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Research articles

Synthesis and Mössbauer study of ^{57}Fe-based nanoparticles biodegradation in living cells

A. Yurenya[a,b,*], A. Nikitin[b,c], A. Garanina[b,c], R. Gabbasov[a], M. Polikarpov[a], V. Cherepanov[a], M. Chuev[a,e], A. Majouga[b,d], V. Panchenko[a,b]

[a] National Research Center "Kurchatov Institute", Moscow, Russia
[b] Lomonosov Moscow State University, Moscow, Russia
[c] National University of Science and Technology MISiS, Moscow, Russia
[d] Dmitry Mendeleev University of Chemical Technology, Moscow, Russia
[e] Valiev Institute of Physics and Technology, Russian Academy of Sciences, Moscow, Russia

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ABSTRACT

Biodegradation of nanoparticles includes the destruction of a stabilizing coating and the accompanying change in interparticle interaction, as well as the direct destruction of the inorganic nuclei of particles. These processes lead to characteristic changes in the shape of the Mössbauer spectra of iron oxide nanoparticles. In this work, we investigated the in vitro biodegradation of ^{57}Fe-based magnetic nanoparticles with the aid of Mössbauer spectroscopy. For this purpose, two types of magnetic nanoparticles enriched with the ^{57}Fe isotope were synthesized. Copolymer Pluronic F-127 and citric acid were used to stabilize nanoparticles in aqueous medium. Moreover, synthesized nanoparticles were analyzed by physicochemical methods and investigated for cytotoxicity. The study of magnetic nanoparticles biodegradation was performed on 4 T1 cell culture (breast cancer). We measured Mössbauer spectra of nanoparticles incubated with 4 T1 cells and spectra of control nanoparticle samples at different conditions. The analysis of spectra was carried out in the many-state relaxation model formalism. The study revealed that after 120-hours incubation of nanoparticles in cells, they did not undergo measurable changes typical of biodegradation processes. Nevertheless, we noted intense intracellular oxidation of ferrous iron of synthesized nanoparticles to the ferric phase. The results obtained indicate the possibility of using the obtained nanoparticles in Mössbauer in vitro studies.

1. Introduction

The applications of nanoparticles and other nanomaterials become more and more common in medicine and biotechnology [1,2]. The intersection of nanotechnology and biomedicine offers the challenge for one of the most exciting and cross-disciplinary developments over the last decade [1,3]. One of the most frequently used nanomaterial is magnetic nanoparticles, which offer some promising possibilities in biomedicine due to unique magnetic properties [4]. The particular applications of magnetic nanoparticles are targeted drug delivery, hyperthermia treatments, magnetic resonance imaging (MRI) contrast enhancement and magnetic separation [5–8].

In addition to research that has already become traditional, in recent years a number of new experimental biomedical methods based on the use of nanoparticles have appeared. One of the promising areas is the study of anomalous diffusion in biological fluids and the viscoelastic properties of the cytoplasm [9]. This research is based on the investigation of the behavior of nanoparticles that are probed inside the cytoplasm [10,11]. We are developing a novel approach to the study of viscoelastic properties with unprecedented resolution (less than 10^{-7}s), which is based on nuclear gamma-resonance spectroscopy. By means of thorough analysis of changes in the spectra of nanoparticles probed into viscous fluid, it is possible to obtain completely new data on the motion on a nanoscale level [12,13]. The principle of our approach is the Mössbauer study of Brownian motion of ^{57}Fe-based magnetic nanoparticles administrated into cells. However, it is well known that nanomaterials can significantly change their properties as a result of interaction with living organisms. Therefore it is crucial to take into account any subtle changes in the spectra of probing nanoparticles arising from their destruction in intracellular environment.

The biodegradation of nanoparticles is a key and at the same time one of the most difficult problems of modern nanotechnology. A conventional

* Corresponding author.
E-mail address: antonyurenya@gmail.com (A. Yurenya).

