Synthesis, Characterization and Blood Based Toxic Effects of Superparamagnetic Nanoparticles

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The objective of this study is to assess the toxicity of superparamagnetic nanoparticles within normal and diabetic human blood groups using complete blood count (CBC). For this purpose, iron oxide nanoparticles (IONPs) were synthesized by co-precipitation method. Samples were characterized by XRD, SEM, EDS, VSM and CBC. XRD confirmed the cubic structure of Fe3O4 with Miller indices (2 0 0), (2 2 0), (3 1 1), (4 0 0), (4 4 0) and average size of magnetic nanoparticles was calculated about 11.13 nm diameter. The morphology of Fe3O4 nanoparticles was investigated by scanning electron microscope (SEM). SEM images of magnetite were found partially smooth. Spectra of EDX depicted the Fe, O and Cl elements in IONP. Magnetic properties were examined by vibrating sampling magnetometer (VSM). The blood toxicity was reported by blood contents in normal and diabetic blood samples for specified intervals using CBC technique. One sample test of variance of hemoglobin, erythrocytes, leucocytes and thrombocytes showed the significant difference (p > 0.05) among 0 hrs to 72 hrs for all normal and diabetic blood samples. This study concluded that presence of iron oxides nanoparticles of size 11.13 nm in human blood induces reactive oxygen species which cause the cell death. Hemoglobin was highly affected content of blood than erythrocytes, leucocytes by toxic effects of IONPs. Reduction of platelets among all content of blood groups posed another signature of this hematology study. Toxic spectrum of IONPs must be considered before the application of MRI contrast agents and drug delivery.

Keywords: iron oxides nanoparticles, complete blood count, hemoglobin, erythrocytes, leucocytes.

1. INTRODUCTION

In biomedical research, superparamagnetic nanoparticles have been approved by Food and Drug Administration U.S.A for in vivo applications such as artificial implants, targeted drug delivery, cell separation, hyperthermia, MRI contrast agents in the imaging of the vasculature and lymph nodes [1, 2]. Iron oxide nanoparticles deal unique chemical, biological and magnetic properties like chemical stability, biocompatibility and high saturation magnetization. The relaxivity of MNPs depends upon on their size concentration and change the saturation magnetization. More than 50 nm particles are emerged out from body through reticuloendothelial system [3]. Therefore, iron oxide nanoparticles less than 20 nm are preferred for MRI contrast agents. However, many nano sized materials are reactive or catalytic and may be potentially toxic. They can easily penetrate through cell membrane and other biological barriers into living organisms and cause cellular dysfunction [4]. Reactive oxygen species (ROS) originate from the transmission of energy or electrons to oxygen is extremely reactive and probably lethal to living organisms [5]. ROS induces oxidative stress which causes cells failure to maintain normal physiological functions of DNA that leads to cell death. This ROS is produced either by Fenton reaction or by Haber-Weiss cycle reaction [6]. Fenton reaction is an important mechanism of nanoparticle toxicity that generates the high ROS. Iron nanostructures containing Fe²⁺ and Fe(II) could directly reduce molecular oxygen dissolved in the aqueous solution to generate ROS or other complexes through a homogeneous or heterogeneous mechanism Fenton reaction, which involves one-electron reduction of hydrogen peroxide by soluble ferrous iron species, generates hydroxyl radicals that are powerful enough to oxidize most organic molecules as shown in Eq. 1 [7]:

\[
\text{Fe}^{2+} + \text{EDTA} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{EDTA} + \text{OH}^- + \text{OH}^-.
\]  

The Haber-Weiss reaction induces hydroxyl radicals from hydrogen peroxide and superoxide by soluble ferric iron species through following reactions as mentioned in Eq. 2 – Eq. 5. The homogeneous Fe(II) autoxidation in the presence of molecular oxygen in the aqueous solution via single-step two electron transfer or stepwise one electron transfer reactions can generate oxidants of ferryl-oxo complexes or a series of ROS [8].

