Original Article

Preparation and Cytotoxic Evaluation of Magnetite (Fe_3O_4) Nanoparticles on Breast Cancer Cells and its Combinatory Effects with Doxorubicin used in Hyperthermia

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Abstract

Background: Magnetic nanoparticles in a variable magnetic field are able to produce heat. This heat (42-45 *°C*) has more selective effect on fast dividing cancer cells than normal tissues.

Methods: In this work magnetite nanoparticles have been prepared via co-precipitation and phase identification was performed by powder x-ray diffraction (XRD). Magnetic parameters of the prepared nanoparticles were measured by a Vibrating Sample Magnetometer (VSM). A sensitive thermometer has been used to measure the increase of temperature in the presence of an alternating magnetic field. To evaluate the cytotoxicity of nanoparticles, the suspended magnetite nanoparticles in liquid paraffin, doxorubicin and a mixture of both were added to the MDA-MB-468 cells in separate 15 *ml* tubes and left either in the RT or in the magnetic field for 30 *min*. Cell survival was measured by trypan blue exclusion assay and flow cytometer. Particle size distribution of the nanoparticles was homogeneous with a mean particles size of 10 *nm*. A 15 *°C* temperature increase was achieved in presence of an AC magnetic field after 15 min irradiation.

Results: Biological results showed that magnetite nanoparticles alone were not cytotoxic at RT, while in the alternative magnetic filed more than 50% of cells were dead. Doxorubicin alone was not cytotoxic during 30 *min*, but in combination with magnetite more than 80% of the cells were killed.

Conclusion: It could be concluded that doxorubicin and magnetite nanoparticles in an AC magnetic field had combinatory effects against cells.

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Introduction

Soft magnetic oxides, MFe_2O_4, where M is a divalent cation, have a spinel structure named spinel ferrites and in the bulk form have many applications in telecommunication and electronics [1]. Nanoparticles of these magnetic oxides have different characteristics in comparison with the bulk ones [2]. The use of magnetic nanoparticles to induce hyperthermia in biological tissues is an important factor for tumor therapy [3,4]. Hyperthermia is a thera-

peutic procedure, which is used to raise the temperature of a region of the body affected by cancer to 42-46°*C* locally. This method involves the introduction of magnetic nanoparticles into tissues, and their subsequent irradiation with an alternating electromagnetic field. Hyperthermia is a promising approach in cancer therapy. The challenge in this method is to restrict local heating of the tumor surrounding [5].

This goal can be partially accomplished by the physical phenomenon of losses when magnetic nanoparticles are injected within the cancer tissue and then heated in an alternating electromagnetic field [6]. Magnetic losses to be utilized for heating arise due to different processes of magnetization reversal in systems of magnetic nanoparticles which depend strongly on structural magnetic particle properties like mean size, width of size distribution, particle shape and crystallinity [4]. This means that by controlling the particle size of a magnetic nanoparticle, the heat generation can be adjusted under an oscillating magnetic field [4]. Hyperthermia has been used in combination with other forms of cancer therapies such as radiation therapy and chemotherapy [7].

On the other hand, hyperthermia and liposomal drug delivery are treatment modalities that have been used to treat cancer over the last two decades [8]. Doxorubicin as a broad spectrum antineoplastic antibiotic has been used for a variety of solid and hematological malignant tissues for many years. However, its usefulness is limited, by its dose dependent cardiotoxicity. Many combination therapies have been introduced to reduce the cardiotoxicity of doxorubicin by reduction of its effective dose. With this in mind, the purpose of this work was to evaluate whether the prepared Fe_3O_4 nanoparticles had a cytotoxic effect on a human breast cancer cell individually or in combination with doxorubicin via hyperthermia.

Materials and Methods

Chemistry

Preparation of magnetite nanoparticles: Magnetite nanoparticles were prepared by co-precipitation method as previously mentioned [9]. Briefly, a 50 *ml* solution containing 0.5 *molar* ferric iron chlorides (Merck Co.), and a 50 *ml* solution containing 0.25 *molar* ferrous iron chlorides (Merck Co.) were prepared. The solutions were then mixed and added to a 6 *molar* NaOH (Merck Co.) solution (pH of this medium was about 14) at room temperature. After stirring at the same temperature for 10 *min*, the obtained precipitate was washed off several times by distilled water while a pH of 7 was obtained. After each washing an ultrasonic bath was used to extract the ions through the precipitates. To dry the washed precipitate, a hot plate magnetic stirrer was used. This procedure was done at 70°*C* for 2 *hr*, so that a black powder was obtained.