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nanoparticle for biological applications comprises of an inorganic core and a coating organic surface. Both coating and inorganic core can biodegrade and change their physicochemical properties through different mechanisms [14]. Iron oxide nanoparticles (IONPs) are thought to be biocompatible, biodegradable and non-toxic material. After their administration *in vivo*, a host of innate immunological mechanisms start to recognize and collect particles as well as direct them to the major elimination pathways of the body [15]. Due to peculiar biological status of iron, IONPs are less toxic for organism than other inorganic nanocrystals since they have the chance to be transformed into endogenous forms of iron by cellular homeostasis [16]. It is also believed that the mechanisms involved in intracellular degradation of any IONPs are very similar to those related to ferritin. In ferritin, the protein shell first gets dissolved by lysosomal proteases and then the internal IONPs get released followed by rapid dissolution in acidic environment of the lysosomes [17]. Under conditions similar to the medium of intracellular lysosomes, intensive destruction of IONPs can occur up to their complete dissolution [18]. Earlier in a number of Mössbauer studies on the biodegradation of magnetic nanoparticles in mammals, we demonstrated that both the biodegradation of a stabilizing organic coating of particles and the biodegradation of their inorganic core alter their physicochemical properties, which is clearly observed on their Mössbauer spectra [19–21]. The process of nanoparticles degeneration *in vivo* occurs with the involvement of a number of organism systems. It is obvious that *in vivo* and *in vitro* biodegradations may differ significantly in terms of mechanism and intensity. Despite the fact that the issue of IONPs biodegradation at the cellular level is highly relevant and still poorly studied, there are very few research studies that have investigated this process using Mössbauer spectroscopy. In this work, we carried out a Mössbauer study of *in vitro* biodegradation of IONPs. For this purpose, two types of aqueous colloids of nanoparticles isotopically-enriched with ^{57}Fe were synthesized. Synthesis methods were chosen in such a way as to obtain samples of biocompatible nanoparticles with different hydrodynamic size. Obtained nanoparticles can be used as nanoprobes for the Mössbauer study of the cytoplasm viscoelastic properties described above. Relaxation Mössbauer spectroscopy methods were used to analyze the biodegradation of ^{57}Fe-nanoparticles after they were incubated in cell culture for several days. We measured the Mössbauer spectra of cell samples and control samples at different conditions. Simultaneous analysis of the spectra allows us to define the lines shape of each component independently. Such analysis makes it possible to investigate the transformations of administered nanoparticles including their degradation and the formation of other iron-containing species [22].

2. Materials and methods

2.1. Nanoparticles synthesis

a) Small nanoparticles (NP1)

The mixture of 0,0356 g $FeCl_2$ and 0,0975 g of $^{57}FeCl_3$ (the ^{57}Fe content was not less than 98%) was placed in a flask and dissolved in 2 ml deionized water. The resulting solution was stirred for 10 min while bubbling with argon and then heated to 95 °C. 500 μL of 28% NH_4OH solution was added to the heated solution with continuous stirring for 1 h at 95 °C. Then 0.041 g of citric acid in 500 μL of water was added to the reaction mixture and the resulting solution was stirred at 95 °C for an additional 2 h. After that, the mixture was cooled to room temperature, centrifuged, and the resulting precipitate was dispersed in 20 ml of citric acid solution (20 mg/ml) with an ultrasonic homogenizer (20 min). The nanoparticles were again separated from the solution by centrifugation (7000 rpm, 10 min) and 20 ml of citric acid solution (20 mg/ml) were added to the resulting precipitate, followed by 5 min sonication. For separation of large nanoparticles, the resulting solution was centrifuged for 10 min at 7000 rpm. The supernatant solution was collected and its pH value was adjusted to 7.4 by adding a 1 M NaOH solution. Resulting solution was dialyzed for 48 h in a dialysis bag with a pore size of 12–14 kDa. Total ^{57}Fe concentration achieved was not less than 65%.

b) Large nanoparticles (NP2)

For obtaining ^{57}Fe(III)-oleate complex 1.294 mmol sodium oleate was dissolved in a mixture of deionized water (10 ml), ethanol (10 ml) and hexane (20 ml) followed by addition of 0,432 mmol $^{57}FeCl_3$. The resulting reaction mixture was boiled for 4 h at vigorous stirring. Than the solution was cooled to room temperature, the upper dark brown layer was separated, washed three times with deionized water and the solvent residues were removed using a rotary evaporator.

