Invitro toxicity test and searching the possibility of cancer cell line extermination by magnetic heating with using Fe₃O₄ magnetic fluid

Pham Hoai Linh¹, Nguyen Chi Thuan¹, Nguyen Anh Tuan¹, Pham Van Thach¹, Tran Cong Yen², Nguyen Thi Quy², Hoang Thi My Nhung², Phi Thi Xuyen², Nguyen Xuan Phuc¹ and Le Van Hong¹

¹Institute of Materials Science, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay Dist., Hanoi, Vietnam
²Biology Faculty, Hanoi University of Science, 334 Nguyen Trai Road, Thanh Xuan, Hanoi, Vietnam

E-mail: honglv@ims.vast.ac.vn

Abstract. A Fe₃O₄ based magnetic fluid with different concentrations ranged between 0.15 ng/cell to 10 ng/cell (nano gram/cell) was used in the invitro toxicity test on several cancer cell lines, Sarcoma 180, HeLa and H358. It shows that the fluid with a concentration of Fe₃O₄ below 1.2 ng/cell is completely non-toxic for these cell lines. Even through in the presence of the highest concentration of 10 ng/cell, the cell viability still reaches more than 60%. The magnetic fluid with Fe₃O₄ concentration of about 0.1 ng/cell was also used to search ex-vivo the possibility of Sarcoma 180 extermination by magnetic heating with an AC field of 1200e and 184 KHz. The result shows that after a heat treatment for 30 min., 40% of Sarcoma 180 cells was killed.

Keywords: Magnetic fluid, hyperthermia, cancer treatment.

1. Introduction
The use of magnetic nanoparticles in cell biology was proposed in the early 1990s in the separation of cells and molecules such as peptides, proteins, and DNA. Magnetic nanoparticles first were used in diagnostics of tumors by using magnetic resonance tomography, in targeting of magnetically–labeled drugs by an external magnetic field [1, 2]. As known, cancer is one of the most dangerous sickness which killed thousands men per year. Humankind has devoted a big endeavor for checking this disaster by using various methods treatment such as ethanol injection [3], radiofrequency ablation [4], laser light and ultrasound waves [5, 6]. In recent years, magnetic nanoparticle hyperthermia has become a potential solution for the treatment of cancers [7]. In this solution tumor cells are selectively killed under heating at the temperature around 45-46°C by exposing the magnetic nanoparticles in an alternating magnetic field. Basically, when exposing to an alternating magnetic field, the nanoparticles can absorb energy via various physical mechanisms including the magnetic hysteresis loss, Néel relaxation and Brownian loss [8]. This energy is considerably high to make the particles as the local nano-fires for the application of magnetic hyperthermia. Till now the most appropriated material using for magnetic hyperthermia treatment is ferromagnetic nanoparticle, especially Fe₃O₄ magnetite.
nanoparticle because of its high specific loss power (SLP) and well proven biocompatibility with life cells [9, 10]. The first study on the magnetic heating induced by an alternating magnetic field was performed by Gilchrist et al. [11], and its result was later reconfirmed by several groups [12, 13]. The recent investigations showed that the capacity in magnetic hyperthermia of liquid magnetic nanoparticles essentially depends on specific loss power (SLP) of the iron oxide nanoparticles, consequently depends on their structural properties and magnetic behaviors [14]. With the aim of improving SLP of Fe₃O₄ nanoparticle several methods were established and their results showed that the SLP of magnetic Fe₃O₄ nanoparticles has much been improved [15, 16]. The highest value of SLP of 960 W/g at the field of 410 kHz and 10 kA/m was found for magnetosomes produced by magnetotactic bacteria [17]. By now several investigations in magnetic hyperthermia using Fe₃O₄ magnetic nanoparticles were attempted. Hilger et al [18], for example, carried out a hyperthermia treatment for breast cancer using a magnetic fluid. In this paper we report recent new results of our study on the toxicity of Fe₃O₄ magnetic nanoparticles tested on several cancer cell lines and also on an ex-vivo test of cancer cell extermination by using magnetic heating. Three following cell lines were used in our study: (1) Hela cells, an epithelial cell line originally derived from human cervix; (2) a human bronchioalveolar carcinoma H358 cells; (3) mouse ascites sarcoma cells named Sarcoma 180. These cell lines were obtained from American Type Culture Collection (ATCC). Hela and Sarcoma 180 cells were maintained in DMEM (Dulbecco’s modified Eagle’s) 1g/l of Glucose (Invitrogen). H358 cells were maintained in RPMI (Roswell Park Memorial Institute) (Gibco). These medium were supplemented with 10% foetal bovine serum (Invitrogen), penicillin-streptomycine 100 IU/ml (Invitrogen), L – Glutamine 2mM (Invitrogen). Cells were grown in a humidified chamber in the presence of 5% CO₂, at 37°C.

