Synthesis, Characterization and *In-Vitro* Toxicity Assessment of Superparamagnetic Iron Oxide Nanoparticles for Biomedical Applications

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Abstract

On the growing clinical demands, superparamagnetic iron oxide nanoparticles (SPIONs) have a vital role due to their infinite physical and chemical properties at the nanoscale. The researchers have started to focus more on the magnetite nanoparticle applications with unique shape and size in various filed such as biomedicine, food and environment to synthesize Fe$_3$O$_4$ NP. Here we synthesized SPIONs-Fe$_3$O$_4$ NP successfully by co-precipitation technique. The prepared nanoparticles were characterized using suitable analytical tools for structural, morphological, elemental, optical and thermogravimetric analysis. Moreover, the genotoxicity and hemolysis assay test has been carried out to estimate the toxicity of SPIONs-Fe$_3$O$_4$ NP at various concentrations. The results found that 25 µg/mL concentration of SPIONs-Fe$_3$O$_4$ NP shows good hemocompatibility than other concentrations. The genotoxicity assay was examined in *Allium cepa* (onion root tips) for chromosomal aberration. Hence, the present study discusses the synthesis characterization, assessing the genotoxicity and hemocompatibility potential for the synthesized Fe$_3$O$_4$ NPs.

Keywords: Superparamagnetic iron oxide nanoparticles (SPIONs); Co-precipitation method; *Allium cepa* test; Hemolysis assay

Introduction

The large number of research work on nanoparticles has resulted in significant advancements in various biomedical applications. The nanoscale materials such as silica, gold, silver, carbonaceous materials, Titanium, magnetic materials etc., have important biological applications like cancer therapeutics [1–10]. Especially, magnetic nanoparticles are at the foundation of nanotechnology-based structures, and they have made a significant difference in nanomedicine, analytical chemistry, electronics, and biosensing [11, 12]. Among several magnetic nanoparticles such as Ni, Co, Fe, and their respective ferrite composition, Fe$_2$O$_3$ and Fe$_3$O$_4$ magnetic nanoparticles are the most prevalent. Extremely magnetic particles such as Co and Ni are responsive to oxidation and are harmful, whereas iron oxide nanoparticles and ferrites are most functional due to their low cytotoxicity and smaller size [12–14].

SPIONs are the most extensively used in nanomedicines application in either conventional or novel advancements. The activity of the Fe$_3$O$_4$ nanoparticles strongly depends on their size, shape, including their crystal phase for its applications.
Various studies have been exposed that the shape has a great impact on the properties of Fe₃O₄ nanoparticles for their potential applications [15, 16]. They are particularly developed for targeted drug delivery, clinical diagnosis, magnetic resonance imaging, bioremediation, hyperthermia, cancer biomarker due to their superior magnetic response property [12, 13]. Also, they demonstrate interesting properties such as high saturation field, superparamagnetism, high field irreversibility, and shifted loops after the field cooling. For these properties, the particles don’t have an extensive demonstration of magnetic interrelation after the external magnetic field is detached [17]. In general, particles are made by minor crystals of iron oxides, normally called magnetite (Fe₃O₄) or maghemite (γ-Fe₂O₃) [18]. They are synthesized by various techniques, such as co-precipitation, sol-gel, hydrothermal, mixture, thermal decomposition, electrochemical and microemulsion synthesis. Among them, the co-precipitation method is the most effective, common and economical method for high-yield synthesis. Attributing to these advantages, the extensive particles size distribution of the subsequent mixture is synthesized by the co-precipitation technique [19–21].

In this research, we synthesized SPIONs-Fe₃O₄ NPs using coprecipitation method. Various characterization analyses were performed and the hemocompatibility of Fe₃O₄ NPs was analyzed using human red blood cells (RBCs). Similarly, a genotoxic study was also performed using Allium cepa (onion root tips).

Materials and Methods
Materials
Ferric chloride tetrahydrate, Ferric chloride hexahydrate and Ammonia solution were procured from Himedia Laboratory Pvt. Ltd. All the used chemicals were used in research grade.

Synthesis of iron oxide nanoparticles (co-precipitation method)

0.1 mol/L (30 mL) of ferric chloride hexahydrate and 0.1 mol/L (15 mL) of ferric chloride tetrahydrate were mixed well, and 3 mL of 5 mol/L ammonia solution was added dropwise and magnetically stirred continuously until a homogeneous solution was attained. Further, the stirring condition was maintained in a nitrogen-gas atmosphere. Finally, the obtained Fe₃O₄ NP precipitate was washed three times by centrifugation (4000 r/min for 15 min at 87 °C), and the supernatant was removed [22]. The resultant product was a pure – Iron oxide nanoparticles, which is shown as a schematic diagram in Fig. 1.

