BIOCOMPATIBILITY STUDY OF MAGNETITE NANOPARTICLE SYNTHESIZED USING A GREEN METHOD

M. TEMELIE1, R.C. POPESCU1,2 *, D. COcioABA1,3, B.S. VASILE2, D. SAVU1*

1Horia Hulubei National Institute of Physics and Nuclear Engineering, Department of Life and Environmental Physics, Magurele, Romania,
2University Politehnica of Bucharest, Department of Science and Engineering of Oxide Materials and Nanomaterials, Faculty of Applied Chemistry and Materials Science,
3Faculty of Physics, University of Bucharest
*E-mail: dsavu@nipne.ro; roxana.popescu@nipne.ro

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Abstract. The aim of our study was to characterize our new synthesized magnetite nanoparticles (NP), obtained by a less pollutant turmeric-assisted method and to evaluate their biocompatibility. Physical-chemical characterization was done using transmission electron microscopy (TEM) and selected area electron diffraction (SAED) in order to prove the high crystallinity of the nanoparticles and to identify the component phase. The in vitro biocompatibility studies were performed on normal fibroblasts (L929 cell line). The magnetite NP that we have synthesized does not induce cellular death or genotoxic effect. Analysis of metabolic function showed a transient decrease in metabolic capacity at 24 hours of incubation with the newly synthesized NP, but the cells regained quickly their normal function. The decrease in metabolic capacity was correlated with a temporary cell cycle block. These data suggest that the cells need a time interval to process the material. To further demonstrate that the NP are not toxic in a biological context we have performed a hemolysis assay showed no erythrocyte lysis. Our experiments conclude that the new synthesized NP obtained by a green method represent a biocompatible material and can be used in various biological applications.

Key words: nanoparticles, magnetite, turmeric.

1. INTRODUCTION

Nanotechnology has recently gained a lot of attention in science and industry due to its multiple new utilizations and improved functionality of the final products. With a large surface to volume ratio, NPs have unique features that made them to be successfully used in various fields: textile production (water-repent materials, antibacterial odor-control textile, antistatic fibers, UV protection) [1, 2, 3], medicine (diagnosis and treatment of various conditions) [4], cosmetic (mostly used for UV protection) [5], electronic [6] etc. Fe3O4 NPs are a particular type of NPs, that have recently grown in interest, due to their magnetic properties and good biocompatibility that make them useful in applications such as medical imaging, targeted drug delivery or hyperthermia induction in cancer treatment [7].
The nano-formulated materials confer several well documented beneficial features – some magnetite nanoparticles formulations received FDA approval for use in humans for iron deficiency and as imaging contrast agents [8]. However, their production involve frequently complicated and expensive procedures, using very high temperatures, or toxic precursors, leading to the formation of pollutant coproducts.

Recently, researchers have been working on the elaboration of new ecological synthesis methods for NPs production. Several methods involving plants [9], algae extracts [10] bacteria [11], or other biological products are described in the literature for the production of magnetite NPs. These lead to production of NPs coated with different types of biomolecules however, not always the methods lead to formation of NP with good characteristics for use in biological studies.

Curcuma longa (Turmeric) represents a plant that has been widely used in cuisine for thousands of years as a spice, and that is now studied for its multiple beneficial effects: antioxidant, antitumoral, antibacterial activity etc. Despite its benefits, the use of turmeric is limited, due to its low solubility in water which makes it hardly accessible for cells [23]. We describe here the production and testing of novel magnetite NPs obtained by co-precipitation in the presence of turmeric (Curcuma longa) extract. Turmeric-assisted NPs synthesis represents an ecological method of production for NPs, and it can be used in order to increase the bioavailability of the substance.

2. MATERIALS AND METHODS

2.1. SYNTHESIS OF NANOPARTICLES

For the synthesis of the nanoparticles we have used the following substances from Sigma Aldrich Chemie GmbH (Munich, Germany): FeSO₄ • 7H₂O, FeCl₃, 25% NH₃. The Turmeric powder of Indian provenience was purchased from Solaris Plant SRL (Bucharest, Romania).

FeCl₃ and FeSO₄ were dissolved in 100 mL deionized water (1:2 molar ratio), resulting the precursor solution which was added dropwise into an ammonia solution [12]. The resulted Fe₃O₄ nanoparticles were washed several times in deionized water by magnetic separation and then suspended in an aqueous Turmeric powder extract, by ultrasound dispersion. The suspension was let under magnetic stirring for 24 h; after this period of time, the nanoparticles were washed again for several times using deionized water.

