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Efficiency analysis of clearance of two types of exogenous iron from the rat brain by Mössbauer spectroscopy

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Abstract Fe_3O_4 based ferrofluid was injected transcranially in the ventricle of the rat brain. At 3 months after the injection the rat was sacrificed and the brain was investigated by Mössbauer spectroscopy and histological Perls Prussian blue method. Joint analysis of histological and Mössbauer data confirms that superparamagnetic nanoparticles Fe_3O_4, which constituted about 91 % of the iron of the ferrofluid, were cleared from the brain, while the concomitant chemical compound containing ferric ion in the high-spin state, remains intact.

Keywords Mössbauer spectroscopy · Magnetic nanoparticles · Brain

1 Introduction

The concept of targeted drug delivery was proposed by Paul Ehrlich more than 100 years ago and consists of a therapeutic agent mixed with drug carrier. The drug carrier can bind selectively to the pathogens or the diseased area within the body. Finding of the carrier which is specific for a particular disease is a very difficult and

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often impossible task. Currently, the achievements in the magnetic resonance imaging (MRI) technology gave rise to a new variant of targeted drug delivery, based on the application of magnetic carriers. In this variant the diseased area in the body is determined by MRI method. Then the delivery or the magnetic fixing of the drug in the target area can be performed by an external magnetic field under the MRI control [1].

The ferrofluids based on the superparamagnetic particles, Fe_3O_4, are considered to be most promising means for such targeted delivery. Their advantage is connected with the biocompatibility assumption of iron. The human body contains about 4 g of endogenous iron, mainly in the form of iron-containing proteins hemoglobin and ferritin. The metabolism of iron in the body is one of the most highly organized process, in which almost all of the iron released by the breakdown of hemoglobin and other iron-containing proteins, re-utilized. Therefore the development of methods to control a clearance or transition of exogenous Fe_3O_4 nanoparticles (NP) in the endogenous forms is important for the introduction of the magnetic drug delivery technology into clinical practice.

Mössbauer spectroscopy is widely used to investigate both the ensembles of superparamagnetic NP [2], and the iron-containing proteins [3]. Based on a multilevel model of the relaxation behavior of ensembles of single-domain magnetic particles [4–6], the authors of this article in the past few years were developing experimental Mössbauer spectroscopic method for the quantitative study of the process of biodegradation of superparamagnetic NP in the liver and spleen [7–11]. This method allows, by evolution of the shape of the relaxation Mössbauer spectra of the investigated body, to control both a process of reducing the concentration of exogenous superparamagnetic NP and the simultaneous increase in the concentration of endogenous iron-containing proteins. In this paper, the task was to assess experimentally the feasibility of the method for solving more challenging problems—control of biodegradation of magnetic NP in the brain. To achieve this goal it was necessary to answer the following questions:

1. What is the maximum amount of ferrofluid that can be injected into the ventricle of the rat brain without irreversible damage to its vital functions?
2. Will the amount of iron administered in this way sufficient to obtain statistically reliable Mössbauer spectra of the brain?
3. Will the magnetic NP of this ferrofluid diffuse in the brain, biodegrade and clear from the brain, or fall out in the ventricle of the brain in the form of an insoluble precipitate?

2 Experiment

Magnetic Fe_3O_4 NP were synthesized using co-precipitation of $FeCl_2{\cdot}4H_2O$ and $FeCl_3{\cdot}6H_2O$ in 30 % NH_4OH. After sedimentation and decanting the particles were washed in 2M HNO_3 and stabilized with dextran.

Adult Wistar male rat weighing 450 g was used for the *in vivo* experiments. The animal care facility was kept under a natural light/dark cycle. The rat was housed individually and was provided with water and food *ad libitum*. All animal care standards and protocols were in compliance with the NIH guide for the care and use of laboratory animals (NIH Publications No. 80–23, revised 1996).