2. Experimental

2.1. Synthesis of Fe₃O₄ nanoparticles
Fe₃O₄ nanoparticles were prepared by the chemical co-precipitation method. Aqueous solution of FeCl₃, FeCl₂ salts in their respective stoichiometry of Fe²⁺ /Fe³⁺ = 1/2 was mixed and kept at 40°C. NaOH was added to the solution under vigorous mechanical stirring for 30 minutes. The precipitation and formation of Fe₃O₄ nanoparticles were taken by a conversion of the metal salts into hydroxides and, in turn, immediately transformed into ferrites. During the reactions, magnetic-stirring speed was kept at 1000 round/min.

2.2. Coating particles
Starch was used for coating the Fe₃O₄ nanoparticles. The coating process was carried out as follows: Starch was dissolved in water with a concentration of 0.1 g/ml at 80°C. After the good dissolving of starch, the solution was immediately placed in a 60°C water bath and dropped into the obtained aqueous of dispersed Fe₃O₄ particles after a co-precipitation reaction at 80°C in 2 h. Around 50 wt.% of water was evaporated during the boiling. The remaining suspension was then cooled to 0°C within 12 h. The formed gels were washed with deionized water until the pH is reduced to less than 8.5. Excess salts and ions were then removed by washing. For cleaving glycoside bond and shortening the polymer chain, starch-coated iron oxide particles were oxidized by H₂O₂ solution

2.3. Characterization
The structure and the morphological characterization of magnetic nanoparticles were taken by using an X ray diffractometer D5000 of SIEMEN and a field-emission scanning electron microscope (FESEM S-4800 of the Hitachi Company). The magnetic properties were checked by using a home made vibrating sample magnetometer (VSM) and a Quantum Design PPMS 6000.

The magnetic heating experiments were performed under an alternating magnetic field with the frequency changed in a wide range from 50 kHz to 400 kHz and its field strength can increase up to maximal value of 12 kA/m. The magnetic field is generated by a 5.5-loop induction coil with a 4.5 cm
diameter and a 6.3 cm length. During the heating experiment, the temperature of the sample was monitored by using a Copper-Constantan thermocouple. The magnetic heating experimental setup is shown in figure 1.

![Copper-Constantan thermocouple](image.png)

**Figure 1.** The experimental set up for SLP values measurements.

All the samples used in magnetic heating experiment contain 50 million cells of Sarcoma 180 line and 3 mg/ml of Fe₃O₄ nanoparticles. It means the concentration of Fe₃O₄ nanoparticles in all the samples is about 0.3 ng/cell.

2.4. Cytotoxicity Assay
Sulforhodamine-B (SRB) assay is performed to assess cell survival. SRB is a water-soluble dye that binds to the basic amino acids of the cellular proteins. Thus, colorimetric measurement of the bound dye provides an estimate of the total protein mass that is related to the cell number. Cells were seeded in 96-well plates with the density of 1x10⁴ cells/well. After 24 h, the culture medium was replaced by the new medium plus different concentrations of magnetic particles. 72 h after the incubation, cultures fixed with 10% trichloroacetic acid were stained for 30 minutes at 4°C with 0.4% (wt/vol) sulforhodamine B (SRB) dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and protein-bound dye was extracted with 10 mM unbuffered Tris base [tris(hydroxymethyl) aminomethane] for determination of optical density in a computer-interfaced, 96-well microtiter plate reader (SpectraMax 250). The absorbance was measured at 540 nm [19, 20]

2.5. Determination of cells viability by trypan blue
Place a suitable volume of a cell suspension (20-200 μl) in appropriate tube add an equal volume of 0.4% trypan blue and gently mix, let stand for 5 minutes at room temperature. Place 10-20 μl of stained cells in a hemacytometer and count the number of viable (unstained) and dead (stained) cells. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells [21, 22].

2.6. Determination of cell death rate after the hyperthermia Sarcoma 180 cells ex vivo
Sarcoma 180 cells were collected from Swiss mice bearing 10 days of Sarcoma 180 ascite. Cells then were washed three times with PBS 1X and centrifuged to a pellet. Magnetic nanoparticles were added on the cells. The mixes of cells and nanoparticles were subjected to the hyperthermia treatment either with different times of heating or different concentrations of particles. Cells viability was determined by trypan blue [23, 24].