2.2. MORPHOLOGICAL AND STRUCTURAL CHARACTERIZATION

The transmission electron microscopy was done using a Tecnai™ G2 F30 S-TWIN HR-TEM (FEI Company, Hillsboro, OR, USA) equipment with selected
area electron diffraction (SAED). The samples were diluted successively in deionized water by ultrasound dispersion, until the resulted suspensions turned clear from black. The samples were then placed on to a holey carbon copper. The equipment was set to transmission mode at 300 kV, with 2 Å point resolution and 1 Å line resolution.

Dimension of nanoparticles was analyzed from TEM images, using ImageJ Software. 3 different images were analyzed, with a total of 135 NP. The data was used to generate a histogram curve and to calculate the average size of NP +/- standard deviation.

2.3. BIOCOMPATIBILITY/GENOTOXICITY TESTS

The experiments were done on L929 – mouse fibroblasts cells used until passage 20. The cells were growth in MEM (Biochrom, Germany), supplemented with 10% FBS (Biochrom, Germany), 2 mM L-glutamine (Biochrom, Germany), 50 μg/mL penicilin, 50 μg/mL steptomycin (Biochrom, Germany) and stored in a humidified incubator at 37°C, 5% CO₂.

For biocompatibility/genotoxicity experiments the cells were seeded in culture vessels adequate for the specific test, at a low density so that in the day of the test the cultures will be at ~80% confluence. One day following seeding of the cells the medium was replaced with NP solutions (or control – culture medium) and incubated for other 24 or 48 hours when several end-points were analyzed.

Nanoparticle treatment. Cells were treated with NP solution at concentrations from 0.078–0.625 mg/mL. The solutions were prepared in growth medium from a stock solution of 8 mg/mL prepared in distillated water.

MTT assay. Viability assay was performed using MTT method. The cells were seeded in 96 well plates, incubated overnight and then medium was replaced with 90 μL of NP solution (or control – medium) for 24 or 48 hours. For every concentration of NP a blank sample (cell free) was prepared. MTT powder was dissolved in PBS at a concentration of 5 mg/mL. The solution was sterile filtered and stored at ~20°C. At the desired time of incubation, 10 μL of MTT stock solution were added to each well. The plates were incubated for 2h in dark at 37°C. The solution was then removed and 100 μL of DMSO were added to each well. Absorbance was measured at 540 nm, using a Mithras spectrophotometer (Berthold Technologies).

Phalloidin staining. The cells were seeded on 10 mm glass coverslips, placed in 24 well plates, incubated overnight and treated with NP as previously described. Phalloidin staining was performed using Phalloidin conjugated with Rhodamin Red (Invitrogen, Life Technologies, USA) following the manufacture protocol. The coverslips were mounted on glass slides using an antifade mounting medium containing DAPI (ProLong Gold antifade reagent with DAPI, Invitrogen, Life Technologies, USA).
Tripan staining. Cells were seeded in 24 well plates, incubated overnight and treated with NP as previously described. The supernatant was collected; the cells were detached with trypsin and the solution was added to the corresponding supernatant. An equal amount of cell suspension and Tripan blue were mixed in a tube and placed on a hemocytometer. Live (unstained) and dead (blue stained) cells were counted using an inverted microscope. For each probe we loaded the hemocytometer 2 times and calculated average live and dead cell number. The live/dead cell number was then expressed as a percentage of the total number of cells on the probe.

Morphological apoptosis. Cells were seeded in 24 well plates, incubated overnight and treated with NP as previously described. Apoptosis staining and scoring was done as described by Temelie et al., 2017 [13].

Micronucleus assay and division index. Cells were seeded on 24 mm coverslips placed in 6 well plates, incubated overnight and treated with NP as previously described. At 24 or 48 hours Cytochalasin B was added to a final concentration of 3 µg/mL; following another 18 hours of incubation the cells were washed with PBS, fixate (acid acetic:methanol 9:1, 20 minutes) and stained with Acridine Orange (10 µg/mL,10 minutes). For division index scoring we counted number of cells with 1 nucleus, 2 nuclei, 3 nuclei or 4+ nuclei. 500 cells per slide were counted. Division index (ID) was calculated using the following formula:

\[
ID = (\% \text{ cells 1 nucleus} + 2 \times \% \text{ cells 2 nuclei} + 3 \times \% \text{ cells 3 nuclei} + 4 \times \% \text{ cells 4 nuclei}) / 100
\]

Micronucleus scoring was done using the criteria described by Fenech [14] in 1000 cells per probe.

Hemolysis assay. Hemolytic potential of the cells was analyzed following ASTM E2524-8 standard [15] by a protocol described by Popescu et al., 2017 [12].

2.4. STATISTICAL ANALYSIS

Mean, standard deviation and standard error of the mean were calculated for each experimental point using data from at least 3 experiments. Statistical significance is evaluated by Student T-Test (p < 0.5).

