOsm) [23].

Voluntary inhalation and clearance: A water-based suspension of 2 mg/ml cobalt ferrite nanoparticles was prepared in physiological saline and inhaled under controlled conditions using the AKITA inhalation device (INAMED GmbH, Gauting, Germany) in combination with a Pari LC Star nebulizer (Pari GmbH, Starnberg, Germany) [24]. Sixteen breaths were performed with a tidal volume of 1.2 L and a flow rate of 0.2 L/s. The mean droplet size of this nebulizer is about 4.5 μm and in combination with the low flow rate we get a high peripheral deposition. The deposition fraction was measured with inhalation and exhalation filters and was 74.5%.

Particle retention and relaxation was detected using a magnetopneumographic system in combination with a SQUID sensor and a magnetically shielded room [25]. Relaxation was measured continuously for 5 min after the particles were aligned in a strong magnetic field pulse ($\approx 100\,\mathrm{mT}$, some milliseconds).

3. Results and discussion

Morphology of magnetic nanoparticles in macrophages: The composition of the suspension medium plays an important role in the behaviour of

the particles and the uptake by cells. In medium w/o FCS the particles tend to aggregate and form chains with a subsequent sedimentation to the bottom of the dish. In medium containing 5% serum (w FCS) the particles keep in suspension for longer times (days). It appears that the absence of serum proteins causes a coagulation of the particles due to a neutralization of the surface charges by the salt ions in the medium, following a destruction of the colloid. The attractive magnetic forces between the particles can then enhance the formation of aggregates.

The formation of aggregates is prevented by the presence of serum proteins. The serum proteins can bind to the particle surface and keep or enhance the particle charges in the medium and prevent the neutralization by salt ions. A proper dispersion of nanoparticles at the presence of serum was also seen with other ultrafine particles (i.e. TiO_2, [9,21]). This also affects the uptake by macrophages and the appearance within the cells. Aggregated particles settle down to the Petri dish (Fig. 1B and D). Therefore the particle density in the vicinity of the cells is higher. In addition, the particles are larger and are more adapted for uptake by phagocytic processes. In the presence of serum the particles keep distributed in the medium and one can find only few aggregates on the bottom of the Petri dish (Fig. 1A and C). The mean density of particles in the vicinity of the cells is much lower. In addition, because of the small size, the process of active uptake by phagocytosis is less effective and other processes, like pinocytosis or passive diffusion, may happen. Suspended single nanoparticles cannot be visualized by light microscopy and the particles seen in Fig. 1A and C show aggregates, which are also present in small fractions despite the presence of serum.

Electron microscopic investigations (Fig. 2) show aggregates of particles within phagosomes and intracellular vacuoles. Only few particles were located within the cytoplasm and no particles were found within the nucleus.

Probes w/o FCS showed reduced viability compared to probes w FCS. This was most obvious after 24 h of incubation. Amounts of

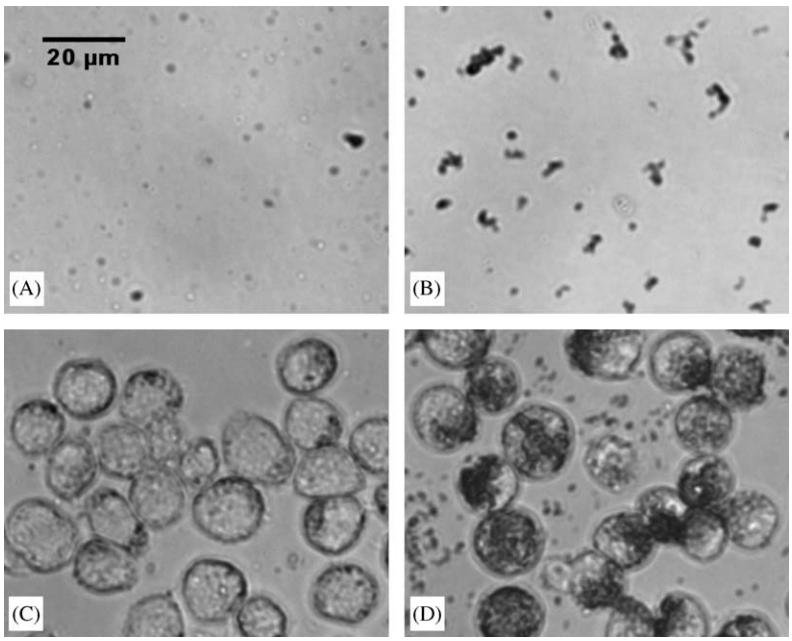

Fig. 1. Light microscopy of 20 µg/ml $CoFe_2O_4$-nanoparticles w FCS (A) and w/o FCS (B) after 3 h of incubation, and of particles together with 1 million J774A.1 macrophages w FCS (C) and w/o FCS (D).