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Fe$^{3+}$+$e^-$ $\rightarrow$ Fe$^{2+}$; 
(2)
O$_2$+$2e^-$ $\rightarrow$ H$_2$O$_2$; 
(3)
Fe$^{3+}$-EDTA+$H_2$O$_2$ $\rightarrow$ Fe$^{2+}$-EDTA + O$_2$ + 2H$_2$; 
(4)
Fe$^{3+}$-EDTA+O$_2$ $\rightleftharpoons$ Fe$^{2+}$-EDTA+O$_2$. 
(5)

A question raises in biomaterial research that how and what type of iron oxides nanoparticles can induce biochemical toxicity [9]. In this perspective, iron oxides nanoparticles pose the long-term effects in accumulation, metabolism and excretion on the base of administration in brain as well as other organs of body. Iron participates in a single oxidation-reduction reaction and it produces highly reactive oxygen species. This cytotoxicity and interferes of iron oxides with the normal components and functions of the cell [10]. Furthermore, many toxicity reports of iron oxides NPs have been presented a profile considering the particle size, type of surface coating, breakdown products, concentration, the degree of opsonization and cytotoxicity in cells [11].

The CBC test offers a comprehensive assessment of pathology and guides the diagnosis and treatment of almost all diseases. A CBC determines any increases or decreases in cell counts. For instance, low levels of hemoglobin and hemocrit are considered as signs of anemia. Similarly, any abnormal increases or decreases in the number of white blood cells could be a sign of infection, inflammation and cancer. Platelets make blood clot and control bleeding. A question raises in biomaterial research that how and what type of iron oxides nanoparticles can induce biochemical reaction. In this study, we have performed at Model PW 3710 operated at 45 kV and 40 mA with a Cu-Kα radiation. Five characteristic peaks marked by their Miller indices (2 0 0), (2 2 0), (3 1 1), (4 0 0), (4 4 0) were identified which confirmed pure Fe$_3$O$_4$ nanoparticles. These peaks were found at 20 degree range from 20° to 80° i.e. 21.23, 30.47, 35.57, 43.37, 62.81 respectively which were mentioned in the Table 1. Scherer’s formula indicated the formation of Fe$_3$O$_4$ nanoparticles with approximately 11.13 nm in diameter which were matched with reported data [15]. The average grain size of the Fe$_3$O$_4$ was calculated in the Table 2 using Scherer’s formula.

2. SYNTHESIS OF IRON OXIDE NANO Particles

Ferric chloride hexa-hydrate (FeCl$_3$•6H$_2$O, 98.0 %), Ferrous chloride tetra-hydrate (FeCl$_2$•4H$_2$O, 99 %), ammonium hydroxide (NH$_4$OH) were obtained from Daejung Chemicals & Metal Co. Korea. Iron oxide nanoparticles were prepared by co-precipitation method. Ferric chloride and ferrous chloride were mixed 2:1 ratio. The solution (0.1 M) of FeCl$_3$•6H$_2$O, > 98.0 % and 0.05 M solution of FeCl$_2$•4H$_2$O, 99 % were prepared. Both the solutions were mixed on magnetic stirrer plate at 40 °C having 400 rpm. Approximately 20 ml of NH$_4$OH with a concentration of 25–28.0 % added by drop up to the pH 9.5. The solution was stirred for additional 40 minutes without nitrogen environment. The solution color altered from orange to black, leading to a black precipitate. Finally centrifuged the solution at 4500 rev/min for 15 min to obtain Fe$_3$O$_4$ nanoparticles and dried in oven at 80 °C for approximately 24 hours. Chemical reaction has been shown in Eq. 6 as

FeCl$_3$+2FeCl$_2$+8NH$_4$OH $\rightarrow$ Fe$_3$O$_4$+8NH$_4$Cl + 4H$_2$O. 
(6)

3. RESULTS AND DISCUSSION

3.1. X-Ray Diffraction (XRD)

X-ray analysis of the magnetic nanoparticles was performed at Model PW 3710 operated at 45 kV and 40 mA with a Cu-Kα radiation. Five characteristic peaks marked by their Miller indices (2 0 0), (2 2 0), (3 1 1), (4 0 0), (4 4 0) were identified which confirmed pure Fe$_3$O$_4$ nanoparticles. These peaks were found at 20 degree range from 20° to 80° i.e. 21.23, 30.47, 35.57, 43.37, 62.81 respectively which were mentioned in the Table 1. Scherer’s formula indicated the formation of Fe$_3$O$_4$ nanoparticles with approximately 11.13 nm in diameter which were matched with reported data [15]. The average grain size of the Fe$_3$O$_4$ was calculated in the Table 2 using Scherer’s formula.