The crystal structure of the powders was characterized by an X-ray diffractometer (Bruker, Advanced D8 model), using CuKα radiation (λ=1.5406 *Å*). Mean crystallite size of the nanoparticle was calculated by Scherrer's formula after applying the necessary corrections. Particle morphology of the sample has been investigated by a Transmission Electron Microscope (TEM), Philips CM12. Magnetization curves of the nanoparticles were obtained by a Vibrating Sample Magnetometer (VSM). To measure temperature increase, a mixture of one *mg* of the magnetite nanoparticles and 100 *ml* distilled water was prepared. Ten *ml* of the mixture was then placed at the center of a 2 turns RF coil and an AC current (f=400 *kHz*) was applied with an rms value of 400 *A/m*. A sensitive thermometer was used to measure the temperature increase.

Preparation of magnetite nanoparticles suspension: Magnetite nanoparticles (average particle size 10 *nm*) were dispersed in edible liquid paraffin according to previously published methods [9]. Its solid concentration was 40% with final density of 1.13 g/cm^3. This dark brown colored suspension had a viscosity of 130 *Pa.s* and shear stress yield of 20 *Pa* at room temperature. Prepared Fe_3O_4 ferro-

fluid showed a very good stability up to 3 years.

Preparation of doxorubicin solution: Doxorubicin hydrochloride (Farmitalia, Italy) was dissolved in distilled water so that the final concentration was 20 *μg/ml*. This solution was used either as positive control or in combination with nanoparticles.

Biological

Cell culture and cell seeding: Human breast cancer cell line (MDA-MB-468) were cultured as monolayer in RPMI 1640 medium [Gibco, UK; each 500 *ml* of RPMI-1640 supplemented with 10% of Fetal Calf Serum (FCS, Gibco, UK), 5 *ml* of penicillin/ streptomycin (Sigma, USA; 50 *IU/ml* and 500 *μg/ml*, respectively), 5 *ml* of sodium pyruvate (1 *mM*), $NaHCO_3$ (1 *g*) and 5 *ml* of l-glutamine (2 *mM*)]. Completed media was sterilized by 0.22 *μ* microbiological filters after preparation and kept at 4℃ before using. Cells were kept in an incubator at 37℃, 5% CO_2 air humidified up to 15 subcultures. Cells were detached, using 0.25% trypsin and seeded (5×10^4 *cell/m1*) in 15 *ml* tubes for biological evaluation.

Samples preparations

Blank: Two *ml* of cell suspension (5×10^4 *cell/ml*) in a 10 *ml* test tube was used as blank. An AC magnetic field (f=450 *kHz* and H=100 *A/m*) was applied on blank for 30 *min*. Then 20 *μl* of it was mixed with 20 *μl* of trypan blue (0.2% *w/v*) and the viability of cells was measured using a hemocytometer.

Negative control

Four *ml* of cell suspension (5×10^4 *cell/ml*) and 0.5 *ml* of edible paraffin oil (nanoparticle carrier) were mixed and divided into 2 equal portions in a 10 *ml* test tube. One test tube was left at room temperature and the second one was left in the magnetic field for 30 *min*. The magnetic field condition and the percent of viability were measured as mentioned for the blank treatment.

Positive control

Four *ml* of cell suspension (5×10^4 *cell/ml*) and 0.5 *ml* of doxorubicin (Farmitalia, Italy) solution (20 *μg/ml*) were mixed and the experiments were carried out as mentioned for the negative control.

Sample 1: Four *ml* of cell suspension (5×10^4 *cell/ml*) and 0.5 *ml* of magnetite nanoparticles suspension were mixed and the experiments were repeated the same as for the negative control. To increase the contact of cells with nanoparticles test tubes were left on shaker either at room temperature or in the magnetic field.