200 μg of magnetic nanoparticles/ml/million cells and above were toxic in the presence of serum. This may be due to soluble cobalt ions.

Néel relaxation: The relaxation behaviour of dried particles (Brownian motion is inhibited) is shown in Fig. 3. About 25% of the remanent magnetization followed the Néel relaxation process, indicating that not all particles are ferromagnetic and behave as being super paramagnetic. The fit of the relaxation curve is a logarithmic function according to Eq. (6) and is consistent with other measurements of comparable particles [13].

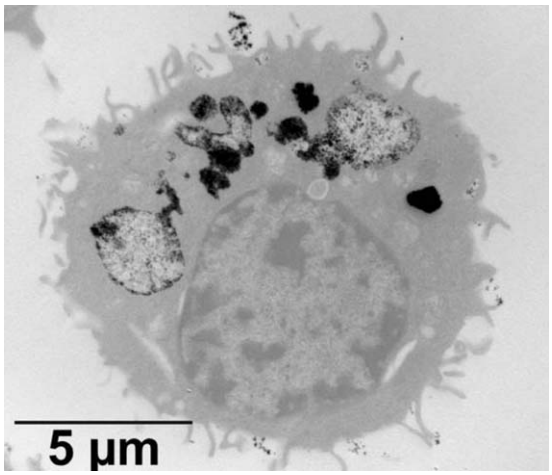

Fig. 2. Transmission electron micrograph of $CoFe_2O_4$-nanoparticles in J774A.1 macrophages.

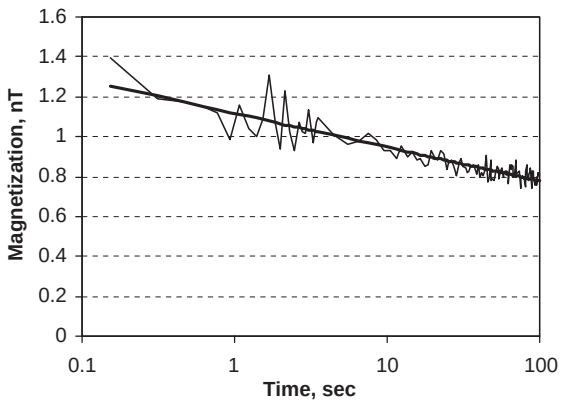

Fig. 3. Relaxation of dried particles and fit with a logarithmic function (bold line) according to Eq. (6).

Relaxation of magnetic nanoparticles in J774A.1 macrophages: The amount of particle uptake by J774A.1 cells and relaxation depended on the presence of serum (FCS) in the culture medium. Fig. 4 shows relaxation curves of 2 μg magnetic nanoparticles/ml/million cells after 3 and 24 h of incubation in culture medium w and w/o FCS. Because of the formation of aggregates, the absence of serum results in a slowed decay, which is in direct agreement with the presence of larger structures and Eq. (1). In addition, longer incubation times cause a slightly retarded relaxation, reflecting increased particle uptake and increased probability of aggregate formation. After discrimination the decay with the Néel relaxation process (Fig. 3), the remaining Brownian intracellular relaxation does not follow a single exponential function. The simplest fit is a double exponential behaviour, where about $\frac{1}{3}$ decays with a time constant of 10 s and the remaining fraction decays with a time constant of 130 s. A comparable behaviour was found with particles in the micron size range, but with larger time constants [2], and the presence of elastic components of the cytoskeleton was found to be the main reason for the deviation from a single exponential (pure viscous) relaxation [26].

Fixation of the probes with glutaraldehyde completely inhibits intracellular transport mechanisms and shows a relaxation, as was recorded with physically fixed particles according to Fig. 3. The data show that the relaxation of incorporated magnetic nanoparticles is not a passive diffusion mechanism, but an active transport process within the cells. The role of the different cytoskeletal components in this transport processes has to be investigated.

The remanent magnetization measured directly after pulse magnetization reflects the amount of particles in the Petri dish. In probes w FCS this is primarily the cell-associated fraction (attached and internalized), because either suspended particles had been removed by medium exchange or thermal relaxation is too fast for the time window of our studies. In probes w/o FCS the signal also may contain free particles being attached to the