Characterization techniques
To confirm the crystallinity of the prepared, the X-ray diffractometer (XRD) was carried out with Cu-Kα radiation (1.5406 Å), the morphological analysis was studied with field emission scanning electron microscope (FESEM) (Quanta-250-FEG), the elemental composition was identified by elemental diffraction spectroscopy (EDS). The functional groups were confirmed by Fourier transformed Infrared (FTIR) spectroscopy (Bruker Tensor 27) with the range from 400 cm⁻¹ to 4000 cm⁻¹. The thermal stability of the material was observed by thermogravimetric analysis (TGA) and STA 8000 (PerkinElmer), the in-vitro hemocompatibility was evaluated by UV-visible spectrophotometer (JoscoV-650, Japan).

Allium cepa test
The genotoxicity effects of Fe₃O₄ nanoparticles were performed by using Allium cepa to analyze the chromosomal aberration. The healthy onion bulbs with roots of about 2 to 3 cm were used with different concentrations of Fe₃O₄ nanoparticles. Fe₃O₄ NPs were exactly quit in the distilled water for various concentrations of control (25, 50, 75 and 100 µg/mL, respectively) [23, 24].

Hemolysis assay
To analyze the hemocompatibility of prepared Fe₃O₄ NPs, a hemolysis assay test was carried out using a human blood cell sample. For this, 5 mL of blood was taken and centrifuged for 5 min at 6000 r/min to isolate the red blood cells from the blood sample. The red blood cell Pellet composed was diluted with 5 mL of PBS and centrifuged for 5 mins at 6000 r/min. Further, the 4 mL of Fe₃O₄ NPs were dissolved with 1 mL of red blood cell diluted at 25, 50, 75 and 100 µg/mL in phosphate buffered saline (PBS) solution and were incubated for 3 h at 36 °C. Subsequently, after 3 hrs, all the tubes were centrifuged at 6000 r/min for 15 min, and the performed supernatant result was gathered to
evaluate the UV-visible absorbance at a wavelength of 540 nm to estimate the haemoglobin release [25, 26].

**Result and Discussion**

Table 1 exhibits various characterization techniques required to confirm the physiochemical properties of synthesized SPIONs-Fe$_3$O$_4$ NPs and their respective outcomes obtained. The FESEM analysis confirmed that the morphology was cubic in nature, and the elements present were also confirmed with their atomic weight percentage by EDS analysis. The grain size of the SPIONs was calculated as 20 nm from XRD by using the Scherrer equation. But from the morphological observation, the particle size was measured as approximately 65 nm. The correlation of grain size calculation between the Scherrer equation and FESEM morphological scale measurement is different, and it may be negligible in range. The UV-visible absorption range of SPIONs was determined as 368 and 399 nm. From TGA analysis, the weight loss of Fe$_3$O$_4$ NPs was observed at about 800 °C, and nearly 70% of the iron oxide was found to be stable [27].

**XRD analysis**

Figure 2 shows the XRD pattern for synthesized Fe$_3$O$_4$ NPs, where main peaks are placed at 30.07°, 35.42°, 43.04°, 53.40°, 57.42° and 63.53° which correspond to (220), (311), (400), (422), (511) and (440), showing a cubic crystalline structure. To verify the crystalline magnetite structure of the synthesized Fe$_3$O$_4$ nanoparticles, XRD analysis was performed with a scan from 20°–80° with a step size of 2° per minute. The analyzed diffraction peak positions with parallel plane orientations of Fe$_3$O$_4$ nanoparticles approve with the standard data (JCPDS Card No: 89-0950). The grain size ($D$) for the Fe$_3$O$_4$ nanoparticles were evaluated by applying the Scherrer formula:

$$D = \frac{n\lambda}{\beta \cos \theta},$$

where $n$ is the integer, $\lambda$ is the wavelength of the incident X-ray, $\beta$ is the full width half maximum and $\theta$ is the Bragg angle.

The calculated grain size for Fe$_3$O$_4$ nanoparticles is 20 nm [28].

**Field emission scanning electron microscopy (FESEM) analysis**

The FESEM images of Fe$_3$O$_4$ nanoparticles are viewed at 2 µm and 500 nm magnification (Fig. 3), and a cubic-shaped morphology was observed. This may be attributed to the incorporation of ammonia and the presence of nitrogen during the synthesis procedure. They have a few graininess structures, which are

<table>
<thead>
<tr>
<th>No.</th>
<th>Particles</th>
<th>FESEM (morphology)</th>
<th>EDS (elements)</th>
<th>UV-visible spectroscopy (absorbance range)</th>
<th>XRD (grain size)</th>
<th>TGA (weight loss percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fe$_3$O$_4$-NPs</td>
<td>Cube shaped; particle size in ~65nm</td>
<td>Fe (61.72%) O (38.28%)</td>
<td>368 and 399 nm</td>
<td>20 nm</td>
<td>70% stability</td>
</tr>
</tbody>
</table>

![Fig. 2 XRD pattern of Fe$_3$O$_4$ NPs](image1)

![Fig. 3 FESEM image of Fe$_3$O$_4$ NPs](image2)
very small particles of the cube structure of Fe$_3$O$_4$ nanoparticles [29].

**EDS analysis**

The energy dispersive X-ray spectroscopy (EDS) was analyzed to confirm the elements present in the Fe$_3$O$_4$ nanoparticles. As shown in Fig. 4, the weight percentage of Fe and O are present at 61.72% and 38.28%, respectively [30].