3. RESULTS

3.1. MORPHOLOGICAL CHARACTERIZATION OF NP

Morphological characterization of Fe₃O₄ nanoparticles obtained by turmeric assisted synthesis showed a spherical particles with a diameter of 13.9 +/- 3.34 (Figs. 1–2).
Fig. 1 – TEM/HR-TEM images showing the morphology and crystallinity Fe₃O₄ NP obtained by turmeric assisted synthesis at different scales.

Fig. 2 – Histogram showing diameter distribution of Fe₃O₄ NP obtained by turmeric assisted synthesis.
We have proved a Gaussian distribution of the particle size (Fig. 2), most of the NP ranging in the interval of 10–14 nm, the smallest NP having 6 nm and the largest 24 nm diameter. As it can be seen (Fig. 1) the particles present a highly crystalline structure. The outer region of the NP is composed by a material with a lower crystallinity, most likely due to the functionalization with the phytochemical compounds present in turmeric.

![SAED spectrum](image1)

**Fig. 3** – SAED spectrum for Fe$_3$O$_4$ NP obtained via turmeric assisted synthesis.

![EDX spectrum](image2)

**Fig. 4** – EDX spectrum for Fe$_3$O$_4$ NP obtained via turmeric assisted synthesis.
SAED spectra showed the diffraction interferences [12] characteristic for the polycrystalline magnetite spinel structured samples (Fig. 3). EDX spectra showed a high composition of Fe, as it was expected and also considerable amount of carbon, due to the organic compounds of the turmeric content (Fig. 4).

3.2. BIOCOMPATIBILITY AND GENOTOXICITY OF NP

**Cellular morphology and interaction with NP.** Following incubation with Fe$_3$O$_4$ or Fe$_3$O$_4$ produced by Turmeric assisted synthesis for 24 or 48 hours cellular morphology did not modify, showing a non-toxic effect. As it can be seen in Fig. 5, both types of NP form aggregates at the surface of the cells suggesting interaction with the biological material.

![Fig. 5 – Morphology of L929 cells incubated for 48 hours with a concentration of 0.625 mg/ml – of a. Fe$_3$O$_4$ or b. Fe$_3$O$_4$ obtained by turmeric assisted method. Staining reveal cytoskeleton (F-actin fibers) in red and nucleus (DNA) in blue. Objective 40x (Color online).](image)

**Cellular viability.** MTT test showed a transient decrease in viability at 24 hours of incubation with the newly synthesized Fe$_3$O$_4$ NP (Fig. 6). The viability restores the control levels at 48 hours of incubation. While MTT test is usually interpreted as an assay of cellular death, we must keep in mind that the actual parameter analyzed here is the mitochondrial oxi-do-reductase activity and a decrease in mitochondrial activity does not always indicate cellular death.

**Cell survival assay.** Trypan blue assay showed no increase in cellular death at 24 or 48 hours of incubation with Fe$_3$O$_4$ or turmeric assisted new synthesized Fe$_3$O$_4$ NP. Living cells represent more than 99% of total cells in all of the samples that we analyzed (control or NP treated at a concentration of 0.625 mg/mL).

**Apoptosis/necrosis test.** Apoptosis/necrosis assay showed no increase in apoptosis or necrosis induction in any of the conditions analyzed: Fe$_3$O$_4$, Fe$_3$O$_4$ obtained by turmeric assisted method at various concentrations from 0.078 to 0.625 mg/mL at
24 or 48 hours of incubation (Fig. 7). The results of the test sustain the previous results of trypan blue test, showing no cytotoxic effect of the new synthesized material.

Fig. 6 – Cellular viability of L929 cells incubated for A. 24 hours or B. 48 hours with Fe₃O₄ or Fe₃O₄ obtained by turmeric assisted method revealed by MTT assay. Bars represent average +/- SEM. * p < 0.05.

Fig. 7 – Apoptosis/necrosis analysis at 24 hours (A, B) and 48 hours (C, D) of incubation with NP at various concentrations. Bars represent mean of minimum 3 experiments; *p < 0.5 (student T-test).
Mitotic index. Mitotic index showed a decrease at 24 hours of incubation of the cells with the newly synthetized NP (Fig. 8), results that correlated with the decrease in viability described earlier by MTT test. Considering that no cellular loss occurred under these conditions, the results suggest a transient decrease of cellular proliferation, probably due to biochemical processes required in order to process the newly added material.

Micronucleus induction. Micronucleus assay showed no changes of micronuclei number in any of the conditions analyzed: Fe$_3$O$_4$, Fe$_3$O$_4$ obtained by turmeric assisted method at various concentration from 0.078 to 0.625 mg/mL at 24 or 48 hours of incubation (Fig. 9). The results indicate no genotoxicity of the materials.
Fig. 10 – Hemolysis induction. A. Visual inspection of Fe$_3$O$_4$ obtained by turmeric assisted method, positive and negative control following hemolysis assay. It can be observed in the case if NP and C− the formation of a compact red pellet, indicating no cellular lysis. B. Hemolysis values calculated for all the tested samples.

