IRON OXIDE NANOSTRUCTURES SYNTHESIS AND ASSESSMENT OF THERAPEUTIC EFFICACY UTILIZING PC-12 CELL LINES

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Abstract

Nanotechnology is an advanced field of research and nanotechnology can be defined as the production of various materials at the magnitude of nanoscale. Nanoparticles are an extensive class of materials that can be classified based on properties, size, and shape. These are very small particles from 1 to 100 nm in the size range. One of the paramount utilization of nanoparticles in biological and biomedical sciences is nanodevices which can be used for drug delivery or compound of reduced size which is currently referred to as "nanomedicine. As nanoparticles have proved to be efficient carriers in drug delivery, currently drug delivery across the blood-brain barrier through nanoparticles is gaining interest. Iron oxide based nanoparticles' magnetic properties have significant potential in the biomedical and clinical fields because of their unique properties. Magnetic nanoparticles have proven to be efficient to cross the blood-brain barrier. In this present research, iron-oxide nanoparticles are synthesized and characterized by infrared spectroscopy, atomic absorption spectroscopy, and field emission scanning electron microscopic studies. PC-12 cell lines were utilized to assess the therapeutic efficiency of synthesized iron oxide nanostructures.

Keywords: Iron oxide nanostructures, therapy, cell lines, PC-12 cells, nanomedicine

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DOI: - 10.31838/ecb/2023.12.si5.046

Introduction

Nanotechnology is defined as an applied science done on a nanometer scale that has applications in real time. It is defined as the recreation of matter in the dimension of 1–100 nm. Nanomaterials have remarkable chemical, and physical properties and noteworthy applications that will be beneficial for the betterment of society. The matter at the nanoscale has different properties in comparison to large-scale materials. The changes in properties of matter can bedistinguished when the size is reduced to at or below 100 nm (Ahire *et al*., 2022). The creation of new materials, and forms at the nanoscale and the improvement of new exploratory methods towards the beginning of the twenty-first century gave new opportunities for the advancement of novel nanosystems and nanomaterials. Nanotechnology provides a novel thrust in science (Bayda *et al*., 2019, Nasrollahzadeh *et al*., 2019).

Nanotechnology has an application in the medicinal field which is defined as "Nanomedicine" (Bobo *et al*., 2018, Ravindran *et al*., 2018, Ravindran *et al*.,2019). In the field of nanomedicine, nanomaterials are utilized for the diagnosis, control, monitoring, prevention, and treatment of diseases in biological entities (Tinkle *et al*., 2014).

American scientist Robert A. Freitas Jr has coined the term nanomedicine in 1999 when he published "Nanomedicine: Basic Capabilities" From then, vast research in this area has continued. It has been also known in Ayurveda the use of 'bhasmas' which has supposed to have dimensions in nanometers also referred to as nanomedicine with less toxicity in therapeutic doses.

Nanoparticles

Nanostructured materials and nanoparticles (NPs) define an acute area of research with the technologically and economically active sector which has full expansion in many domains. Wettability, melting point, thermal and electrical conductivity, catalysis, photon scattering, and absorption are notable properties of nanostructures. Nano particles have gained notability in technological advancement and in

biomedical sciences (Jeevanandam *et al*., 2018).

The science of synthesis and use of nanoparticles has been known for more than several years (Heiligtag and Niederberger, 2013). The synthesis of metallic nanoparticles by using the chemical method have been known since the 14th and 13th century BC when making glass using metals was started by Egyptians and Mesopotamians (Schaming and Remita, 2015) but in those days, there were no characterization tools to confirm they are nanoparticles. Nanoparticles have been used in healthcare products and biomedical purposes for a long, and the use of nanoparticles in cosmetics and sunscreen as an antioxidant is well known. The incorporation of engineered nanoparticles in many chemical, physical and biological processes has been studied. E.g. the use of nanoparticles in treating muscle wounds asit is used as plaster, the use of silver nanoparticles (Ag-NP) as an antimicrobial agent has been very well studied and used in medical applications like wound healing in the past decade(Jeevanandam *et al*., 2018) (Kharisov *et al*., 2016)

Classification of nanoparticles

Nanoparticles have been categorized into 4 classes based on materials (Stone et al., 2010) they are derived from:

1. Carbon-based nanoparticles: Carbon is a fundamental molecule found in all living organisms, hence the nanomaterial made of carbon possesses morphologies such as hollow tubes and spheres.
2. Inorganic-based nanoparticles: Nanostructures originating from metals and metal oxides are considered inorganic nanostructures. Some ofthe metal and metal oxide nanostructures are used as biomaterials and a few of them as semiconductors.
3. Organic-based nanoparticles: Nanoparticles made of organic matter, which have non-covalent interactions. These have morphologies unlike carbon-based like dendrimers, liposomes, polymers, etc.
4. Composite-based nanoparticles: Nanoparticles that have one phase and are combined with different NPs or which are formed by different composites such as metal and or organic composition and or carbon-based, metal-based etc. These can be formed by two different materials or with different morphologies (Jeevanandam *et al*., 2018).