\[ D = \frac{k \lambda}{\beta \cos \theta} \] 
(7)

Fig. 1. XRD of superparamagnetic iron oxide nanoparticles [15]
different elements with weight percentage. The
was in the range of 3, 21 (temperature as shown in Fig. 3.4. Magnetization curve of nanoparticles were at
confirmed the pure iron oxide nanoparticles.
3.3. Energy Dispersion X-ray Spectroscopy (EDX) Analysis
Iron oxide magnetic nanoparticles were scrutinized
using VEGA3 TESCAN (SEM) and Oxford EDX detector. EDX study was used for quantitative analysis of MNPs (Mishra et. al, 2014). The EDX result showed the atomic percentage of different elements with weight percentage elements present in the sample. EDX technique explained the obtained peaks of each sample [17]. Fig. 3 showed Fe, O, C and Cl peaks with 62.2, 26.3, 5.4 and 5.9 weight % and 31.24, 47.86, 12.39 and 8.51 atomic % which confirmed the pure iron oxide nanoparticles.

3.4. Energy Dispersion X-ray Spectroscopy (EDX) Analysis
The magnetic properties of MNPs were investigated by using a Dexing Magnet Tech Co, Limited (VSM-100). Magnetization curve of nanoparticles were at room temperature as shown in Fig. 4. Saturation magnetization (Ms) of Fe3O4 was recorded 45.01 emu/g. This value of saturation magnetization confirmed the results reported by Anbarasu et al. and Mahdavi et al. [18, 19].

Table 2. Calculation of average grain size of Fe3O4

<table>
<thead>
<tr>
<th>Peak</th>
<th>Plane</th>
<th>(λ) Å</th>
<th>FWHM(B) Rad</th>
<th>θ1</th>
<th>θ2</th>
<th>θ12</th>
<th>cosθ12</th>
<th>Grain size</th>
</tr>
</thead>
<tbody>
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<td>(200)</td>
<td>1.54</td>
<td>0.015</td>
<td>20.8</td>
<td>21.7</td>
<td>21.3</td>
<td>0.93</td>
<td>9.72</td>
</tr>
<tr>
<td>2</td>
<td>(220)</td>
<td>1.54</td>
<td>0.017</td>
<td>29.9</td>
<td>31.0</td>
<td>30.4</td>
<td>0.86</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>(311)</td>
<td>1.54</td>
<td>0.021</td>
<td>35.0</td>
<td>36.3</td>
<td>35.6</td>
<td>0.81</td>
<td>7.79</td>
</tr>
<tr>
<td>4</td>
<td>(400)</td>
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<td>0.015</td>
<td>42.8</td>
<td>43.7</td>
<td>43.2</td>
<td>0.72</td>
<td>11.9</td>
</tr>
<tr>
<td>5</td>
<td>(440)</td>
<td>1.54</td>
<td>0.018</td>
<td>62.3</td>
<td>63.4</td>
<td>62.9</td>
<td>0.45</td>
<td>16.2</td>
</tr>
</tbody>
</table>

Average grain size of iron oxide nanoparticles was 11.13 nm which was closer to the result calculated by Kulkarni SA. et al. [15.] The Miller indices showed the cubic structure of superparamagnetic iron oxides nanoparticles.

3.2. Scanning Electron Microscopy (SEM)
The morphology of the iron oxides nanoparticles was studied by using VEGA3 TESCAN, SEM machine at 20 KV. Samples were taken in powder form for SEM analysis. SEM images illustrated the spherical like particles shape. Surface of MNPs showed little aggregation on images as shown in Fig. 2. The basic chemistry of agglomeration in pure magnetite was due to the Van-der Walls forces among the particles [16].