3. Result and discussion
Our research is an interdisciplinary science mission in physics, biology and medicine, therefore the research was concentrated on both the preparation of magnetic liquid containing Fe₃O₄ nanoparticles as well as the test research on its biocompatibility and the possibility in cancer cell lines treatment by
using magnetic heating. Preparation of the magnetic liquid containing Fe₃O₄ nanoparticles has been presented in details elsewhere in other our papers. Here in this paragraph we would like to show its magnetic characterization, especially its response to an alternating magnetic field, possibility of controlling the heating temperature. So we can see in figure 2 which presents the heating curve by using the liquid with various concentrations of Fe₃O₄ nanoparticles exposed in an alternating magnetic field with a frequency of 184 kHz, and an intensity of 12 kA/m. As seen in figure 3 the initial heating rate and the saturation temperature of heating process can be controlled by changing concentration of Fe₃O₄ nanoparticles in the liquid.

![Figure 2. The heating curves of the magnetic fluid with various particles concentration.](image1)

![Figure 3. The dependence of the initial heating rate and saturation temperature on particles concentration.](image2)

The most decisive parameter in verifying the application possibility of the magnetic liquid in cancer cell treatment by using magnetic heating is a specific loss power (SLP), which was defined as follows [8]:

\[
SLP = \frac{(C * m_s * \frac{dT}{dt})}{m_i}
\]

where \(m_s\) is the total mass of magnetic liquid, \(\frac{dT}{dt}\) is the initial heating rate, which was defined at the moment of about some seconds after starting the heat, \(C\) is the specific heat capacity of magnetic liquid, which was accepted equal to that of water, because of the mass of Fe₃O₄ nanoparticles containing in the liquid \(m_i\) is much smaller than the total mass \(m_s\). The estimated SLP values are presented in table 1.

| Concentration of particles (mg/ml) | \(\frac{dT}{dt}\) (°C/s) | Saturation temperature (°C) | SLP values (W/g) |
|-----------------------------------|-----------------|-----------------|-----------------|
| 3                                 | 0.092           | 45              | 129             |
| 6                                 | 0.141           | 53              | 97              |
| 7                                 | 0.159           | 62              | 94              |
| 12                                | 0.240           | 73              | 83              |
| 15                                | 0.310           | 80              | 86              |

As mentioned in the Introduction, several research groups devoted their endeavour for looking for the magnetic liquid having high SLP. R. Muller et al [15] using a glass crystallization method received a mix of iron oxides with a SLP of 100 W/g, Q. Zeng et al. [16] carried out manufacture the monodispersed Fe/Fe₃O₄ composite nanoparticles with an improved SLP of 345 W/g, and recently R.
Hergt et al received very high SLP of 960 W/g by using bacterial magnetosomes [17]. According to Hergt [25] the ferrofluids with a SLP of 50 W/g are suitable in application for cancer tumors not smaller than about 4 mm diameter. Conclusively the ferrofluid containing Fe₃O₄ nanoparticles manufactured by us could be applicable in hyperthermia applications for cancer cell treatment.

According to the law of health care we have first to take researches on toxicity test and biocompatibility of the ferrofluid containing Fe₃O₄ nanoparticles. Two cell lines, Sarcoma 180 and H358 were taken in the toxicity test with a ferrofluid containing Fe₃O₄ nanoparticles coated with starch. Using the trypan blue method for Sarcoma 180 and SRB method for cell line H358 the proliferation rate of the cell lines was estimated and presented in table 2.

| Cell lines     | Concentration of Fe₃O₄ nanoparticles (ng/cell) |
|---------------|---------------------------------------------|
|               | 0.1            | 0.5            | 1.0            | 5.0            | 10.0           |
| Sarcoma 180   | 95.5 ± 2       | 94.5 ± 5       | 96.3 ± 6       | 92.0 ± 3       | 89.5 ± 5       |
| H358          | 84.5 ± 5       | 82.5 ± 7       | 85.0 ± 4       | Not determined | 78.2 ± 4       |

As was shown in table 2, the proliferation of two cell lines slightly decreases after 72 h of growth for the test concentration of Fe₃O₄ nanoparticles above 1.0 ng/cell. Fe₃O₄ nanoparticle not equally respects to the cell lines, Sarcoma 180 line has higher proliferation compared with that of the H358 cell line. Probably Fe₃O₄ nanoparticles are more toxic for the adherent cell lines compared to the suspension line. This suggestion has been proven by other experiment which allows us to evaluate the adherent possibility of Fe₃O₄ nanoparticles onto surface of the cancer cells. Observing microscopic images (figure 4) we confirmed that the lower proliferation of the adherent cell line, Hela, is caused because the Fe₃O₄ nanoparticles more strongly adhere to surface of the cells in comparison with the suspension cell line, Sarcoma 180.

Figure 4. Microscopic images exhibiting the adherence of Fe₃O₄ nanoparticles onto Hela cell membranes (a) and with enlargement (b).