Sample 2: Four *ml* of cell suspension (5×10^4 *cell/ml*) and 0.25 *ml* of magnetite nanoparticles suspension plus 0.25 *ml* of doxorubicin were mixed and the experiments were repeated the same as mentioned for the sample 1.

Flow cytometry

Five *μl* of propidium iodide dye (PI; 1 *mg/ml* in deionized water, Sigma) was added to 3 *ml* of all treated cell suspension (*i.e.* blank, negative control, positive control, samples 1 and 2) and the cell viability was evaluated. Each sample was analyzed in a PAS/Dako flow cytometer (Partec, Denmark) with the use of acquisition/analysis program Flo Max 2.4 (Partec). Cells were plotted according to forward scatter and side scatter profiles (a measure of size and granularity of an event, respectively) and gated to include cells only. Cells were located using these parameters and a live gate analysis was set around this population. Data were acquired from 10,000 cells (events) and the cells which showed high fluorescent in channel FL2 were regarded as dead (stained with PI).

Results

Chemistry results

Figure 1 shows XRD pattern of the co-precipitated magnetite nanoparticles. As can be seen all main peaks are related to a single-phase spinel structure. A mean crystallite size of about 5 *nm* has been obtained for the nanoparticles using Scherrer's Equation.

Figure 2 shows TEM photograph of the single-phase nanopowders and as can be seen there is a uniform distribution of particles

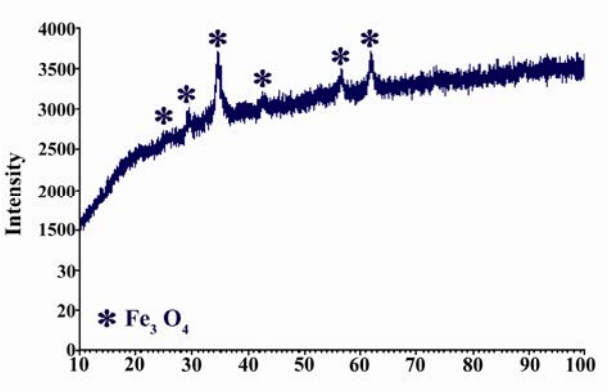

Figure 1. XRD pattern of the co-precipitated magnetite nanoparticles

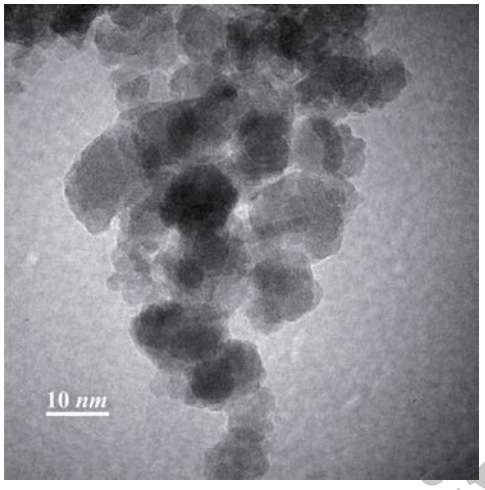

Figure 2. TEM photograph of the single phase magnetite nanopaticles

with particle sizes between 3 and 10 *nm*, which is in agreement with the Scherrer's result.

Figure 3 shows room temperature hysteresis loop of the co-precipitated magnetite nanoparticles. As seen in figure 3 the saturation magnetization of the nanoparticles is 32 *emu/g*, which is less than the saturation magnetization of bulk magnetite (92 *emu/g*) [10]. This difference can be explained by core-shell model that explained elsewhere [11]. In this model, it is supposed that each particle consists of a core with ferrimagnetic order and a constant thickness nonmagnetic shell with spin glass phase. It is obvious that by decreasing the particle size the surface-to-volume ratio of a particle will increase, which leads to a reduction in magnetization of the particles [8]. Temperature increase measurement due to a mixture of the nanoparticles and distilled water (10 *mg/l*) in presence of an AC magnetic field (f=400 *kHz* and H=400 *A/m*) show that a 15°*C* temperature increase is achievable after 30 *min* radiation. As the magnetic nanoparticles have super paramagnetic behavior, this can be due to relaxation losses [6].