0.432 mmol of obtained ^{57}Fe(III)-oleate complex, 0.05 g oleic acid, 0.05 g NaOL and 5 ml of 1-octadecene were placed in a flask equipped with a thermometer, a supply of argon and reflux. The mixture was heated to 140 °C in an argon flow with continuous stirring (4000 rpm) and held at this temperature for 1 h. Then, the reaction mixture was heated to the boiling point at a rate of 4 °C/min and held at this temperature for 30 min. The resulting solution was cooled to room temperature followed by addition of isopropanol (20 ml), than nanoparticles were separated from the solution by ultracentrifugation (14000 rpm) and dispersed in hexane. 1 ml of resulting particles solution (total $^{57}Fe_3O_4$ concentration = 4.0 mg/ml) was mixed with 4 ml of an aqueous solution of Pluronic F-127 (4 mg/ml) and sonicated for 30 min. Coated nanoparticles were collected from the resulting emulsion by centrifugation (14000 rpm, 20 min) and redispersed in deionized water. The procedure described above was repeated three times to completely purify obtained ^{57}Fe-nanopartices from the unreacted polymer. Total ^{57}Fe concentration achieved was not less than 98%.

2.2. Characterization of nanoparticles

Transmission electron microscopy (TEM) images of synthesized particles were taken on JEOL JEM-1400 (120 kV) anmicroscope. All samples were prepared by dropping 10 μL NPs dispersion of synthesized samples onto a formvar-coated copper grid and subsequently evaporating of the solvent. The average diameter of the samples and size distribution were evaluated by using ImageJ software. At least 1000 NPs were analyzed for each sample.

The results of size measurement using DLS showed that the mean hydrodynamic diameter of the NP1 was about 40 nm and that the mean hydrodynamic diameter of the NP2 was about 140 nm after coating with Pluronic F-127.

MTS assay was performed to estimate the toxicity of synthesized nanoparticles and to specify max particle concentration we could use in our experiments. 4 T1 mouse breast cancer (ATCC, Manassas, VA, USA) cells were incubated with different particle concentrations in culture medium for 72 h. The medium with nanoparticles from each well was replaced by 120 μL of new culture medium/MTS reagent solution at a rate of 20 μL MTS (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay) reagent per 100 μL culture medium. Cells were incubated with MTS for 4 h at 37 °C in darkness. After this wells were placed on the permanent magnet in order to remove nanoparticles from solution, and 80 μL of the obtained solution were carefully replaced in new 96-well plate. The absorbance of the solution was measured at 490 nm using Thermo Scientific Multiskan GO spectrometer. The percentage of live cells in the MTS-assay was represented as mean ± SD (for four repeats in each experiment). Percentage of live cells, cultivated in full culture medium in incubator, was taken as 100%. Plotting and calculation of the standard deviation value were made using Microsoft Excel software. P value was calculated using oneway ANOVA calculator. P values < 0.01 were considered significant.

2.3. Nanoparticle biodegradation in vitro experiments

4 T1 were cultured in RPMI-1640 medium (gibco) supplemented

with 10% fetal bovine serum (FBS) (gibco) and 2 mM L-glutamine (gibco) at 37 °C in a humidified incubator supplied with 5% CO_2. Two cell samples with NP1 and NP2 were made. Cells were seeded in T75 flasks at concentration 60 000 cells/ml and cultivated for 48 h. After this culture medium was removed from the flasks and replaced by a new portion containing ^{57}Fe-based nanoparticles (60 μg/ml). Cells were incubated with NPs for 120 h at 37 °C and 5% CO_2 in the presence of 0.2 kOe magnetic field to enhance nanoparticles uptake [23]. The field was applied perpendicular to the flask surface by using permanent flat magnet. An incubation period was chosen based on data from our previous studies on the *in vivo* biodegradation of IONPs [20,22]. After co-cultivation cells were carefully washed with PBS three-four times by centrifugation to remove particles, which were not associated with cells. Then cells were detached from the substrate with TrypLE (gibco), resuspended in culture medium and counted using automatic cell counter EVE. After that cells were collected and dried with rotary evaporator. At the same time some initial NP1 and NP2 particles were dried in the same way for further study (Control samples).