![EDS spectra of Fe$_3$O$_4$ NPs](image)

**UV visible spectroscopy analysis**

In the UV visible spectroscopy analysis (Fig. 5), the absorption peak hump for Fe$_3$O$_4$ nanoparticle was found in 360–420 nm UV range. However, the minute sharp peak at 368 nm and 399 nm correspond to the oxide group present in the Fe$_3$O$_4$ NPs and the magnetite presence. In this way, UV-Visible analysis further confirmed the formation of Fe$_3$O$_4$ nanoparticle [31].

![UV visible Spectrum of Fe$_3$O$_4$ NPs](image)

**FTIR analysis**

The functional group confirmation for synthesized Fe$_3$O$_4$ NPs was revealed by FTIR analysis which is shown in Fig. 6. The strong absorption band at 622 cm$^{-1}$ and 583 cm$^{-1}$ shows the formation of magnetic nanoparticles. The stretching vibration of tetrahedral sites of spinel structure of Fe—O was confirmed at 583 cm$^{-1}$. The band at 447 cm$^{-1}$ is attributed to octahedral and tetrahedral sites; also, the peak at 3 637 cm$^{-1}$ corresponds to the O—H stretching on the surface of the Fe$_3$O$_4$ NPs.

![FTIR spectrum of synthesized Fe$_3$O$_4$ NP](image)

**TGA analysis**

The thermal stability of the synthesized Fe$_3$O$_4$ NPs was analyzed by TGA analysis under nitrogen atmosphere at the heating rate of 10 °C/min. As exhibited in Fig. 7. The removal of physically trapped water molecules caused the initial mass loss of around 3% at temperatures below 200 °C, and the thermal degradation of the organic parts grafted on the surface of the nano-catalyst caused the second mass loss in the temperature range of 200–600 °C. Further, the weight loss of Fe$_3$O$_4$ NPs was nearly 30% at 800 °C, which was due to the volatilization of the water. This proves that 70% of the iron oxide has thermal stability [32].

![TGA analysis of Fe$_3$O$_4$ NPs](image)

**Chromosomal aberrations assay**

The genotoxic effect of Fe$_3$O$_4$ NPs is studied in the
root tips of *Allium cepa*. From the optical microscopy analysis, the results were comprehensive for the various concentrations of Fe₃O₄ NPs. As shown in Fig. 8, the several mitoses phases, specifically the prophase and interphase of the exposed chromosomes, are quite evidently seen and are individual of the phase at control, i.e., 25 µg/mL. The sticky metaphase showed chromosomes aberration at 50 µg/mL, and the anaphase showed chromosomes development to opposite poles in an even way at 75 µg/mL [33]. The telophase stage in which the chromosomes were prepared at opposite poles, waiting for cytokines was observed at 100 µg/mL respectively.

**In-vitro hemocompatibility assay**

A hemolysis assay was executed to analyze the hemocompatibility for the prepared Fe₃O₄ using human red blood cells with series of Fe₃O₄ concentrations such as 25, 50, 75 and 100 µg/mL. From the outcomes, as shown in Fig. 9, Fe₃O₄ nanoparticles showed propagated hemolytic results based on increasing concentration, evaluated on the human RBCs. From the statistical analysis, the significant error was shown as < 0.05, which is relatively considered. The low-level concentration of Fe₃O₄ 25 µg/mL shows better results compared with higher concentrations (100 µg/mL). The reason for the above results is due to the erythrocytes level on the blood may be damaged and enhance the free haemoglobin level, leading to reduce the transport of oxygen to the tissues. Our research resolved that Fe₃O₄ NPs are non-hemolytic at lower concentrations against human RBCs, confirming biocompatibility and non-hazardous nature of Fe₃O₄ NPs.

**Conclusion**

The superparamagnetic iron oxide nanoparticles (SPIONs) were synthesized by the facile co-precipitation technique. The XRD and FESEM studies confirmed the formation of grain size and morphology. Adopting the Scherrer equation, the grain size was found to be 20 nm, and a cubic morphology was observed by FESEM analysis. The coherence proportion of Fe and O elements present in the Fe₃O₄ NP was identified by EDS analysis. The UV visible absorption peak for Fe₃O₄ NPs was observed at 360 nm. The thermal stability of the SPIONs was analyzed by thermogravimetric analysis. The chromosomal aberration was analyzed by using the *Allium cepa* root tip. From the hemocompatibility analysis, the toxic level for the synthesized Fe₃O₄ NP was analyzed. The mitotic index marker of increasing and decreasing the cells through mitosis for genotoxicity effects indicated that the synthesized Fe₃O₄ NPs are the potential transporter of chromosomal aberration. Furthermore, the hemocompatibility for the Fe₃O₄ NPs towards human RBCs was observed from lower to higher concentrations and confirmed that the lower concentration level of 25 µg/mL Fe₃O₄ NPs is hemocompatible. Here we concluded that the synthesized superparamagnetic iron oxide nanoparticles are a promising material for biomedical...
applications.

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Conflict of Interests

The authors declare no conflict of interest to the manuscript.

Reference


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