Synthesis, Characterization and Blood Based Toxic Effects of Superparamagnetic Nanoparticles

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The objective of this study is to the assess 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 Fe_3O_4 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 Fe_3O_4 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 Fe° 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]:

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + OH^- + OH. \quad (1)$$

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)

$O_2 + 2H^+ \, 2e \rightarrow H_2O_2$; (3)

$Fe^{3+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{2+}\text{-EDTA} + O_2^- + 2H_2$; (4)

$Fe^{3+}\text{-EDTA} + O_2^- \leftrightharpoons Fe^{2+}\text{-EDTA} + O_2$. (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 spices. 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 [12 – 14]. However, toxicity of magnetic nanoparticles varies with size, uncoated and coated concentration of material in biochemical reaction. In this study, we have made an effort to assess the toxic effects of superparamagnetic nanoparticles in blood samples counting hemoglobin, erythrocytes, leucocytes and thrombocytes in healthy as well as diseased blood samples using CBC test which are commonly used in biomedical applications as MRI contrast agents and drug delivery.

2. SYNTHESIS OF IRON OXIDE NANOPARTICLES

Ferric chloride hexa-hydrate ($FeCl_3 \cdot 6H_2O$, 98.0 %), Ferrous chloride tetra-hydrate ($FeCl_2 \cdot 4H_2O$, 99 %), ammonium hydroxide (NH_4OH) 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 $FeCl_3 \cdot 6H_2O$, > 98.0 % and 0.05 M solution of $FeCl_2 \cdot 4H_2O$, 99 % were prepared. Both the solutions were mixed on magnetic stirrer plate at 40 °C having 400 rpm. Approximately 20 ml of NH_4OH 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_3O_4 nanoparticles and dried in oven at 80 °C for approximately 24 hours. Chemical reaction has been shown in Eq. 6 as

$FeCl_2 + 2FeCl_3 + 8NH_4OH \rightarrow Fe_3O_4 + 8NH_4Cl + 4H_2O$. (6)

3. RESULTS AND DISCUSSION

3.1. X-Ray Diffraction (XRD)

XRD 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_3O_4 nanoparticles. These peaks were found at 2θ 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_3O_4 nanoparticles with approximately 11.13 nm in diameter which were matched with reported data [15]. The average grain size of the Fe_3O_4 was calculated in the Table 2 using Scherer's formula.

$D = k\lambda / b\cos\theta$. (7)

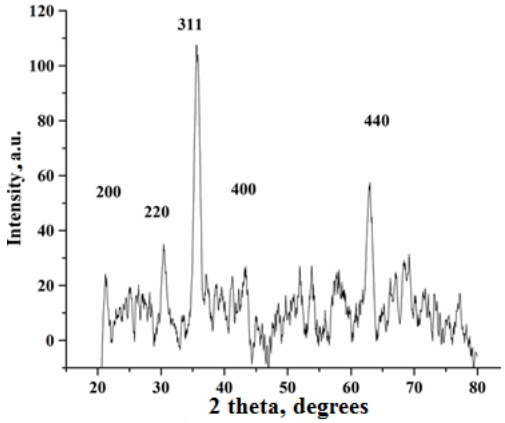

Fig. 1. XRD of superparamagnetic iron oxide nanoparticles [15]

Table 1. Miller indices calculation of Fe_3O_4

S. No	2θ	Θ	$\sin^2\theta$	$\sin^2\theta/\sin^2\theta_{mini}$	$2x$ $\sin^2\theta/\sin^2\theta_{mini}$	$3x$ $\sin^2\theta/\sin^2\theta_{mini}$	$4x$ $\sin^2\theta/\sin^2\theta_{mini}$	$h^2+k^2+l^2$	hkl
1	21.23	10.62	0.0339	1	2	3	4	4	200
2	30.47	15.24	0.0690	2.0	4	6	8	8	220
3	35.57	17.79	0.0940	2.77	5.54	8.31	11	11	311
4	43.37	21.74	0.1371	2.00	8	12	16	16	400
5	62.81	31.40	0.2714	8.00	16	24	32	32	440