2.4. Mössbauer spectroscopy

For our experiments, the ^{57}Fe Mössbauer spectra of lyophilized cell samples and the spectra of control samples were measured at different temperatures with electro-dynamical type spectrometer CMS-1104Em, working in the constant acceleration mode. ^{57}Co in a rhodium matrix was used as a source of the resonant gamma-irradiation. Isomer shifts were determined in relation to the absorption line of α-Fe.

The analysis of all Mössbauer spectra was carried out in the many-state relaxation model formalism [24,25] with assumption of two different magnetite sublattices, which corresponds to the two sextets in the spectra.

3. Results and discussion

The size and the morphology of obtained samples were studied by TEM (Fig. 1). From TEM images of synthesized particles it was found that NP2 particles have a rod-like shape. However, a large number of small spherical nanoparticles are also presented in the sample, which indicates a high polydispersity of NP2 sample. The average size of nanoparticles was 21 nm, and the value of the MSD was ± 10 nm. NP1 nanoparticles have a spherical shape and high monodispersity. The average size of these particles was 10 ± 1.5 nm. The results of size measurement using DLS show that the hydrodynamic diameter of NP1 particles is about 40 nm and the hydrodynamic diameter of NP2 particles after coating with Pluronic F-127 is about 140 nm. Such a high value is due to the fact that NP2 particles are collected in small micelles.

From MTS assay it was found that concentration up to 60 μg/ml was permissible for 4 T1 cells with both nanoparticle samples.

The main task of the work was to evaluate the change in the hyperfine structure of the spectral lines of nanoparticles as a result of their interaction with the cell culture. It should be mentioned that any cell culture contains endogenous iron. The iron content in the cells varies, and usually does not exceed 0.1% by weight of dry matter, which corresponds to 0.001% ^{57}Fe-isotope content [26]. We used isotopically enriched nanoparticles and for this reason, the initial content of the ^{57}Fe in cell samples was considered negligible. Mössbauer absorption spectra of ^{57}Fe nuclei of initial NP1 particles cultured with cells as well as control sample spectra are given in Fig. 2. The profile of these spectra reflects qualitatively the standard behavior of an ensemble of iron-oxide nanoparticles with magnetic anisotropy.

A group analysis of the spectra demonstrated that the prolonged residence of citrate-coated particles within the cell culture hardly changes their hyperfine structure. A resembling picture with some differences is observed in the experiment with larger nanoparticles coated with Pluronic F-127 (Fig. 3). The first difference is the appearance of a pronounced singlet in NP2 particle spectra incubated in cell culture. This singlet appears in the low-temperature spectrum, and in the spectrum collected at room temperature. The specific chemical shift of the order of −0.1 mm/s at room temperature and the character of the singlet, which is extrinsic to biological forms of iron, suggests that an impurity, such as stainless steel shavings having similar Mössbauer parameters, could get into the cell sample. The final nature of this singlet is not established at the moment.

Another sigh of the interaction of NP2 particles with cell culture can be observed on their Mössbauer spectra (Fig. 3). Thermal decomposition method allowed us to obtain particles with a phase composition close to bulk magnetite. On the spectra it is manifested as two sextet components, associated with the A and B sublattices of the inverse spinel structure (Fig. 3 highlighted blue and red). There is also the Verwey transition which can be observed on the 100 K spectrum [27]. On the other hand, Mössbauer spectrum of the cell culture already at room temperature shows a low content of ferrous iron, which is indicated from the small chemical shift of the B-sublattice component (i.e. near 0.3 mm/s). This effect can be explained by the intensive oxidation of ferrous iron of NP2 particles inside the cells as a result of the Fenton reaction [28].

However, in the case of both samples, no spectral transformations are observed that are peculiar to changes associated with the biodegradation of nanoparticles in the organism. Such a result was quite unexpected. In accordance with a number of recent studies on the biodegradation of magnetic nanoparticles, the properties of an ensemble of IONPs are undergoing significant changes almost immediately after ingestion. So, for example, an hour after the introduction of similar iron-oxide nanoparticles into the mammalian organism,

