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AC Magnetic susceptibility study of *in vivo* nanoparticle biodistribution

L Gutiérrez¹, R Mejías², D F Barber², S Veintemillas-Verdaguer¹, C J Serna¹, F J Lázaro³ and M P Morales¹

¹Instituto de Ciencia de Materiales de Madrid, ICM-CONIC, Sor Juana Inés de la Cruz 3, Cantoblanco 28049, Madrid, Spain

²Centro Nacional de Biotecnología, CNB-CONIC, Darwin 3, Cantoblanco 28049, Madrid, Spain

³Departamento de Ciencia y Tecnología de Materiales y Fluidos, Universidad de Zaragoza, María de Luna 3, 50018, Zaragoza, Spain

E-mail: lucia@icmm.conic.es

Abstract. We analysed magnetic nanoparticle biodistribution, before and after cytokine conjugation, in a mouse model by AC susceptibility measurements of the corresponding resected tissues. Mice received repeated intravenous injections of nanoparticle suspension for two weeks and they were euthanized one hour after the last injection. In general, only 10% of the total injected nanoparticles after multiple exposures were found in tissues. The rest of the particles may probably be metabolised or excreted by the organism. Our findings indicate that the adsorption of interferon to DMSA-coated magnetic nanoparticles changes their biodistribution, reducing the presence of nanoparticles in lungs and therefore their possible toxicity. The specific targeting of the particles to tumour tissues by the use of an external magnetic field has also been studied. Magnetic nanoparticles were observed by transmission electron microscopy (TEM) in the targeted tissue and quantified by AC magnetic susceptibility.

1. Introduction

Nanosized materials are already providing novel tools that are contributing to improve healthcare in the 21st century [1]. Opportunities include superior diagnostics and biosensors, improving imaging techniques and innovative therapeutics to enable tissues regeneration and repair. One of the main challenges is the design of nanosized drug delivery systems able to target specific diseases or to allow the transport of drugs across biological barriers. In this sense, magnetic nanoparticles offer the advantage of an efficient carrier for drug delivery in the presence of a magnetic field that can be combined with Magnetic Resonance Imaging (MRI) detection [2].

However, there is an urgent need to improve the understanding of toxicological implications of nanomedicine. One of the top priorities is the determination of the distribution of nanoparticulate carriers in the body following systemic administration through any route [3]. Although pictorial evidence of nanoparticle accumulation *in vivo* can be obtained by radiological imaging methods, the only technique that can give quantitative mass-balance information at this time is through radionuclide labelling [4,5].

Magnetic measurements are sensitive and offer substantial information in magnetic nanoparticles biodistribution studies, as they are specific for transition metals and allow

characterization of a big portion of tissue samples. Recently, magnetization curves of lyophilized samples of liver, spleen and kidney, enabled quantitative estimation of the amount of magnetic material in those organs by comparison of the normalized saturation magnetization of the organs to that of the nanoparticles alone [6]. In the intravenous repeated exposure protocol, the percentage of magnetic nanoparticles uptake per organ was an average of 35% for liver, 1.5% for spleen and 0.5% for the kidney [6]. In that case, magnetisation curves failed in quantifying magnetic nanoparticles in other organs and tissues containing much lower amounts of magnetic nanoparticles since this technique it is not able to distinguish magnetic nanoparticles from other iron species present in the body.

Another approach to measure magnetic nanoparticles biodistribution is the measurement of the AC (alternating current) magnetic susceptibility. In this technique an AC magnetic field (the excitation) is applied to the sample and the resulting AC magnetisation (the response) is recorded. From the experiment, which quite often is carried out from cryogenic temperatures up to room temperature, the in-phase (χ') and the out-of-phase (χ'') susceptibility components are determined. Quantitative analysis has been done before for biological tissues that did contain both ferritin iron and iron oxide magnetic nanoparticles [7], and is based on the treatment of the temperature dependent out-of-phase susceptibility profile. Both species (magnetic nanoparticles and iron-containing ferritin cores) have a $\chi''(T)$ maximum located at different temperatures, due to their different average particle size and crystalline structure, that allows their differentiation by AC susceptibility measurements. Eventually, as highly magnetic nanoparticles (as, e.g., magnetite) may show some degree of magnetic interaction some distortion of the $\chi''(T)$ profiles may occur. However, this phenomenon is also systematically contemplated by magnetic analysis of agar diluted nanoparticle samples with different concentrations, and the resulting $\chi''(T)$ profiles, now with careful control of the particle concentration, are also incorporated into the quantitative analysis protocol as a collection of standards.