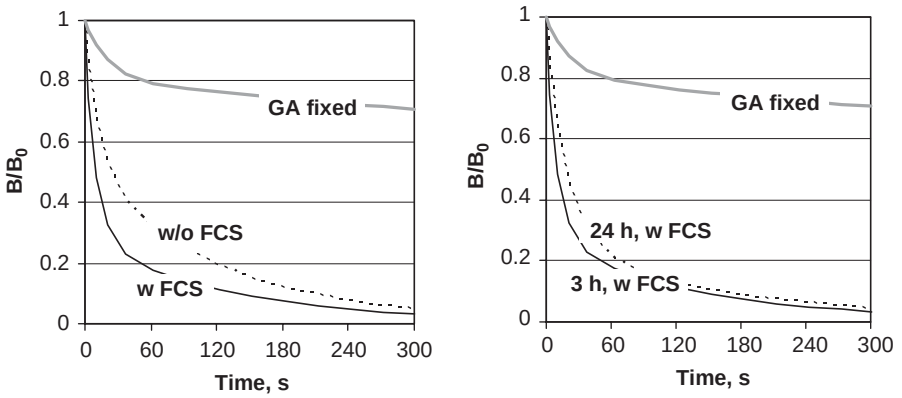

Fig. 4. Relaxation of 2 μg magnetic nanoparticles/ml/million macrophages after 3 and 24 h of incubation in culture medium with (w FCS) and without (w/o FCS) serum. After fixation of the cells in glutaraldehyde (GA fixed), intracellular relaxation is inhibited.

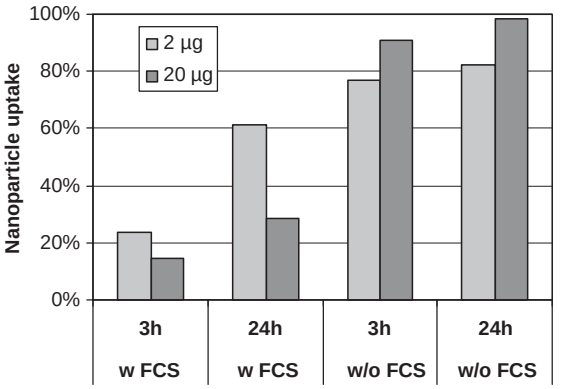

Fig. 5. Effectiveness of magnetic nanoparticle uptake by J774A.1 macrophages after 3 h and 24 h of incubation in culture medium with and without serum (w and w/o FCS).

Petri dish, but not being internalized by the cells, as can be seen by microscopy (Fig. 1). The fraction of free particles on the Petri dish is low after 24 h of incubation. Fig. 5 shows that the absence of FCS gives a higher fraction of particle uptake, which is primary due to the formation of aggregates in combination with a more effective phagocytosis.

Behaviour of inhaled magnetic nanoparticles: The clearance of inhaled nanoparticles from the lung periphery was followed for 2 weeks and is shown in Fig. 6. The clearance half time is 4.5 days. Leaching tests in deionized water and in physiological saline showed no degradation of the

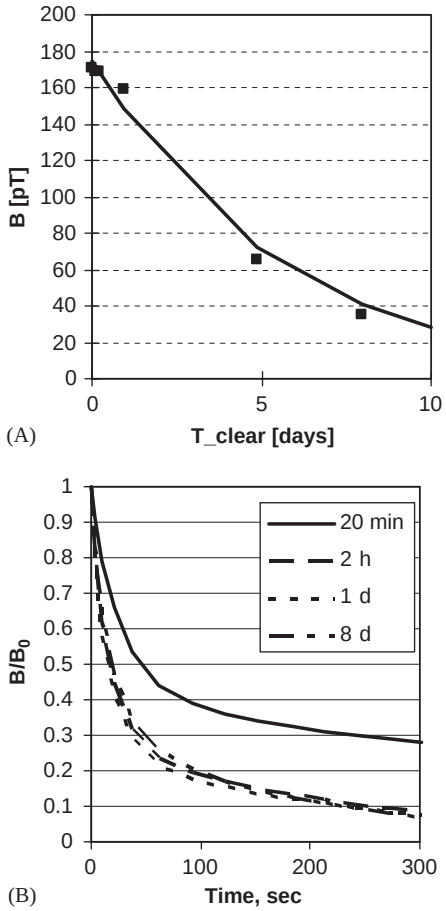

Fig. 6. Clearance of CoFe-ferrite nanoparticles from the lung periphery (A) and relaxation of inhaled CoFe-ferrite nanoparticles after different times of particle deposition (B).

particles; therefore, the interpretation of the clearance curve is not clear because mechanical clearance of insoluble micron-sized particles from the lung periphery lasts much longer and has half times of several hundred days [27,28]. The data may suggest for additional clearance mechanisms of the nanoparticles, such as systemic translocations.

During all times after inhalation relaxation of the nanoparticles was faster in comparison to dried (fixed) particles (Néel relaxation, see Fig. 3), which shows Brownian relaxation of the particles and active transport within cells, as was demonstrated in J774A.1 macrophages. Two hours after inhalation and later relaxation is constant and independent on clearance time. The slower relaxation time immediately after inhalation (20 min) may reflect a fraction of particles not being internalized by macrophages and being located freely on the epithelium.

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