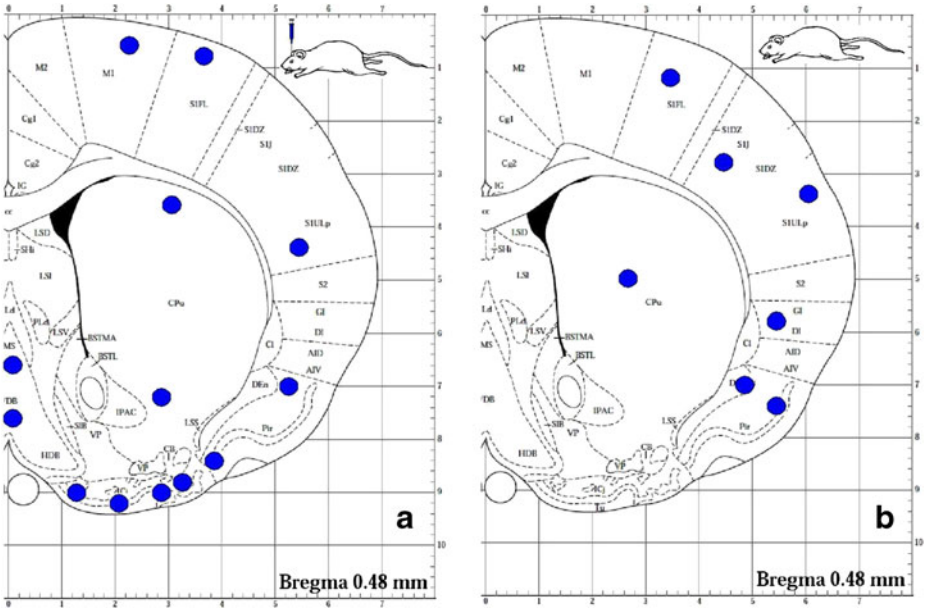

Fig. 1 Location of the *blue*-colored areas on the cross section of the brain of the rat at 3 months after injection of ferrofluid (**a**) and of the control rat without injection (**b**)

Five mg of the magnetic NP suspended in 5 μl of water were injected transcranially in the ventricle of the brain (AP = −0.8, L = 1.5, H = 3.8) according to atlas [12]. Injection was performed by Quintessential Stereotaxic Injector "Stoelting" under chloral hydrate anesthesia (400 mg/kg). After 3 months, the rat was sacrificed, brain extracted and divided into two parts. One part of the brain was used for the histological study by Perls Prussian blue method. Other part was lyophilized, grounded and the powder sample was prepared for Mössbauer studies.

^{57}Fe Mössbauer spectra of the powder obtained after grinding of the lyophilized rat brain and the spectra of magnetic NP were measured at 77 and 300 K with electrodynamical type spectrometer, working in the constant acceleration mode. ^{57}Co in a chromium matrix was used as a source of gamma-irradiation. Isomer shifts were determined in relation to the absorption line of α-Fe.

3 Results and discussion

Ventricular volume of a rat brain is about 5 μl. Transcranial injection into the ventricle of the brain 5 μl of ferrofluid has not led to any changes in the natural behavior of the animal during 3 months period. Total amount of Fe injected in the brain was about 5 mg, which is much higher than the amount needed for the Mössbauer measurements. For example, the spectra of the initial NP in Fig. 2 was measured on the sample containing only 0.5 mg Fe.

Three months after transcranial injection of the ferrofluid the rat was sacrificed and part of the brain was cut into histological slices and stained with Prussian blue for detection of iron. Histological examination showed the presence of blue areas in parenchyma and on the dura mater of the brain (Fig. 1a). The brain of the control rat

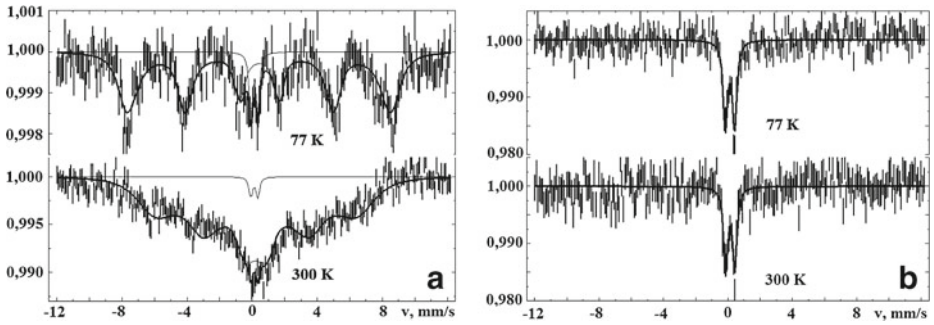

Fig. 2 ^{57}Fe Mössbauer spectra for initial NP (**a**) and a rat brain in 3 months after the NP injection (**b**). The results of simultaneous treating are represented by *solid lines*. These are partial relaxation spectra of NP, additional non relaxation doublet as well as the resulting spectra

demonstrated the presence of similar blue areas in parenchyma too (Fig. 1b). This indicates the presence in the brain of the endogenous iron-containing proteins. On the other hand the concentration of such blue areas in the parenchyma and especially on the dura mater of the brain of the experimental rat was higher compared to control rat. The observed pattern of the iron distribution in the brain of experimental rat indicates complete removal of iron from ventricle and diffusion of ferric ions deep into the parenchyma.