In this paper, magnetic nanoparticles designed in terms of magnetic core, coating material and functional ligands to improve their magnetic properties and tested as in vivo drug delivery system for tumour immunotherapy [8] have been used. Uniform magnetic nanoparticles 9 nm in size were coated with dimercaptosuccinic acid (DMSA) and functionalized with a cytokine. The whole compound was injected to mice and the biodistribution of the particles was studied by AC magnetic susceptibility measurements. Quantitative data of the particle concentration in different organs were obtained with the help of particulate agar dilution standards. Furthermore, the specific targeting of the injected magnetic nanoparticles to tumour sites by using a magnetic field was also studied. The study of the biodistribution of magnetic nanoparticles within the different organs of an animal model is of paramount importance for biomedical applications and the main scope of this paper.

2. Material and methods

2.1. Synthesis of magnetic nanoparticles

Monodisperse dimercaptosuccinic acid-coated magnetite nanoparticles (DMSA-MNP) (9.2 nm-diameter $\pm$ 13% SD) were prepared following the protocol described by Roca et al [9]. Nanoparticles were synthesized in organic medium using oleic acid as a surfactant and DMSA was used to displace oleic acid from the nanoparticle surface. The coated particles were then stable in water at pH 7 with hydrodynamic sizes <100 nm and a negative surface charge (-40 mV) over a wide pH range (4-12). DMSA-MNP were bound to murine recombinant interferon- γ (IFN- γ) (Peprotech) as described by Mejías et al [10] producing functionalized particles (IFN- γ -DMSA-MNP).

2.2. Agar dilutions preparation

Agar (Fluka ($C_{12}H_{18}O_9$)_x, Mw = 3000–9000) gel solutions (1% w/v) were prepared using four different dilutions (D1-D4) of the original particle suspension in MilliQ water. The mixtures of

the agar powder and the particle suspensions were heated up to ≈ 55 °C and allowed to cool down to room temperature in a warm water ultrasonic bath, what provides a homogeneous distribution of the particles in the agar gel. The iron concentration in the agar gels ranged from 2 to 0.0001 mg Fe/ml. These gels were then freeze-dried during 24 h and the resulting products were used for the magnetic measurements. Finally, the iron concentration achieved in the freeze-dried samples was D1 = 154.5, D2 = 47.9, D3 = 1.1 and D4 = 0.011 mg Fe/ g dry product.

2.3. Mouse model

To study the magnetic nanoparticles biodistribution among the different organs, twelve-week-old female C57BL/6 (Harlan Laboratories) mice received intravenous injections of DMSA-MNP (300 μ g Fe/injection) twice a week for two weeks. Another batch of animals was treated in the same way but using IFN- γ -DMSA-MNP, and the same iron concentration. Some animals were also treated with 3-methylcholanthrene (MCA) in order to induce a tumour in their right leg and were afterwards exposed to a magnetic field of 0.4 T on the right flank for one hour after each particle injection. During the whole study, animals were maintained in the Centro Nacional de Biotecnología (CNB) animal facility and the study was approved by the Ethics Committee for Animal Experimentation at the CNB in compliance with European Union legislation.

One hour after the last injection mice were euthanized, perfused to remove rest of blood in the tissues, and spleen, liver, heart, brain, lungs, kidneys and the tumour tissue (in the corresponding animals) were harvested for analysis.

2.4. Magnetic characterization

The magnetic characterization was carried out in a Quantum Design MPMS-5S SQUID magnetometer with an AC susceptibility option. The measurements were performed with an AC amplitude of 0.45 mT, in the temperature range between 1.8 and 300 K and at a frequency of 1 Hz.

2.5. Transmission Electron Microscopy characterization

Transmission electron microscopy observations were performed in a 200-KeV JEOL-2000 FXII microscope. A part of the tumorous tissue was fixed, washed with buffer, dehydrated in graded series of acetone, and embedded in epoxy resin (TAAB 812 resin) following standard protocols for TEM observations. Staining with heavy metals was avoided in order to facilitate the particle observation in the tissues [11].

3. Results and discussion

3.1. Dipolar interactions study in magnetic nanoparticles agar dilutions

The temperature dependence of both components of the magnetic susceptibility, χ' and χ'' , is shown in figures 1 and 2 for the four different dilutions of the magnetic nanoparticles in agar. All these dilutions exhibit a single peak in both the in-phase and the out-of-phase components, indicative of blocking of the particle magnetic moments.

Figure 1. Temperature dependence of the (a) in-phase and (b) out-of-phase components of the susceptibility, per mass of sample, of the freeze-dried agar dilutions of the magnetic nanoparticles.

