Journal of Magnetism and Magnetic Materials 427 (2017) 111–113

Contents lists available at ScienceDirect

Journal of Magnetism and Magnetic Materials

journal homepage: www.elsevier.com/locate/jmmm

# The magnetic introduction of magnetite nanoparticles into live cells for radiosensibility enhancement

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ARTICLE INFO

*Keywords:*
Magnetite nanoparticles
Radiotherapy enhancement
Magnetofection
Nanoparticle uptake
Endocytosis

ABSTRACT

Earlier we proposed a new radiotherapy enhancement method that entails the administration of $^{57}$Fe iron-oxide nanoparticles into the cells [5]. Within this work we were prompt to investigate the capability of iron oxide nanoparticles with monolayer coating to penetrate into live cells. Magnetite particle samples were synthesized and stabilized with HCl or citric acid. The cells were incubated in the presence of nanoparticles for 1 h, washed and dried. To distinguish inside-cell particles from outside ones a set of experiments with low temperature incubation was carried out. Several cell samples were prepared in the presence of an external magnetic field in order to study the possibility of the nanoparticle uptake enhancement. To evaluate the amount of particles in each cell sample we used a SQUID-magnetometer. The nanoparticle suspension with HCl stabilization turned to be inadequate for intracellular introduction. Approximately $2{\cdot}10^5$ particles with citric acid covering conjugated with each cell after incubation at normal conditions. An application of an external magnetic field increased this amount up to $10^7$ particles/cell. Most probably much of these particles penetrated into cells.

## 1. Introduction

The applications of iron oxide nanoparticles have been expanding to biological fields such as MRI contrast enhancement, drug delivery and hyperthermia [1]. Magnetite nanoparticles are relatively non-toxic, biocompatible [2,3] and exhibit magnetic properties. Moreover, the magnetic nanoparticles either solely or in combination with a drug are internalized into cells via endocytosis [4].

Earlier we proposed a new radiotherapy enhancement method that entails the administration of magnetic nanoparticles into the cells with its further irradiation [5]. The amplification of cell radiosensibility is based on the Mills' idea [6]. He suggested that the energy of an external monochromic 14.4 keV gamma-ray beam can be converted into secondary radiation in form of low-energy electrons by its scattering on $^{57}$Fe-isotope agents embedded into the cells. It is thought that low-energy electrons exhibit very high biological efficiency (in comparison with gamma radiation) [7]. According to the calculations [6], this approach could reduce the magnitude of the standard dose of a radiotherapy treatment on several orders. Our method implies the use of isotopically-enriched iron oxide nanoparticles as $^{57}$Fe carriers and its introduction into the malignant cells. However this method imposes some constraints on the particle coating. The fact is that electrons with energy less than 10 keV possess a very low free path length [8]. For this reason during irradiation of particles with massive polymer coating much of the secondary radiation energy can dissipate in it. In this paper the magnetite nanoparticles with two different types of stabilization were studied.

We used a magnetometer based technique for nanoparticle detection in the cell culture [9]. It was found that the presence of the magnetic nanoparticles in the cell culture after its incubation in nutrient solution with ferrofluid can be detected using a SQUID-magnetometer. We used this technique to estimate the capability of our nanoparticles to reach inside the cells. In order to study the possibility of the nanoparticle uptake enhancement, some cell incubations with magnetic particles were carried out in the presence of a magnetic field. This procedure was borrowed from the magnetofection technique [10]. Magnetic field attracts the particles toward the cells. This effect leads to increase of particle concentration close to culture surface so that more particles contact with the cells. According to the measurement results, we estimated the amount of particles in each cell sample. To distinguish the particles non-specifically attached to the cell surface from the inside-cell particles we carried out a set of experi-

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http://dx.doi.org/10.1016/j.jmmm.2016.11.025
Received 26 June 2016; Received in revised form 3 November 2016; Accepted 5 November 2016
Available online 09 November 2016
0304-8853/ 

ments with low-temperature incubation (low temperatures block endocytosis process [11]).