Figure 4 presents microscopic images, exhibiting the adherence of Fe₃O₄ nanoparticles onto the cell membrane of Hela cancer cells. It shows that Fe₃O₄ nanoparticles have tendency to adhere at the surface point where connected to the matrix material, therefore prevents proliferation of the Hela cells.

As shown in table 2, the experimental test with concentration below 1 ng/cell was given higher proliferation for both two cancer cell lines in comparison to other concentrations and actually the proliferation of Sarcoma 180 cell with the Fe₃O₄ nanoparticle concentration below 1 ng/cell was estimated of about 96%.
Next step in searching the application possibility of Fe$_3$O$_4$ ferrofluid in biomedicine was an attempt of establishing experiments on cancer treatment by magnetic heating with Fe$_3$O$_4$ ferrofluid in an alternating magnetic field. According to the above results we have chosen ferrofluids having Fe$_3$O$_4$ concentration below 1 ng/cell for the ex-vivo experiment in hyperthermia. Actually, we set up such an experiment: to observe the effects of the heating time on extermination of Sarcoma 180 cancer cell which was heated with Fe$_3$O$_4$ ferrofluid by its exposing to an alternating magnetic field of 12 kA/m in intensity and 184 kHz in frequency. Note here that the intensity of alternating magnetic field, its frequency and Fe$_3$O$_4$ concentration in the ferrofluid were chosen how to receive a saturation temperature of heating of about 47°C ± 1°C. As was known at this temperature the cancer cells probably were killed after keeping for a time such that, conversely the life cells were still saving. Figure 5 presents the number of killed Sarcoma 180 cells in dependence on time of heating at 47°C by an alternating magnetic field with an intensity of 12 kA/m and a frequency of 184 kHz. It shows that the Sarcoma 180 cancer cells were successfully killed after being heated for not shorter than 100 minutes. Evidently, after stopping of the heating Sarcoma 180 cells were continuously themselves killed. This domino effect in the extermination of cancer cells keeps a time that its length depends on the heating time. For example we present in figure 6 the ratio of killed Sarcoma 180 cells in dependence of time after stopping the heating process.

**Figure 5.** The ratio of the killed cells of Sarcoma 180 line versus time of heating by exposing of Fe$_3$O$_4$ magnetic liquid in an alternating magnetic field with an intensity of 12 kA/m and a frequency of 184 kHz.

**Figure 6.** Ratio of the killed cells of Sarcoma 180 line in dependence of time after stopping heating.

For illustrating the progress of the experiment on the extermination of the cancer cells we present microscopic images as shown in figure 7 and 8. Two control samples have almost the same ratio of killed cells of Sarcoma 180 line. It confirmed that the Sarcoma 180 cells survived at least not shorter than 72 hours. In addition it also confirmed that the alternating magnetic field is not harmful for the cancer cell line as well as for the life cells. So it is inferred that the magnetic heating is acceptable method used in vivo cancer treatment for human body. Figure 9 presents two microscopic image of the sample containing 50 millions of Sarcoma 180 cells and Fe$_3$O$_4$ nanoparticles after 75 minutes heating by an alternating magnetic field with intensity of 12 kA/m and frequency of 184 kHz. It shows that a large number of cancer cells were killed, shown in the blue colour, resulting from using the trypan blue method in determination of cell viability. The extermination of Sarcoma 180 cells continuously occurred after stopping the heating process. As can be seen in figure 10, number of the killed cells increased very clearly. Among the cells only a few still were alive. This process extended for very long time. As shown in figure 6 the needful time for the completely killed all the Sarcoma 180 cells extended to 500 minutes.
4. Conclusion
Magnetic fluid containing Fe$_3$O$_4$ nanoparticles with different concentrations ranged between 0.15 ng/cell to 10 ng/cell was successfully prepared and used in the in vitro toxicity test on several cancer cell lines, Sarcoma 180, Hela and H358. The fluid with a concentration of Fe$_3$O$_4$ below 1.0ng/cell is completely non-toxic for these cell lines. The magnetic fluid with Fe$_3$O$_4$ concentration of about 0.3ng/cell was used in ex vivo searching the possibility of Sarcoma 180 extermination by magnetic heating with an AC field of 120 Oe and 219 KHz. The result shows that the heat treatment around 47°C by exposing Fe$_3$O$_4$ magnetic liquid in an AC magnetic field is successfully exterminated Sarcoma 180 cells. Probably, after heat treatment in a duration time of 100 minutes almost 100% Sarcoma 180 cells was completely killed. This result opens a large prospect in researching the hyperthermia applications by using magnetic liquid containing Fe$_3$O$_4$ nanoparticles.
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