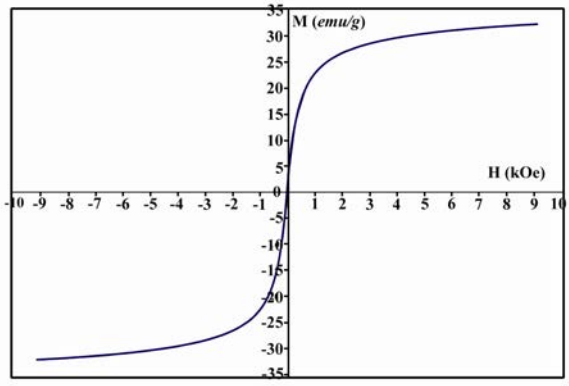

Figure 3. Room temperature hysteresis loop of the co-precipitated magnetite nanopaticles

Biological results

Trypan blue exclusion assay: Trypan blue exclusion results are summarized in table 1.

Table 1. The effect of magnetite nanoparticles suspended in liquid paraffin alone or in combination with doxorubicin incubated 30 *min* either in the lab (room temperature) or in the presence of an AC magnetic field (f=400 *kHz* and H=100 *A/m*). MDA-MB-468 breast cancer cells were used in all the experiments at a concentration of 50000 *cell/ml*. Cells were counted by hemacytometer applying trypan blue exclusion assay

Sample	Contents	Mean no. of *cell/ml* after 30 *min* incubation		p-value
		Magnetic field	No fields applied	
Blank	Cell suspension (4 *ml*)	47000 ± 1000	48000 ± 800	0.3
(-) Control	Cell suspension (4 *ml*) + paraffin (0.5 *ml*)	46000 ± 1000	47000 ± 700	0.1
(+) Control	Cell suspension (4 *ml*) + Doxo. 0.02 *mg/ml* (0.5 *ml*)	25000 ± 900	45000 ± 700	0.05
Sample 1	Cell suspension (4 *ml*) + magnetite in paraffin (0.5 *ml*)	2000 ± 40	43000 ± 600	0.05
Sample 2	Cell suspension (4 *ml*) + magnetite in paraffin (0.25 *ml*) +Doxo. 0.02 *mg/ml* (0.25 *ml*)	1000 ± 80	42000 ± 600	0.05

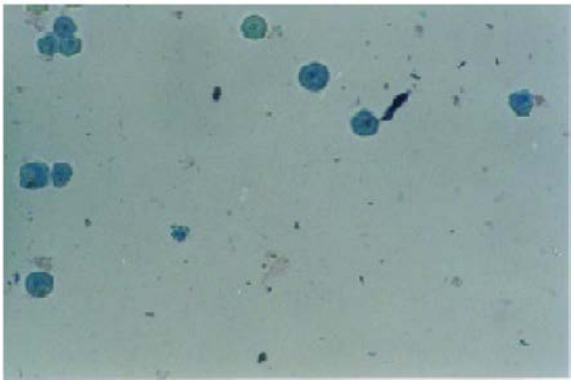

Figure 4. The effect of co-administration of magnetite nanoparticles in edible paraffin oil and doxorubicin (see sample 2 in Table 1) against MDA-MD-468 cells in the presence of an alternate magnetic field for 30 *min*. All cells were dead (stained with trypan blue)

Trypan blue exclusion assay showed that incubation of cells with a combination of doxorubicin and magnetite nanoparticles for 30 *min* in the magnetic field stained almost all the cells. Therefore, this treatment would be considered as a cytotoxic combination (Figure 4).

Flow cytometry results

As seen in figure 5 (A-C) more than 99% of cells were alive before any treatment. Therefore, these cells were used as control to setup the flow cytometer and other treatments were compared to this control. Thirty *min* incubation in magnetic field was not cytotoxic for cells (Figure 5D). In this study, edible paraffin oil was used as a carrier to make nanoparticle suspensions which had no effects on the cell viability either in RT (Figure 5E) or in the magnetic field (Figure 5F). Magnetite nanoparticles (Figure 5G) and doxorubicin (Figure 5H) alone or in combination (Figure 5I) at RT had no significant cytotoxic effects on cells. When cells were incubated with magnetite nanoparticles and magnetic field was applied for 30 *min*, 55.5% of cells died (Figure 5J). As seen in figure 5K, 28% of cells which were incubated with doxorubicin at magnetic field were also dead; whereas dead cells reached up to 80% when incubated with both doxorubicin and magnetite nanoparticles together for 30 *min* in magnetic field (Figure 5L).