## 2. Materials and methods

### 2.1. Preparation and analysis of particles

We prepared two different samples of magnetite nanoparticles with a coprecipitation method. This method allows obtaining stable water suspension of nanoparticles. Furthermore, it will be convenient to synthesize particles from isotopically-enriched iron salts for further work.

The coprecipitations were performed according to the method described by Massart [12]:

$$FeCl_3+FeCl_2+8NH_4OH=Fe_3O_4+8NH_4Cl+4H_2O$$

Iron salts were mixed in a ratio of 20 mL of 1 M $FeCl_3$ solution to 5 mL of 2 M $FeCl_2$ solution. The resulting solution was poured into 250 mL of 0.7 M $NH_4OH$ and stirred vigorously for 30 min at room temperature (23 °C). Then obtained nanoparticles were precipitated in the presence of magnetic field, washed and stabilized with aqueous solution of hydrochloric acid. This sample was named NP1. NP1 particles are most stable at 3.6 pH. Experiments with these particles were performed to estimate whether it is possible to administrate uncoated magnetic nanoparticles into the cells using our technique. The uncoated particles could be useful for further radiobiological experiments as reference targets without any dissipation of secondary radiation in its covering. Another particle sample - NP2 - was prepared in the same way, but stabilized with citric acid. Particles with citric acid stabilization are most stable at pH=7 so NP2 is more suitable for cell experiments.

Obtained nanoparticles were investigated with Mössbauer spectroscopy and X-ray diffraction analysis in the same manner as in our previous paper [13]. The mean diameter of both types of particles equaled 10 nm.

### 2.2. Cell samples preparation

MTT assay was performed to estimate the toxicity of particles and to specify max particle concentration we could use in our experiments. HeLa cells were incubated with different particle concentrations in culture medium at 37 °C for 72 h. In this work some of our cell samples were incubated with particles in the presence of 3 kOe magnetic field. As the application of magnetic field could change particle-cell interaction we supplemented MTT assay with 1 h incubation on magnet. It was found that concentration up to 16 μg/cm$^2$ was permissible for HeLa cells with both types of particles.

For both types of particles we prepared four Petri dishes with HeLa cells. The cell culture was cultured with DMEM containing 10% FBS and 50 μg/mL gentamicin at 37 °C in atmosphere of 5% $CO_2$. HeLa cells were seeded at $3{\cdot}10^5$ cells at each 5 cm Petri dish for 48 h at 37 °C before incubation with nanoparticles. Sequentially, each dish was incubated with 16 μg/cm$^2$ of nanoparticles for 1 h under different conditions (Fig. 1). The cell samples were carefully washed from culture medium with PBS, harvested by EDTA treatment, collected into small diamagnetic plastic vials and dried.

### 2.3. Magnetometer measurements

The measurements of the DC magnetic moment were performed with MPMS XL7 Quantum Design SQUID magnetometer using reciprocating sample option (RSO). The samples were mounted in plastic containers and were put to the straw without any glue. The measurements of magnetic moment field dependences were performed at T=300 K in applied magnetic field up to 7 T. Three main magnetic

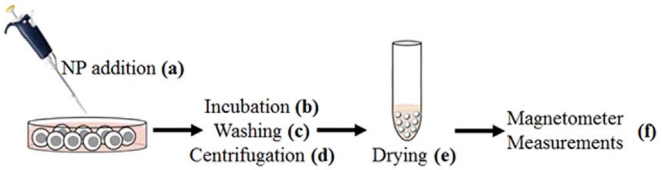


**Fig. 1.** Preparation of the cell samples. (a) Addition of the nanoparticles to HeLa cells. Each 5 cm Petri dish contained 3.5 mL of culture medium. Particles were added in form of a stable water suspension. The amount of particles was chosen on the basis of 16 μg of particles per cm$^2$ (320 μg/dish). According to MTT assay such concentration of both types of particles was proved to be permissible for the cell experiments. (b) Incubation for 1 h. For each type of particles four cell samples with different incubation conditions were prepared: (1) incubation at 37 °C (2) incubation at 37 °C in the presence of a magnetic field (3) incubation at 4 °C (4) incubation at 4 °C in the presence of a magnetic field. The field was applied perpendicular to the dish surface by using permanent magnet (3 kOe). (c) Thorough washing of cell cultures in PBS and counting the number of cells in each sample. (d) Centrifugation at 100*g* for 5 min. (e) Removal of the supernatant. The cell samples were suspended in 100 μL PBS and carried into special plastic vials for magnetometer measurements. The samples were dried at 37 °C for 72 h. (f) Fixation of the samples in plastic tubes. The application of the samples in a SQUID magnetometer.