In figure 1, where the magnetic data are presented per mass of sample, the increase in the maxima height follows the increase of iron concentration of the samples. However when representing the susceptibility per mass of iron (figure 2) it becomes visible that for increasing particle concentration the maxima get lower and shift to higher temperatures as previously observed for these systems [7,12–16]. This phenomenon results from the progressive increase of the interparticle dipole-dipole interaction strength. The out-of-phase susceptibility per mass of iron of the magnetic nanoparticles dilutions is of key interest, as it will be used as the calibration standard for the subsequent magnetic determination of the concentration of the same particles in tissues [7].

Figure 2. Temperature dependence of the (a) in-phase and (b) out-of-phase components of the susceptibility, per mass of iron, of the freeze-dried agar dilutions of the magnetic nanoparticles.

The pre-exponential factor (τ_0) that appears in the Arrhenius expression for the relaxation time ($\tau = \tau_0 \exp(E_a/k_B T)$) has been calculated to study the interparticle interactions of the nanoparticles dilutions, following a protocol previously described [7] (figure 3a). For increasing iron concentration, τ_0 values show (figure 3b) a progressive deviation from the negligible interaction regime (10^{-9} – 10^{-12} s) [17] towards unphysically low values, as previously observed [7]. For the quantitative magnetic determination of the particles in the tissues, agar dilutions of the same particles with a similar degree of dipolar interactions would be the optimum calibration standard.

Figure 3. (a) Example of τ_0 determination, based on the method described by López et al [7], for sample D1, and (b) dependence of the τ_0 prefactor on the particle concentration. For increasing particle concentration a progressive τ_0 decrease takes place, leaving only the most diluted sample within the negligible dipolar interaction range (10^{-9} – 10^{-12} s).

3.2. Quantitative analysis of the particle biodistribution among different tissues

The use of AC magnetic susceptibility measurements as a quantitative tool for the determination of magnetic nanoparticles in biological tissues was previously described [7]. Basically, it was demonstrated that it is possible to determine the iron concentration in the form of magnetic nanoparticles of a given sample from the proportionality factor that relates its mass out-of-phase susceptibility to that of a reference sample with the same type of particles, same particle size distribution and same degree of dipolar interaction strength. The use of the out-of-phase susceptibility, as an alternative to the in-phase one, is especially practical as it is insensitive to diamagnetic and paramagnetic contributions from other species in the sample [18]. Roughly speaking, the height of the tissue samples χ'' maxima, when located at the same temperature as that of the injected particles, is a surrogate measure of the iron concentration in the form of particles within the tissue and, as in this case the particle size distribution is very narrow, the χ'' maxima height is also a surrogate measure of the number of particles in the tissue. Then, just by looking at the height of these maxima we will be able to investigate the magnetic nanoparticles biodistribution in the different tissues. It has to be pointed out, also, that this technique informs us just about the magnetite nanoparticles, independently if they are carrying the IFN- γ or not.

Previous studies on magnetic nanoparticles biodistribution have shown that particles are usually recognized and taken up by the macrophages of the mononuclear phagocyte system and subsequently accumulated in the liver, spleen and lungs [19–21]. The possible accumulation of particles in the lungs was of especial interest for us when studying the biodistribution of magnetic nanoparticles, as it has been previously reported that the DMSA coating targets the particles to the lungs [22]. The attachment of another molecule to the DMSA-MNP, in this case the IFN- γ to be used for tumour treatment, drastically affects both the surface charge and the hydrodynamic size of the particles which may lead to significant changes in the biodistribution [23]. For this reason, we explored the possibility of significant changes in the biodistribution when adding the IFN- γ to the DMSA-MNP. In figure 4 the out-of-phase magnetic susceptibilities of lung and liver tissues from three animals that were treated with DMSA-MNP and two animals that were treated with IFN- γ -DMSA-MNP are shown. The single maxima around 80 K indicates the presence of particles in the tissues, being the lungs the organs that have a higher concentration of particles in comparison with the liver. It can be observed that while the χ'' height of the liver tissues in both treatments remains very similar, indicating a similar iron concentration, the iron concentration in the lungs is significantly reduced when adding the IFN- γ . Then, it can be concluded that the IFN- γ attachment to the DMSA-MNP reduces almost three times the amount of particles that reach the lungs.