**Hemolysis assay.** Hemolysis test showed values under 5% hemolysis for both Fe$_3$O$_4$ and Fe$_3$O$_4$ obtained by turmeric assisted method considered non-hemolytic by the ASTM standard. Values for control samples (C+, C−, PBS) respected ASTM standard values. The materials were tested at concentrations starting from a 1:5 dilution of the stock NP solution resulting in 1.6 to 0.064 mg/mL (Fig. 10). The visual observation indicate a compact red pellet in both probes and negative control indicating that we do not encountered a false-negative reaction (as may happen in some cases if the nanoparticles adsorb the free hemoglobin released upon erythrocyte lysis).

<table>
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<th>Hemolysis</th>
<th>mg/mL</th>
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<th>0.064</th>
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<td>2.30</td>
<td>2.30</td>
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</tr>
<tr>
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<td>4.12</td>
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4. DISCUSSION

Magnetite (Fe$_3$O$_4$) NPs are now intensively studied due to their unique properties: (small volume to surface ratio, magnetic properties, good biocompatibility) that made them a useful tool in several fields of nanomedicine [7].

Many of the well documented methods of magnetite NP synthesis involve use of high temperature or toxic chemicals, leading to environmentally pollutant waste products. New less pollutant methods of magnetite NP are now investigated by many scientists. First, the synthesis of superparamagnetic NP produced by simple protocol using room temperature, and normal atmospheric pressure was done in 2010, using soya bean [16]. Several Fe$_3$O$_4$ NP production methods using plant extracts, algae or other biological materials were later described, but not all of the protocols lead to synthesis of materials with proper physico-chemical characteristics for a good biocompatibility. Many authors have been used tea extracts for production of magnetite NP [17–21]. Nadagouda used different tea extracts generating NP with various characteristics. His studies showed better biocompatibility for the small radius NP [22].
Our study offers a new synthesis method for magnetite NP, using turmeric assisted co-precipitation method. The protocol represents a simple, rapid, reproducible method with a high Fe₃O₄ synthesis rate.

Turmeric was previously used in production of nanomaterials, mainly in the form of nanocapsules with diameters of 400–800 nm [23] or large nanoparticles in the range of 100 nm [24]. Our NPs have a crystalline structure, spherical shape and a size of ~10–14 nm with Gaussian diameter distribution. The radius of our NP is comparable with that of other materials described in the literature using similar co-precipitation green methods [25].

The high crystallinity of the nanoparticles was evidenced here by high resolution TEM imaging, and confirmed by the SAED spectrum that showed mono-phase composition of the polycrystalline compound being spinel structured magnetite. HR-TEM emphasizes the presence of an organic phase around the nanoparticles, which is probably due to the functionalization with Turmeric-derived phytochemicals.

The materials interacts with the cells, forming microscopic aggregates and presents a good biocompatibility even at high concentration (we tested turmeric assisted NP up to 0.625 mg/mL). Incubation with the new synthesized material lead to a transient lower level of mitochondrial endoreductase activity measured by MTT test, associated with a slightly decrease of cellular proliferation but the cells regain quickly the normal levels of these parameter. MTT assay measures the activity of mitochondrial oxidoreductase and is normally used as a viability assay. It is still known that mitochondrial metabolism can be in some cases decreased without cellular death [26]. The process was observed for example, by Schirmer et al., in a cellular viability test using Alamar Blue, a method also based on colorimetric measurement of mitochondrial reductases activity. Following incubation with their substance of interest, the authors have observed a reduction in absorbance values followed by regaining the normal control values, phenomena described as transitory metabolic disruption in their cells [27].

Considering all the other parameters (viability and genotoxicity) analyzed, and the fast recovery of the cellular physiological functions, we hypothesize that these changes represent a time-lapse needed due to the interaction of the cells with the phytochemical material present on the surface of NP.

At 48 hours of incubation with Fe₃O₄ NP obtained turmeric-assisted method we could not found any changes in any of the cellular parameters analyzed resulting in no cytotoxicity or genotoxicity.

Hemolytic assay indicate no human erythrocyte lysis at concentration up to 1.6 mg/mL of material.

5. CONCLUSION

The new Fe₃O₄ NP synthesized by a turmeric assisted co-precipitation method represents spherical particles with a good crystallinity and a size of ~10–14 nm.
The NP are biocompatible (non-cytotoxic and non-genotoxic) and do not induce lysis of human red blood cells.

The study revealed a new synthesis method for magnetite NP adequate for use in various biological applications.

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