Considering the data presented in table 1 and figure 5, the following results can be concluded:

1) MDA-MB-468 cells were kept under magnetic field for 30 *min* and no significant reduction in the cell viability was seen.

2) Paraffin oil as carrier agent for nano-Fe_3O_4 was not cytotoxic at all.

3) Magnetite nanoparticles alone were not cytotoxic in the RT.

4) Magnetite nanoparticles under magnetic field reduced the cell viability to less than 10%.

5) Doxorubicin alone reduced the number of viable cells to 80% after 30 *min*.

6) Trypan blue exclusion assay results showed that applying AC magnetic field on the combination of doxorubicin and magnetite nanoparticles killed the cells up to 100% in 30 *min* (Figure 4); although using this combination in flow cytometry showed more than 80% of cells were killed (Figure 5L).

Discussion

Synthesis, protection, function, and application of magnetic nanoparticles, as well as the magnetic properties and their size, shape and characterization of nanostructured systems have been progressing during the last 50 years. From the synthesis point of view, methods such as co-precipitation, thermal decomposition and/or reduction, micelle synthesis, and hydrothermal synthesis have been developed [12]. A major challenge still is protection against corrosion and agglomeration, and therefore suitable protection strategies will be emphasized. For example, surfactant/polymer coating, silica coating and carbon coating of magnetic nanoparticles or embedding them in a matrix/support [13]. Here in these studies, we applied co-precipitation as a facile and convenient method [13] to synthesize magnetite nanoparticles. The particle sizes of synthesized magnetite nanoparticles were in 3-10 *nm* range. This is in agreement with others who suggest that nanoparticles of about 5-10 *nm* diameters should form the ideal particles for most application, and with this size suspend-