Table 2. Calculation of average grain size of Fe_3O_4

Peak	Plane	(λ) Å	FWHM(B) Rad	θ_1	θ_2	θ_B	$cos\theta_B$	Grain size
1	(200)	1.54	0.015	20.8	21.7	21.3	0.93	9.72
2	(220)	1.54	0.017	29.9	31.0	30.4	0.86	10.0
3	(311)	1.54	0.021	35.0	36.3	35.6	0.81	7.79
4	(400)	1.54	0.015	42.8	43.7	43.2	0.72	11.9
5	(440)	1.54	0.018	62.3	63.4	62.9	0.45	16.2

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-dar Walls forces among the particles [16].

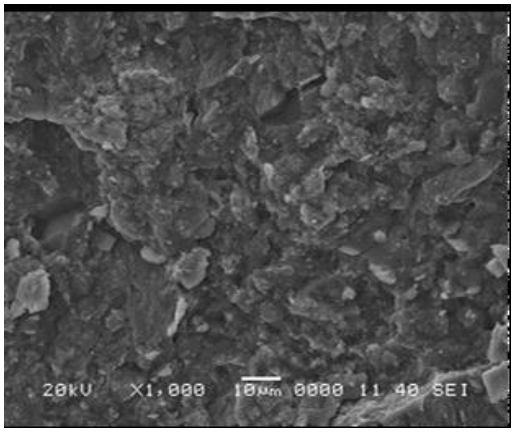

Fig. 2. SEM image of Fe_3O_4

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 (*M*s) of Fe_3O_4 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].

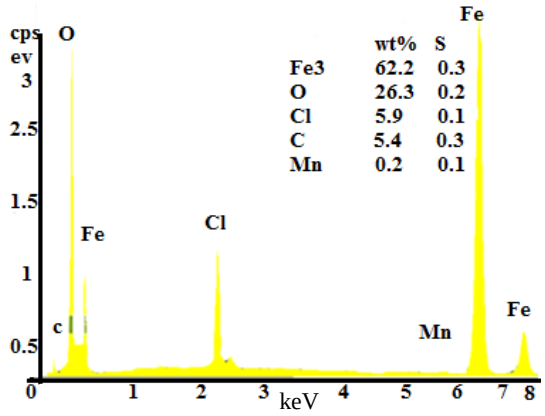

Fig. 3. EDX spectrum of Fe_3O_4

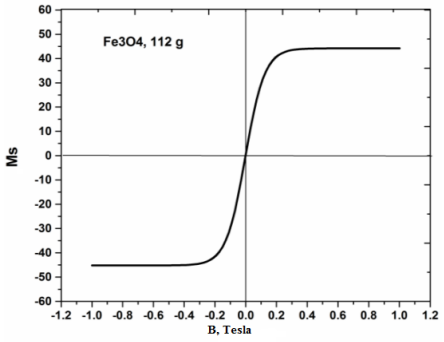

Fig. 4. Saturation magnetization *M*s versus *B*

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 K_2-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 4.74×10^{12} /dL at 0hrs that found in normal range of $3.50 - 5.50 \times 10^{12}$/L. Platelet counts were 309×10^9/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