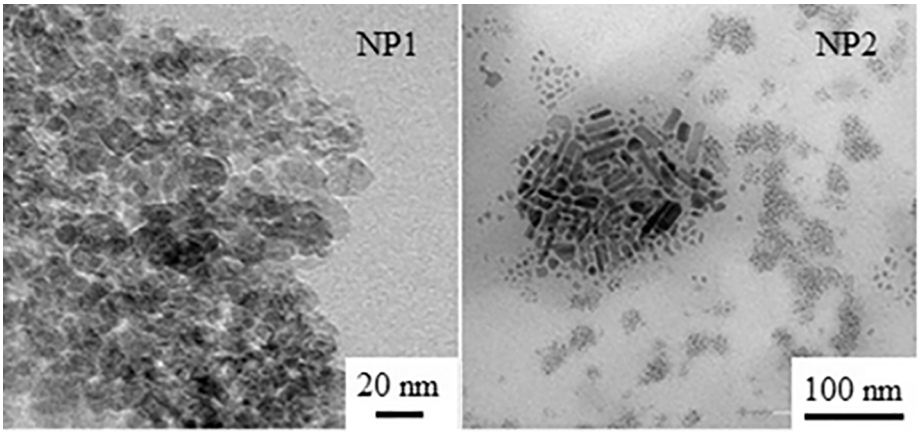

Fig. 1. TEM images of NP1 (left) and NP2 (right).

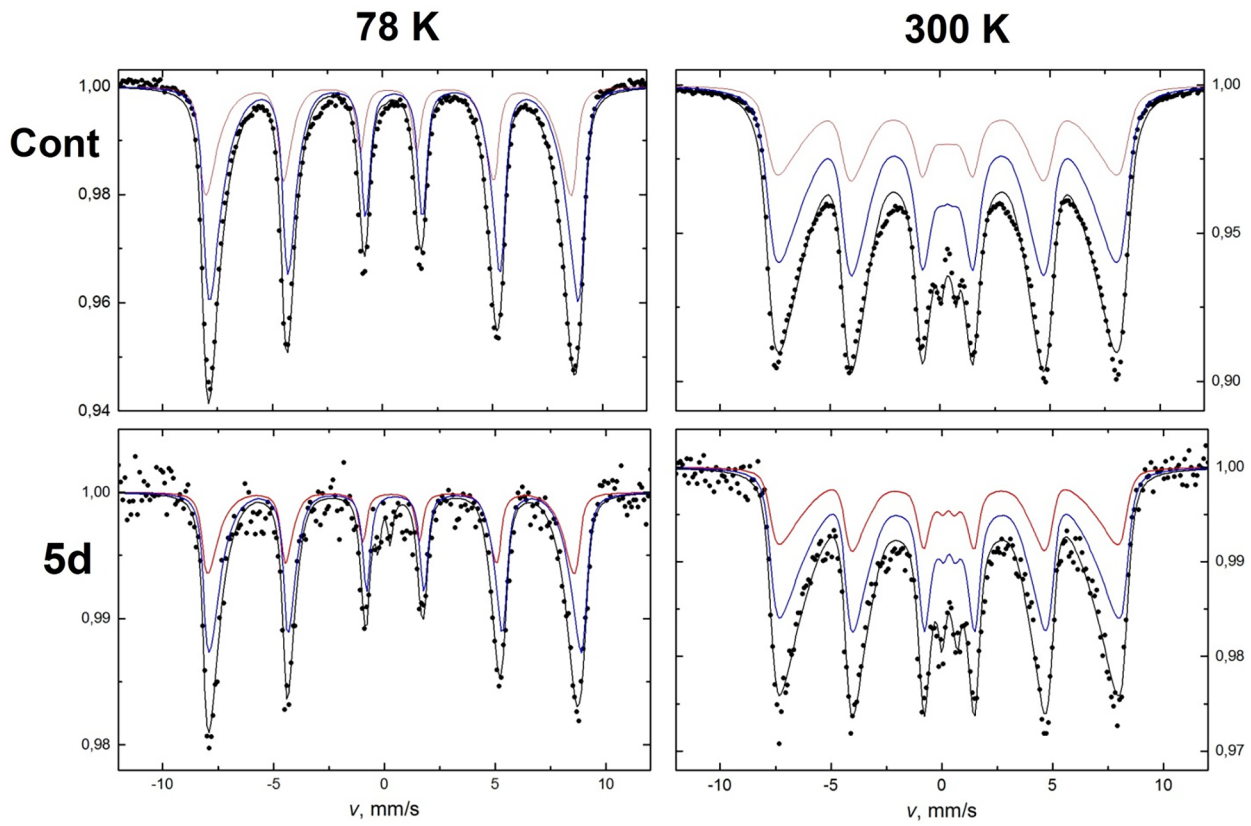

Fig. 2. ^{57}Fe Mössbauer spectra of dried 4 T1 cells cultured for 5 days with NP1 administrated (bottom) and their control sample (top) collected at temperatures 78 K and 300 K. Black lines: spectra of an ensemble of iron-oxide particles calculated with multilevel relaxation models [30]. Colored lines: the partial contributions from ferrimagnetic sublattices A and B.