![SEM image of Fe3O4](image1)

**Fig. 2. SEM image of Fe3O4**

![EDX spectrum of Fe3O4](image2)

**Fig. 3. EDX spectrum of Fe3O4**

![Saturation magnetization Ms versus B](image3)

**Fig. 4. Saturation magnetization Ms versus B**

3.5. Hematology analysis
In this study, CBC test of normal and diabetic blood samples was performed at hematology analyzers for 0 hrs, 2 hrs, 4 hrs, 8 hrs, 24 hrs, 48 hrs and 72 hrs respectively in Medical Lab Technology, Haripur, Pakistan. Blood samples were collected from normal and diabetic patients in K2-EDTA tubes, which were stored at 4 °C. For precise testing manufacturer linearity ranges were also verified for erythrocytes, leucocytes and thrombocytes and hemoglobin. To start test, the blood was warmed at room temperature. Magnetic nano particles in fine powder 3 mg were mixed in 5 dl of each blood sample in EDTA tubes before CBC test. A significant decrease in hemoglobin, erythrocytes, leucocytes and thrombocytes was found due to toxic effect of the iron oxide in blood samples. The oxygen based radicals including hydroxyl radicle, singlet oxygen and hydrogen peroxide interacted with the cell membrane and caused to cell death [20, 21]. In fact, hydroxyl radicals were generated due to Haber-Weiss reaction of iron which initiated the lipid peroxidation to blood cells and finally cell death [22].

Hematology histogram of erythrocytes, leucocytes and thrombocytes histograms was shown in Fig. 5. The vertical axis showed the cell count and horizontal axis presented the cell volume. RBCs measured for normal O-ve subjects were $4.74 \times 10^{12}$/dl at 0hrs that found in normal range of 3.50–5.50 $\times 10^{12}$/L. Platelet counts were $309 \times 10^{9}$/dl for AB+ healthy subject at 0hrs which was in the range of 150–450 $\times 10^{9}$/dl. WBCs count for B-ve diabetic subject
were $9.6 \times 10^9/L$ at 0hrs which lies in the range $4.0 - 10 \times 10^9/dL$.

The vertical axis showed the cell count and horizontal axis presented the cell volume. RBCs measured for normal O -ve subjects were $4.74 \times 10^{12}/dL$ at 0hrs that found in normal range of $3.50 - 5.50 \times 10^{12}/dL$. Platelet counts were $309 \times 10^9/dL$ for AB+ healthy subject at 0hrs which was in the range of $150 - 450 \times 10^9/dL$. WBCs count for B -ve diabetic subject were $9.6 \times 10^9/L$ at 0hrs which lies in the range $4.0 - 10 \times 10^9/L$. Diabetic Blood Groups B- (287 mg/dL), B+ (282 mg/dL), O- (335 mg/dL), O+ (331 mg/dL), AB+ (372 mg/dl) were pathologically evaluated [23, 24].

Table 3 and Table 4 have demonstrated the decrease in blood samples of healthy subjects as well as diabetic blood samples that were shown in Fig. 6.
Table 4. CBC results of diabetic blood samples, mg/dL.

<table>
<thead>
<tr>
<th>S.N</th>
<th>DBG</th>
<th>CBC</th>
<th>0 hr</th>
<th>2 hr</th>
<th>4 hr</th>
<th>8 hr</th>
<th>24 hr</th>
<th>48 hr</th>
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<td>Hb</td>
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<td>10.4</td>
<td>9.8</td>
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<td>8.9</td>
<td>8.2</td>
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<td>Wbc</td>
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<td>8.5</td>
<td>8.1</td>
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<td>7.3</td>
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<td>5.11</td>
<td>5.05</td>
<td>4.78</td>
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<td>4.31</td>
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<tr>
<td></td>
<td>Plt</td>
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<td>133</td>
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<td>108</td>
<td>99</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>B+</td>
<td>Hb</td>
<td>13</td>
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<td>11.3</td>
<td>10.4</td>
<td>8.7</td>
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<tr>
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<td>Hb</td>
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4. CONCLUSIONS

One sample test of variance revealed a significant reduction of hemoglobin, erythrocytes, leucocytes and thrombocytes in complete blood count within normal and diseased blood groups at specified time period. This study concluded that presence of iron oxides nanoparticles of size 11.5 nm in human blood induces reactive oxygen species which cause the cell death. Hemoglobin was highly affected content of blood than erythrocytes, leucocytes by toxic effects of iron oxides nanoparticles. Reduction of platelets among all content of blood groups posed another signature of this hematologic study. Toxic spectrum of iron oxides nanoparticles must also be considered before the fruitfull application of MRI contrast agents and drug delivery.

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