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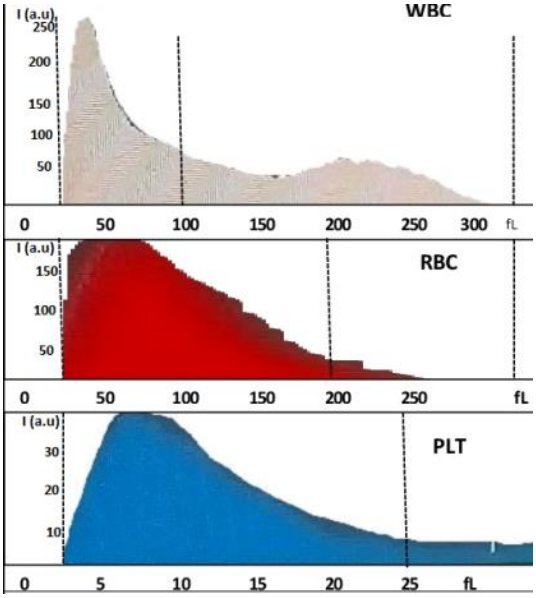
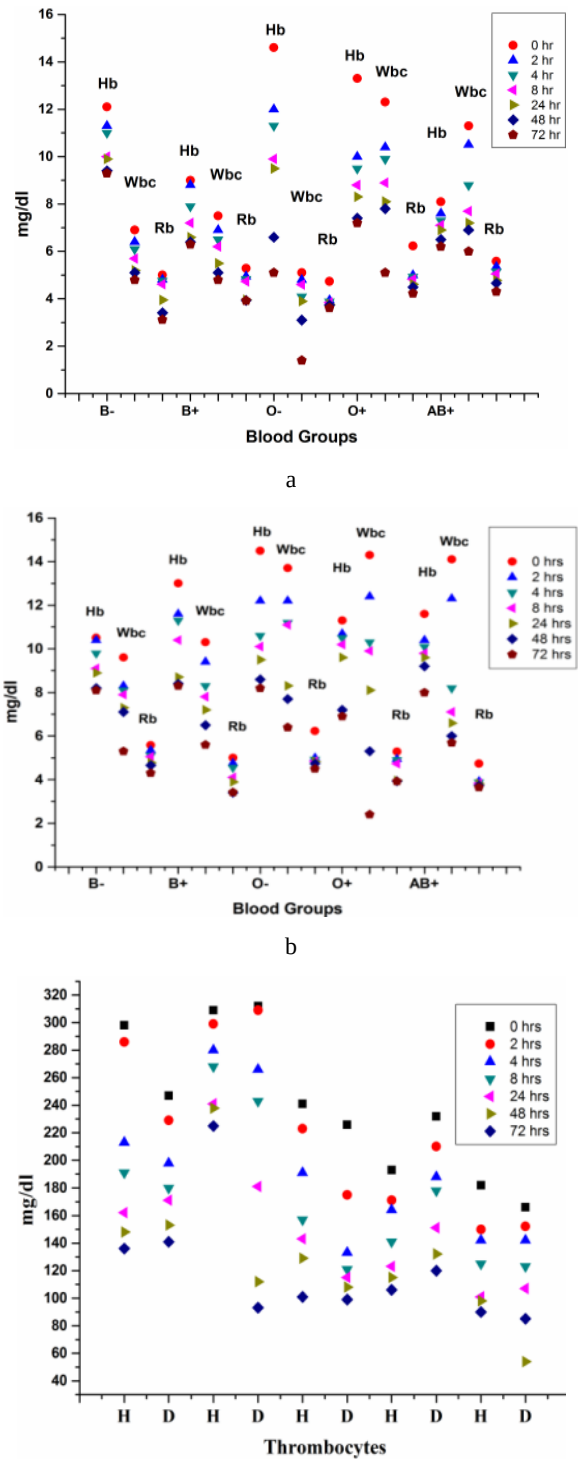


Fig. 5. Hematology histogram of White Blood Cell (WBC), Red Blood Cell (RBC) and Platelets (PLT)