a measurable modification of their spectrum is observed [19,21]. It is believed that rapid change in the spectrum is a consequence of the biodegradation of the particles coating. As a result, the relaxation frequency of the magnetization vector is increased due to weakening of dipole interactions between the particles, which leads to the transformation of the spectral line shape from the sextet to doublet [29]. Another mechanism of the evolution of the hyperfine structure of the spectra of nanoparticles administrated in a living organism is associated with the direct biodegradation of the magnetic core leading to the appearance of paramagnetic iron forms such as iron-containing proteins [19,20]. This effect is expressed in the appearance of a central quadruple doublet in the spectra of iron-oxide particles collected at both room and liquid nitrogen temperatures. The intensity of the doublet increases over time and becomes significant in a few days after the introduction of particles into living organism.

In contrast to the intensive biodegradation of nanoparticles which can be observed in the mammalian organism, here we observed a completely different picture. A five-day culturing of both NP1 and NP2 particle samples introduced into 4 T1 cells did not lead to the measurable evolution of the spectrum. The absence of changes peculiar to biodegradation in the spectra collected at low temperature and room temperature attests to the absence of *in vitro* biodegradation of the synthesized nanoparticles or its highly slow rate. In other words, in samples with cells that were incubated with particles for 120 h, no fractions from biodegraded nanoparticles or newly formed forms of iron were found using Mössbauer spectroscopy. The standard transformation of the shape of the spectral lines resulting from Brownian motion of particles in viscous media is much more pronounced [31]. Consequently, there is no need to take into account the change in the spectra of synthesized nanoparticles arising from their interaction with cells for further Mössbauer *in vitro* studies.

Although both types of nanoparticles were used in the same concentration, the cell sample with NP2 particles showed a much lower probability of the effect in comparison with another sample, which is a consequence of a smaller ^{57}Fe content in the sample. This is due to the fact that Pluronic-coated particles have a larger hydrodynamic size and their cell uptake is difficult even in the presence of an external field. Either small NP1 or large NP2 nanoparticles meet the requirements we set for probes. This will allow us to study the cytoplasm viscoelasticity on a different scale using Mössbauer spectroscopy.

Despite the fact that the study of biological samples is almost always difficult, it has been demonstrated that by using the isotopically-enriched precursor it is possible to perform an adequate Mössbauer study of dried cell samples which contain about 10^{-5}–10^{-4} g of nanoparticles [23]. It is expected that Mössbauer probing of the cytoplasm *in vitro* is associated with a significant decrease in the probability of the nuclear resonance effect. For instance, such decrease was observed even on larger particles suspended in viscous solution simulating the cytoplasm [32]. Consequently, the ^{57}Fe-enrichment is essential.

4. Conclusion

We synthesized two types of ^{57}Fe-enriched magnetic nanoparticles. It was observed that even after a 5-days incubation of synthesized nanoparticles administrated into cells the shape of their spectral lines hardly changes. It suggests that the absence of *in vitro* biodegradation of the synthesized IONPs or its highly slow rate. Nevertheless, we were able to observe the intense intracellular oxidation of NP2 nanoparticles that had a phase composition close to bulk magnetite. During the 5-day incubation in the cell culture, a considerable part of the ferrous iron was converted to ferric phase.