Figure 4. Temperature dependence of the out-of-phase component of the susceptibility, per mass of sample, of lung and liver tissues from (a) three different animals treated with DMSA-MNP and (b) two different animals treated with IFN- γ -DMSA-MNP. As both plots use the same quantitative scale, it is concluded that DMSA coating targets the particles to the lungs but that this effect is reduced by the attachment of the cytokine, which alters both the size and surface charge of the injected compound.

Figure 5. Temperature dependence of the out-of-phase component of the susceptibility, per mass of sample, corresponding to freeze-dried tissues from different organs: (a) mouse treated with DMSA-MNP and (b) mouse treated with IFN- γ -DMSA-MNP.

The biodistribution of particles among other tissues (spleen, brain, heart and kidneys) was also magnetically studied. The results have been incorporated in figure 5 for both treatments (DMSA-MNP and IFN- γ -DMSA-MNP). Apart from the previously discussed cases of lungs and liver, the presence of particles is also clearly observed in the spleen. In particular, a significant difference is found when comparing the concentration of particles in spleen tissues from animals treated with IFN- γ -DMSA-MNP and with DMSA-MNP, being higher the amount of particles that arrive to the spleen if the particles carry IFN- γ . The presence of particles in the rest of the characterised tissues (brain, heart or kidneys) was, in general, negligible under the detection limits of the technique, (see data from perfused animals in figure 6). This result is the same as in previous biodistribution studies of magnetic nanoparticles with different coating and size [24]. The detection limit of the DMSA-MNP was $0.005 \text{ mg Fe / g dry tissue}$. This value was estimated from the iron concentration in the form of particles, whose signal would be twice as the average noise found in the measurements.

It is of especial interest to mention the effect of the presence of blood in the tissues in biodistribution studies. In our case, tissues were collected from the animals one hour after the

last injection, so the presence of particles in the blood after such a short time is still a possibility. For this reason, our study was performed with animals that were perfused with phosphate buffered saline (PBS) solution in order to remove the blood from all the tissues before their characterisation. However, it is also interesting to quantify how much the magnetic susceptibility of a given tissue may be altered by the presence of blood. In figure 6, the out-of-phase susceptibility of different tissues coming from two animals, one perfused before tissue extraction and the other non-perfused, is shown. It is striking how the height of the out-of-phase susceptibility may increase, up to 8 times for the liver, if the tissues are not perfused. The presence of rests of blood in the non-perfused tissues is also responsible for the maxima observed in heart and kidney in which no signal of the particles is found in the perfused tissues. This fact, confirms that part of these particles are still circulating one hour after the injection [8].

Figure 6. Temperature dependence of the out-of-phase susceptibility, per mass of sample, of freeze-dried (a) liver, (b) brain, (c) heart and (d) kidney tissues from two different animals. One animal (red circles) was perfused before tissues removal. Circulatory vessels of the non-perfused one (black squares) still contained in-blood-dispersed nanoparticles.

The location in temperature of the tissue samples out-of-phase susceptibility maxima has been considered to choose the optimum agar dilution standard for magnetic quantification of the particles. It has previously been found that the degree of magnetic nanoparticles aggregation in the different tissues is manifested in the out-of-phase susceptibility as a change of shape and temperature location of the χ'' maxima as predicted by the increased dipolar interactions [7]. In this case, the χ'' profiles of the liver, spleen and lung tissues of the same animal show rather good superimposition, with small differences when the data is plotted scaled to their maxima (figure 7a) indicating a similar degree of aggregation in all tissues where particles were found.

When comparing the shape and temperature location of the out-of-phase susceptibility of a given tissue with that of the agar dilutions (figure 7b) slight differences can though be observed. The tissue $\chi''(T)$ maximum is located at slightly higher temperatures than those of the magnetic nanoparticles dilutions meaning that, in the tissues, a higher degree of aggregation was present. These differences agree with the obtained τ_0 values for several tissue samples that yielded values lower than 10^{-23} s, in that a τ_0 decrease associated to the increase of particle aggregation is expected (figure 3).

Figure 7. Temperature dependence of the out-of-phase susceptibility scaled to its maximum for (a) different tissues from the same animal where magnetic nanoparticles have been identified and (b) the agar dilutions of the magnetic nanoparticles and a liver tissue.

Table 1. Ranges of iron concentration (in the form of particles) in the different tissues obtained from the AC susceptibility measurements.