Prussian blue staining shows localization of ferric ions only. Its main advantage is that it is not sensitive to blood erythrocyte containing divalent iron ions. Unfortunately, it does not allow distinguishing of sources of ferric iron, which can be both exogenous Fe_3O_4 NP, and endogenous iron-containing proteins. Therefore, to clarify the origin and chemical state of these blue areas, we further used the method of Mössbauer spectroscopy.

The Mössbauer spectra of the control brain without injection, measured at 77 and 300 K, showed no signs of the presence of iron compounds. This contradicts the data of histological studies (Fig. 1b) and demonstrates a weaker sensitivity of Mössbauer spectroscopy compared with histological methods. The Mössbauer spectra of the initial NP measured at 77 and 300 K are shown at Fig. 2a. The spectrum at 300 K has a specific "five-step pedestal" shape studied recently in detail in [13]. This shape is typical for an ensemble of monodispersed superparamagnetic NP with narrow size distribution of particles and the weak magnetic dipole interactions between the particles. At 77 K the superparamagnetic component of the spectrum, associated with the presence of Fe_3O_4 NP, is splitted into six lines. As a result, an additional doublet of lines becomes clearly visible. Normally, such a doublet associated with the presence in the ensemble of smaller superparamagnetic NP. In the latter case, reducing the size of the particles decreases the relaxation time of their magnetization vectors, leading to a collapse of the hyperfine magnetic structure to the quadrupole doublet. However, the simultaneous processing of these spectra within multilevel relaxation model shows the fundamental difference between the Mössbauer parameters of the sextet and the doublet, indicating different chemical state of iron for these two components (Table 1). In particular, the treatment of partial spectral areas at two temperatures for each chemical phase within the Debye model allows us to estimate the Debye temperatures and the recoilless fractions

Table 1 Mössbauer data for initial NP and injected rat brain at 3 months after the NP injection: average magnetic anisotropy energy KV, quadrupolar splitting $2q$, hyperfine field H_{hf}, isomer shift δ, line width Γ, Debye temperature T_D, ^{57}Fe concentration n (in brackets we point errors in the least significant digit)

T, K	78	300
NP, Sextet		
KV, K	300 (80)	
$2q$, mm/s	0.35 (7)	
H_{hf}, kOe	530 (6)	500 (10)
δ, mm/s	0.44 (2)	0.22 (3)
T_D, K	280 (20)	
n, 10^{19} cm^{-3}	46 (3)	
NP, Doublet		
$2q$, mm/s	0.42 (4)	0.34 (4)
δ, mm/s	0.14 (2)	0.19 (2)
Γ, *mm/s*	0.24 (6)	0.24 (6)
T_D, K	143 (9)	
n, 10^{19} cm^{-3}	4.3 (6)	
Brain, Doublet		
$2q$, mm/s	0.568 (2)	0.555 (3)
δ, mm/s	0.122 (1)	0.137 (2)
Γ, *mm/s*	0.309 (3)	0.301 (4)
T_D, K	600 (300)	
n, 10^{16} cm^{-3}	1.7 (1)	

for both phases. From these values one can evaluate concentrations of the resonant isotope in both observable components. As a result, we conclude that about 91 % of the iron in the sample is in the form of superparamagnetic NP Fe_3O_4, the Mössbauer parameters of which exhibit the usual relaxation behavior with temperature [14]. Simultaneously, about 9 % of the iron in the sample is characterized by the different chemical shift 0.14 mm/s at 77 K and 0.19 mm/s at 300 K and does not show the relaxation behavior in this temperature range.

The Mössbauer spectrum of the brain at 3 months after injection, measured at 77 and 300 K (Fig. 2b) proved to be different from the spectrum of the intrinsic ferrofluid. It is a doublet of narrow lines with the values of chemical shift and quadrupole splitting very close to parameters of the anomalous doublet in the spectrum of intrinsic NP.

Comparison of the Mössbauer spectra of the original ferrofluid and of the brain 3 months after injection shows that the superparamagnetic component of the spectrum, which splits at low temperature, completely disappears from the spectrum. This proves that the superparamagnetic NP Fe_3O_4, which constituted about 91 % of the iron in the intrinsic ferrofluid, have been completely removed from the brains in 3 months. The nature, chemical composition and spin state of the iron remains in brain are yet to be investigated.

4 Conclusion

Our experiment with the direct transcranial injection has proved that up to 5 mg Fe_3O_4 can be introduced in the ventricle of the brain of adult male rat without irreversible damage to it's vital functions. This amount of iron proved to be sufficient to obtain statistically reliable Mössbauer spectra of the brain. Joint analysis of histological and Mössbauer data confirmed that that this amount of NP was cleared

from the brain during 3 months. Based on these results the authors plan to fulfill more detailed experimental study of the magnetic NP biodegradation process in the brain within this 3 months period.

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