contributions to the magnetic moment were observed: $M(H)=M_{FM}+\chi_D H+\chi_p H$. The linear in field diamagnetic $\chi_D H$ and paramagnetic $\chi_p H$ terms were subtracted to obtain the ferromagnetic moment $M_{FM}$. The saturated value of ferromagnetic moment $M_s$ was determined at H >1 T with absolute accuracy better than $10^{-5}$ emu. The influence of demagnetization field was neglected. The automatic background subtraction (ABS) procedure was used in the case if $M_{FM}$ was comparable with linear terms $\chi_D H+\chi_p H$.

## 3. Results and discussion

It was found that magnetometer could detect 0.1 μg of the magnetic sample with $M_s$=50 emu/g. For dried sample with $10^6$ cells less than 0,1 pg/cell of nanoparticles can be registered. At first we measured the magnetic background noise of the plastic vial and dried cell culture (approximately $10^6$ cells without magnetic nanoparticles). The contribution from the dried cell culture turned out to be negligible. The contribution from the plastic vial equaled approximately 6 μemu. To estimate the specific saturation magnetization $M_s$ of our nanoparticles we measured 100 μg of both dried particle samples. The following results were obtained for $M_s$: 64 emu/g for the NP1 particle sample and 51 emu/g for the NP2 particle one. These parameters are lower than that for the bulk $Fe_3O_4$ (90 emu/g) but they are in accordance with literature data on $M_s$ of magnetite nanoparticles [14]. The difference between specific $M_s$ of NP1 and NP2 is due to the presence of some solid nonmagnetic citric acid in dried NP2.

On the basis of $M_s$, cell quantity and mean particle size we estimated the specific amount of particles/cell in our samples.

The experiments at 4 °C were performed to reveal a mechanism of the interaction of our nanoparticles with cells. Lowering the temperature of culture medium to 4 °C blocks endocytosis process so cells can no longer uptake nanoparticles from extracellular medium. Therefore we believe that the difference in the amount of magnetite material in the cell samples with 37 °C and 4 °C incubation temperatures should be predominantly due to the cell uptake.

In the NP1 samples the incubation temperature did not affect the number of nanoparticles: we obtained 3.1 pg/cell after incubation at 37 °C and 3.0 pg/cell after incubation at 4 °C for the experiments with magnetic field. The situation is similar for the experiments without field: we obtained 1.4 pg/cell after incubation at 37 °C and 1.2 pg/cell after incubation at 4 °C. Apparently NP1 nanoparticles hardly penetrate into cells. When injected into the culture medium these particles become unstable due to pH change. The most probably the rapid formation of large aggregates of nanoparticles does not allow penetrating into the cells. The presence of the magnetic substances in these samples may be due to: non-specific bindings with the cell surface,

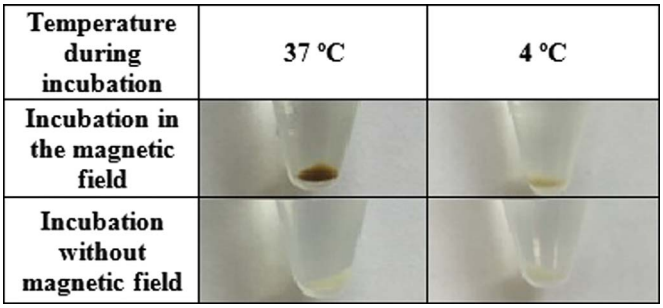


**Fig. 2.** Centrifuged cell samples after its 1 h incubation in culture medium with the NP2 nanoparticles. For samples rich in particles change in color is noticeable. The application of an external magnetic field has changed the picture of nanoparticle interaction with the cells: we observed more than 50-fold increase in the amount of the particles for 37 °C incubation and 13-fold increase for 4 °C incubation.