Treatment	[Fe _{particles}] (mg Fe/g dry tissue)		
	Liver	Spleen	Lung
DMSA-MNP	0.062 – 0.084	0.046 – 0.061	1.22 – 1.90
IFN- γ -DMSA-MNP	0.090 – 0.092	0.14 – 0.36	0.40 – 0.50

The iron concentration in the form of particles in the tissues was calculated (table 1) choosing as reference material for the quantification protocol the most concentrated gel (D1) as this is the one that fits better the tissue samples data. It should be taken into account that these iron concentration values correspond in this case only to iron in the form of particles, although other iron-containing species may also coexist in the tissue. Once the iron concentration was obtained, the total amount of iron in the form of the magnetic nanoparticles in each organ was calculated taking into account the weight of the whole freeze-dried organ. The average amount of iron in the form of magnetic nanoparticles in each organ was calculated with data from three different animals of each type. The total amount of iron in the form of MNP found in the animals, resulting from the addition of the quantities found in all the characterised organs are in the range between 90 and 140 μ g, which is around 10% of the total injected iron (1200 μ g). This value also corresponds to around 30% of the iron administered in the last injection to the animal (figure 8). Although there may be still a big percentage of particles from the last injection in the blood (figure 6), these data indicate that there is not a significant accumulation of the particles from previous injections in tissues. The injected particles are probably dissolved and assimilated by the body, especially in the form of ferritin, as observed for intramuscularly injected iron oxyhydroxide nanoparticles [25]. The "unaccounted" portion of iron in the form of magnetic nanoparticles is around 90% of the total injected during 2-week-treatment (4 injections of 300 μ g Fe each). Therefore, the low amount of particles found in the tissues after

the treatment supports their use in biomedical applications, as it means that these particles do not accumulate in the organism.

Figure 8. Percentage of the magnetic nanoparticles from the last MNP administration found in each organ from animals treated with (a) DMSA-MNP and (b) IFN- γ -DMSA-MNP. Values have been calculated from the total amount of iron found in each whole tissue in the form of MNP and the total amount of injected iron in the last administration.

3.3. Targeting of magnetic nanoparticle to tumour tissues by magnetic fields

As one of the most important applications of magnetic nanoparticles in biomedicine is their use as magnetic carriers for drug delivery, the specific targeting of the IFN- γ -DMSA-MNP to tumour tissues under local magnetic field created by placing a magnet next to them was tested. The presence of magnetic nanoparticles in the tumour tissue was confirmed both by TEM observations and AC magnetic susceptibility measurements.

Figure 9. Transmission electron micrographs of magnetic nanoparticles aggregates found in the tumour tissues from mice treated with IFN- γ -DMSA-MNP and an external magnetic field at two different magnifications (a) higher and (b) lower.

Magnetic nanoparticle aggregates with sizes in the range between 120 and 300 nm were observed in the tumour tissues (figure 9). The unstained sections allowed identifying the presence of the particles although the histological localization was difficult to resolve due to the low contrast in the different cell structures. The presence of these aggregates was found by TEM in tissues treated with IFN- γ -DMSA-MNP either with or without magnetic field application. Although qualitatively it could be said that it was easier to find the particles in the tissues when the magnetic field was applied, indicating a higher particle concentration, it is

really hard to quantify their amount from the TEM micrographs. AC magnetic susceptibility measurements in order to calculate the iron concentration in the tumour tissues were also performed (figure 10). In mice treated with IFN- γ -DMSA-MNP, the iron concentration in the form of magnetic nanoparticle in the tumours was in the range of 0.08 – 0.1 mg Fe/g dry tissue and the mean volume size was 2408 nm³. The mean tumour volume of animals treated with IFN- γ -DMSA-MNP plus the external magnetic field was significantly smaller (mean volume size = 446 nm³) than that of those not treated with the external magnet. As the size of the tumours decreased, a higher concentration of particles (figure 10 a) was found in the tumours, accumulating up to 10 mg Fe/g dry tissue in the form of the magnetic nanoparticles for the smallest tumour (figure 10 a).

Figure 10. (a) Temperature dependence of the out-of-phase susceptibility per mass of sample of three tumour tissues coming from the following animals: control, animal after IFN- γ -DMSA-MNP injection and animal after IFN- γ -DMSA-MNP injection under applied magnetic field. (b) Same data as in (a) but with a different vertical scale in order to better appreciate the significant differences between control and injected animal under no magnetic field.

4. Conclusions

We have used AC magnetic susceptibility measurements to study the magnetic nanoparticle biodistribution in a murine model. Changes in the biodistribution of the particles have been found after their functionalization with Interferon- γ . Specific targeting to tumour sites has been achieved by the use of an external magnetic field. Our findings show that just around 10% of all the injected particles were found intact within the tissues one hour after the last injection, which means that these particles do not accumulate in the organism even after repeated injections and therefore may be easily metabolized or excreted.

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