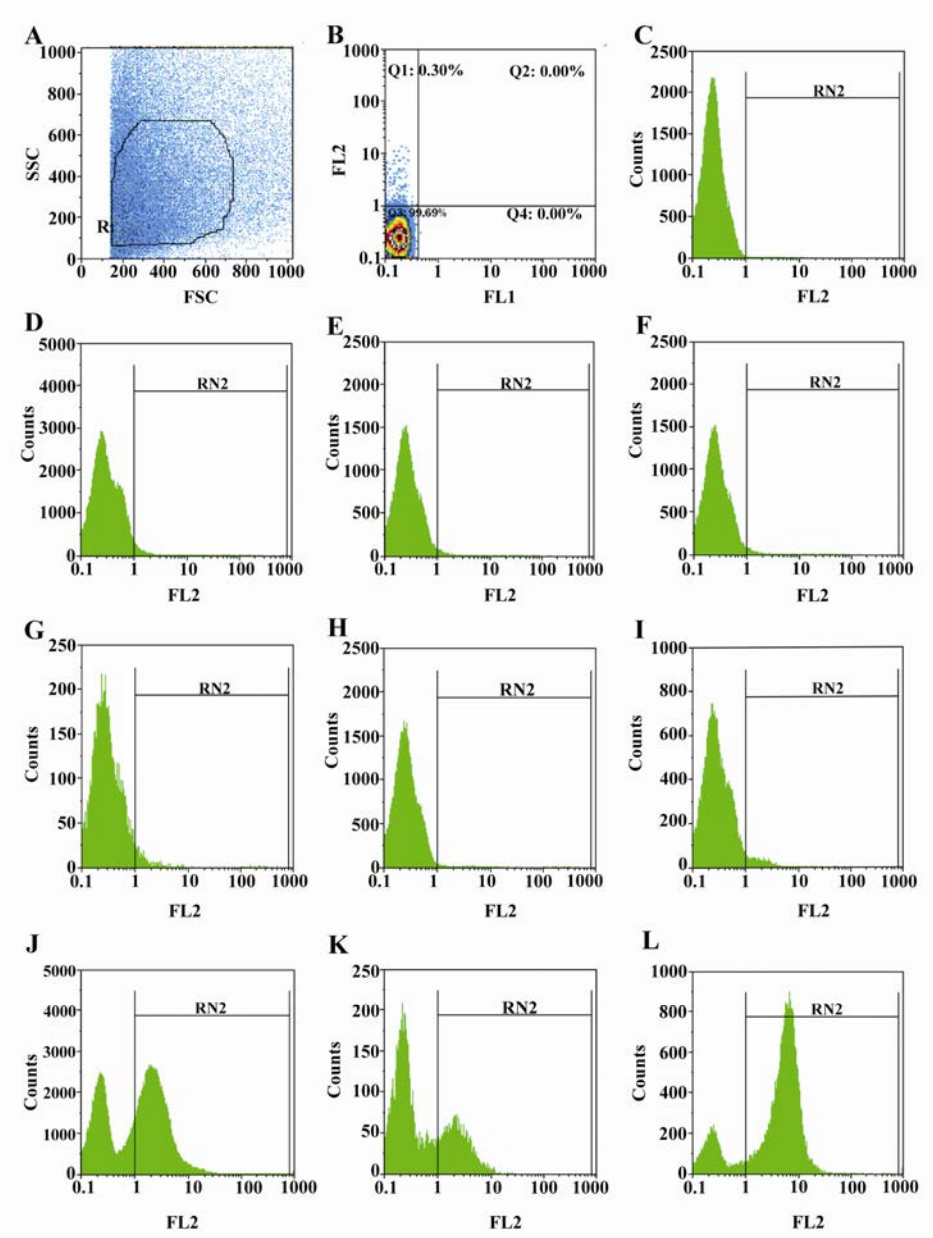

Figure 5. Comparison of cytotoxic effects of different treatments on MDA-MB-468 breast cancer cell line. A, B, C) MDA-MB-468 cells at a density of 50000 *cells/ml* in RT without any treatment were used as negative control; D) Cells incubated in magnetic field for 30 *min* did not show any significant change; E) The effects of paraffin oil (nanoparticle vehicle) on the cells in RT, which shows no changes in cell pattern and viability; F) The effects of paraffin on the cells in magnetic field for 30 *min*, showing no change; G) The effects of magnetite nanoparticles on the cells in RT shown no significant change; H) The effects of 30 *min* doxorubicin treatment on cells in RT shown no significant change; I) The effects of magnetite nanoparticles plus doxorubicin on the cells in RT shown no significant change; J) The effects of magnetite nanoparticles on the cells in magnetic field for 30 *min*; K) The effects of doxorubicin on the cells in magnetic field for 30 *min*; L) The effects of magnetite nanoparticles plus doxorubicin on the cells in magnetic field for 30 *min* (n=3). RT: room temperature or no magnetic field was applied

ing and distribution of them would be easier [12].

To stabilize and disperse the magnetite nanoparticles we used edible paraffin oil, although some researchers used oleic acid for stabilization of such nanoparticles [14].

The first reported use of magnetite nanoparticles in hyperthermia was by Gilchrist *et al* in 1960, and since then there has been tremendous progress in this field. Later on selectivity of this procedure was achieved by direct injection of nanoparticles into the tumors. If sufficient quantity of the magnetite nanoparti-

cles injected into the tumor to maintain a temperature of more than 42℃ for 30 *min*, tumor cells could be destroyed and adjacent healthy cells remain unaffected [15]. Although magnetite nanoparticles loaded with doxorubicin and tetrandrine were used against K562 leukemia cells as a new strategy to inhibit drug resistance [16,17]. Water dispersible Fe_3O_4 nanoparticles carrying doxorubicin [18] or docetaxel [19] have been prepared for cancer therapy.

To the best of our knowledge there were no data to show the combinatory effects of magnetite nanoparticles as a biocompatible and physiologically well tolerated with no significant toxicity compound [20,21] and doxorubicin as a cytotoxic agents against breast cancer cells. In agreement with these researchers our results showed that magnetite nanoparticles in combination with doxorubicin in an alternate magnetic field could have an effect as a cytotoxic remedy on breast cancer cells via hyperthermia.

Conclusion

Upon analyzing the results of these studies it could be concluded that co-administration of doxorubicin and magnetite nanoparticles in an AC magnetic field had combinatory effects against cancer cells.

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