Table 3. CBC results of healthy blood groups, mg/dL

S.n	BG	CBC	0 hr	2 hr	4 hr	8 hr	24 hr	48 hr	72 hr	P=0.05
1	B-	Hb	12.1	11.3	11	10	9.9	9.4	9.3	0.71
		Wbc	6.9	6.4	6.1	5.7	5.2	5.1	4.8	0.79
		Rb	5.01	4.81	4.76	4.61	3.95	3.41	3.12	0.22
		Plt	241	223	191	157	143	129	101	0.92
2	B+	Hb	9	8.8	7.9	7.2	6.6	6.4	6.3	0.23
		Wbc	7.5	6.9	6.5	6.2	5.5	5.1	4.8	0.86
		Rb	5.29	4.95	4.83	4.74	3.96	3.93	3.92	0.12
		Plt	193	171	164	141	123	115	106	0.69
3	O-	Hb	14.6	12	11.3	9.9	9.5	6.6	5.1	0.93
		Wbc	5.1	4.8	4.1	4.6	3.9	3.1	1.4	0.23
		Rb	4.74	3.94	3.88	3.84	3.76	3.73	3.62	0.007
		Plt	182	150	142	125	101	98	85	0.75
4	O+	Hb	13.3	10	9.5	8.8	8.3	7.4	7.2	0.22
		Wbc	12.3	10.4	9.9	8.9	8.1	7.8	5.1	0.97
		Rb	6.23	5	4.93	4.82	4.61	4.49	4.23	0.100
		Plt	298	286	213	191	162	148	136	0.26
5	AB+	Hb	8.1	7.6	7.3	7.1	6.9	6.5	6.2	0.99
		Wbc	11.3	10.5	8.8	7.7	7.2	6.9	6	0.56
		Rb	5.58	5.35	5.15	5.05	4.78	4.66	4.31	0.99
		Plt	309	299	280	268	241	238	225	0.60

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.



a

b

c

Fig. 6. Hematology test analysis: a – hemoglobin, RBC, WBCs in healthy blood samples; b – hemoglobin, RBC, WBCs in diabetic blood samples; c – thrombocytes in healthy and diabetic blood samples

Table 4. CBC results of diabetic blood samples, mg/dL

S.N	DBG	CBC	0 hr	2 hr	4 hr	8 hr	24 hr	48 hr	72 hr	P=0.05
1	B-	Hb	10.5	10.4	9.8	9.1	8.9	8.2	8.1	0.40
		Wbc	9.6	8.3	8.1	7.9	7.3	7.1	5.3	0.77
		Rb	5.58	5.35	5.11	5.05	4.78	4.66	4.31	0.99
		Plt	226	175	133	121	115	108	99	0.32
2	B+	Hb	13	11.6	11.3	10.4	8.7	8.4	8.3	0.35
		Wbc	10.3	9.4	8.3	7.8	7.2	6.5	5.6	0.98
		Rb	5.01	4.75	4.55	4.11	3.9	3.41	3.42	0.52
		Plt	232	210	188	178	151	132	120	0.05
3	O-	Hb	14.5	12.2	10.6	10.1	9.5	8.6	8.2	0.49
		Wbc	13.7	12.2	11.2	11.1	8.3	7.7	6.4	0.69
		Rb	6.23	5	4.93	4.85	4.82	4.74	4.5	0.01
		Plt	166	152	142	123	107	54	85	0.10
4	O+	Hb	11.3	10.7	10.5	10.2	9.6	7.2	6.9	0.11
		Wbc	14.3	12.4	10.3	9.9	8.1	5.3	2.4	0.92
		Rb	5.29	4.95	4.9	4.74	3.96	3.93	3.92	0.11
		Plt	247	229	198	180	171	153	141	0.05
5	AB+	Hb	11.6	10.4	10.1	9.8	9.6	9.2	8	0.95
		Wbc	14.1	12.3	8.2	7.1	6.6	6.0	5.7	0.08
		Rb	4.74	3.92	3.88	3.84	3.82	3.73	3.65	0.003
		Plt	312	309	266	243	181	112	93	0.10

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 hematology study. Toxic spectrum of iron oxides nanoparticles must also be considered before the fruitful application of MRI contrast agents and drug delivery.

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