It was demonstrated that the use of isotopically-enriched nanoparticles allowed to perform an adequate Mössbauer study of dried cell samples which contain a very small amount of nanoparticles. The

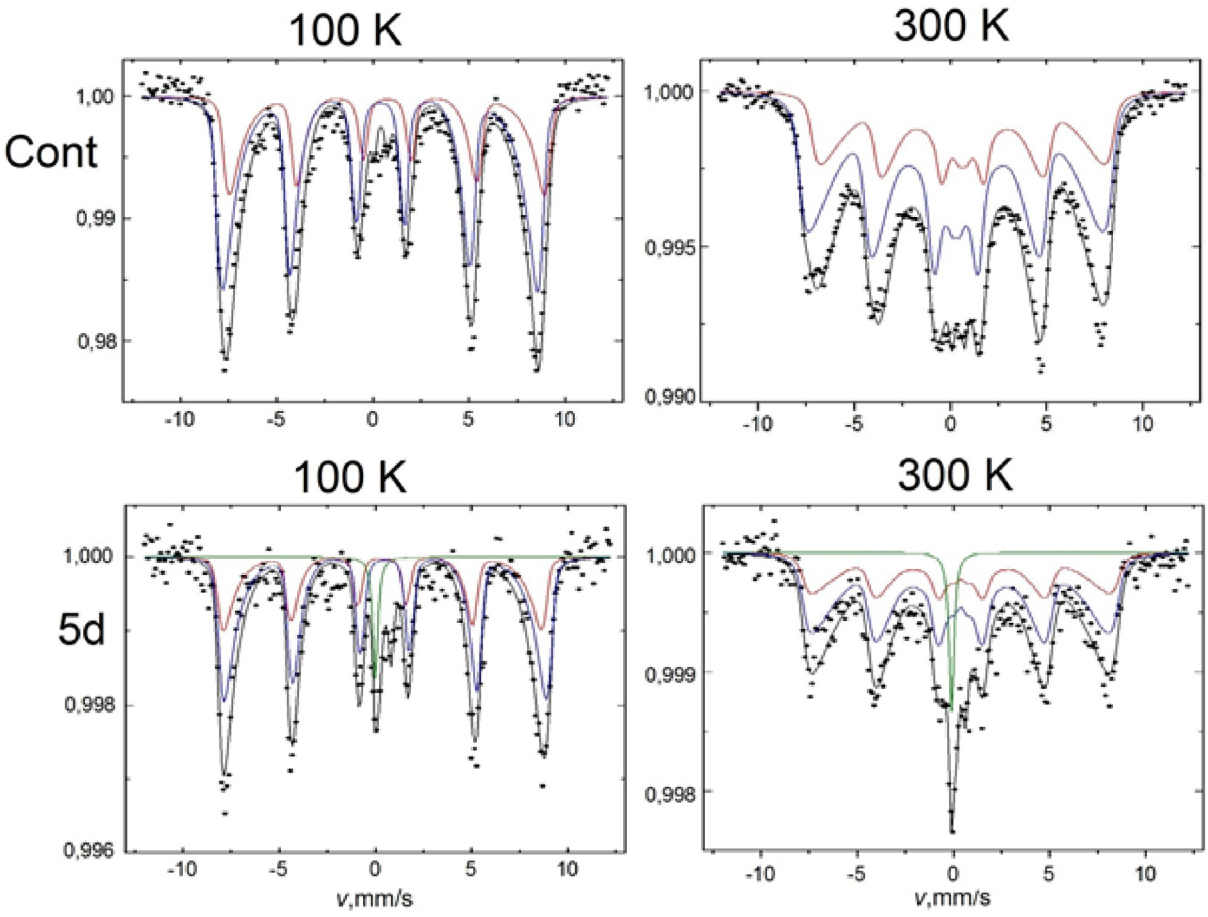

Fig. 3. ^{57}Fe Mössbauer spectra of dried 4 T1 cells cultured for 5 days with NP2 administrated (bottom) and their control sample (top) collected at temperatures 100 K and 300 K. Black lines: spectra of an ensemble of chaotically oriented particles, calculated with multilevel relaxation models [30]. Red and blue lines: the partial contributions from ferrimagnetic sublattices A and B. The green color represents a singlet of unidentified nature.

methods were used to synthesize the nanoparticles proved to be suitable for the fabrication of IONPs intended for Mössbauer *in vitro* studies.

We believe that the Mössbauer study of living organisms using ^{57}Fe-based IONPs as probes opens up new opportunities for biological science. However, this type of research is rather complicated in implementation and therefore has not yet been put into practice.

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