**Table 1**
Specific amount of nanoparticles in the cell samples depending on its coating and conditions during incubation. Number of cells in each cell sample was counted after its washing. The results for different samples vary from 27 pg/cell to 0.2 pg/cell. Four series of experiments for each sample were performed. Data are presented as the mean value ± standard deviation.

| Sample | Presence of the external magnetic field during inc. | Temperature during incubation (°C) | Particles per cell (pg/cell) |
|---|---|---|---|
| NP1 | With field | 37 | 3.1 ± 0.6 |
| | Without field | 37 | 1.4 ± 0.4 |
| | With field | 4 | 3.0 ± 0.5 |
| | Without field | 4 | 1.2 ± 0.4 |
| NP2 | With field | 37 | 27.0 ± 3.0 |
| | Without field | 37 | 0.5 ± 0.1 |
| | With field | 4 | 2.7 ± 0.4 |
| | Without field | 4 | 0.2 ± 0.1 |

large aggregates of particles which can remain after rinsing or diffusion [15]. The samples incubated in the presence of magnetic field contained more particles. This is due to local increase of particle concentration close to cells so the process of the particles aggregation accelerates as well as the particles conjugation to cell surfaces.

A different situation is observed with the NP2 particles stabilized with citric acid. The experiments with the NP2 samples showed a significant difference in the specific amount of particles in the cell samples after its incubation in the presence of a magnetic field at various temperatures: 27 pg/cell for 37 °C and 2.7 pg/cell for 4 °C. In the experiments without magnetic field lowering the incubation temperature led to 2.5-fold reduction in the specific amount of the particles (Fig. 2).

The application of a magnetic field during incubation with NP1 particles resulted in an increase in the number of particles in the sample of about 2 times. The situation is similar for both 37 °C and 4 °C experiments. It is our belief that the magnetic field accelerates the aggregation process of NP1 particles in culture medium. The NP1-particles cell uptake is virtually absent.

The application of a magnetic field during the incubation with the NP2 particles (stabilized with citric acid) led to much more significant effect (Table 1). In the samples with 37 °C incubation temperature we obtained 54-fold increase of specific amount of particles. In the samples with 4 °C incubation temperature the effect is somewhat weaker: the amount of particles increased by an order.

In this work we relied on our development of the radiotherapy enhancement method based on introduction of $^{57}$Fe iron oxide nanoparticles into malignant cells. For this purpose we synthesized the magnetite nanoparticles with monolayer coating and investigated its interaction with HeLa cell culture under the different conditions. We also investigated uncoated particles (stabilized with HCl) in the hope of its cell penetration. The experimental data revealed that the NP1 particles are not suitable for intracellular introduction. These particles hardly penetrate into cells irrespective of the presence of an external magnetic field. The results of experiments with more stable particles are more promising. The application of an external magnetic field increased the amount of particles in the cell samples by almost 2 orders of magnitude. After incubation at 4 °C, the amount of particles in the sample is significantly decreased in comparison with normal conditions. This result gives the right to assume that a considerable amount of particles introduced into the cells during incubation due to endocytosis. We suppose that this effect cannot be explained only by temperature effects of particle-particle interaction: there is no difference between samples with 37 °C and 4 °C incubation temperatures for unstable NP1 particles.

## 4. Conclusions

Particles synthesized with coprecipitation and stabilized with citric acid turned out to be suitable for our further experiments in development of radiotherapy enhancement. These particles can internalize into cells at relatively high concentrations without any vectors. Magnetite particles without any coating are unsuitable for introduction into live cells. An application of an external magnetic field was found to be extremely effective for increasing the amount of particles absorbed by cells.

## Acknowledgements

The work was supported in part by the Russian Science Foundation under Grant 14-15-01096. The magnetic measurements were done at the resource center of electrophysical methods, NRCKI.

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