Classification of nanoparticles based on their origin

1. Natural nanoparticles: Nanomaterials that are made up of natural processes and biological phenomena with no human interference.
2. Synthetic nanoparticles: Nanoparticles that are made by mechanical engineering or produced through physical methods, biological, chemical processes, or hybrid technologies (Wagner *et al*., 2014).

Classification of nanoparticles based on the sources

1. Incidental nanoparticles: The name itself suggests that the NPs are made incidentallyor as a by-product of any industrial or chemical process such as a combustion process or natural process like a forest fire.
2. Engineered nanoparticles: Nanoparticles that are made by humans with required properties and size etc (Singh *et al*., 2017).
3. Naturally produced nanoparticles: These are the NPs that are produced by any natural entities like insects, plants, and microorganisms.

These are some categories based on which nanoparticles are classified.

Factors influencing synthesis and characterization of nanoparticles

Nanobiotechnology is obtaining enormous stimulus in this time to convert metal into the nano size by changing its chemical and physical properties. There are many methods viz chemical and biological for the synthesis of nanoparticles with various compositions and sizes. Several factors influence the biosynthesis, production, and characterization of nanoparticles (Suthar *et al*., 2017). Several factors such as the methods used for synthesis, pH, temperature, pressure, time, particle size, environment, and proximity. The characterization of nanoparticles is necessary for their biomedical application and drug delivery studies (Patra and Baek, 2014).

1. Method of synthesis: There are several methods for the synthesis of nanoparticles with different procedures including chemical, mechanical processes and biological processes (Rai and Yadav, 2013). Each procedure has both advantages and disadvantages, but the biological procedures also known as green synthesis have proven environment friendly, traditional methods were

also in practice to produce nanomaterials with desired yield (Kharissova *et al*., 2013).

2. pH: The pH of the solution medium was studied as an effective factor in the synthesis of nanoparticles, asthe pH has a direct effect on the morphology of thenanostructures (Armendariz *et al*., 2004)

3. Temperature: The optimum temperature required for the synthesis of nanoparticles vary depending on the procedure, like the physical method requires a temperature higher than 350℃, though chemical procedures need less than 350℃. Biological processes require very less temperature i.e. less than 100℃, as the other parameter's temperature is one of the most important factors for synthesis of the nanoparticle.

4. Pressure: The pressure obtained in the process of synthesis of NPs can greatly decide the configuration of the nanoparticles. Pressure applied to the reaction varies from process to process (Baer, 2011).

5. Time: The time required for the synthesis of nanoparticles, light exposure time, and the period for which nanoparticles are stored are time-dependent. Therefore, time is yet another important factor for nanoparticle synthesis (Sharma *et al*., 2018)

Besides this particle size, environment, pore size, etc are a few other factors that determine the synthesis and characterization of nanoparticles (Patra and Baek, 2014).

Iron Oxide Nanoparticles

The synthesis and study of nanoparticles have been done for a while. Over the last decade, the synthesis of combinations of nanoparticles has been studied for desirable characteristics and varied performance. Iron exists in two forms ferrous (II), and ferric (III) which are the natural formsof iron. Iron plays an important role in many areasincluding agriculture, industry, geology, soil science, biology, and medicine (Kharisov *et al*., 2016). Iron oxide nanoparticles have very distinct properties which distinguish iron nanoparticles from other nanoparticles, Fe_3O_4 and Fe_2O_3 are the two main chemical formulas of iron oxide which exhibit paramagnetic properties and biomedical applications as in the case of treatmentof tumors and magnetic resonance imaging(Kostyukova and Chung, 2016).

There are other potent applications of iron oxide nanoparticles in information storage, color imaging, magnetic refrigeration, gas sensors, ferrofluids, and photocatalysis (Ni *et al*., 2022, Cao *et al*., 2016). Magnetic nanoparticles are verywell studied for their application in which combination of organic and inorganic materials inbiomedicine as they are approved by the FDA for human use. The magnetic nanoparticles were alsoproved to be an important therapeutic tool i.e. "theranostic". The use of iron oxide nanoparticles can cross the blood-brain barrier and is therefore studied to treat neurodegenerative diseases (Busquets *et al*., 2014).

Materials And Methods

Materials

The precursors are ferrous sulphate ($FeSO_4.7H_2O$) purchased from sisco research laboratories pvt. ltd. Ferric nitrate ($Fe(NO_3)_3.9H_2O$) was purchased from thermo fisher and Iron chloride was purchased from sigma Aldrich. The precipitating agent usedwas sodium hydroxide (NaOH) from sisco research laboratories Pvt. ltd. PC-12 cell lines were purchased from the National centre for cell science (NCCS), Pune India

Methods

Synthesis of iron oxide nanostructures

Three precursors have been used viz. Ferric nitrate (Mol. Wt.: 404g/mol) of 8g in 100ml Mili- Q® water, Ferrous sulphate (Mol Wt. 278g/mol) of 13.5g in 50ml Mili-Q® water, Iron Chloride (Mol. Wt.: 162.20g/mol) 8g in 50ml Mili-Q® water was used as the precursor for the following reaction to optimize the conditions. Maximum yield was obtained for iron chloride.

20g of Sodium hydroxide (Mol. Wt.: 40g/mol) from Sigma Aldrich was dissolved in Mili-Q® water and a solution of 100 ml was used as the reducing agent or precipitating agent. Both the solution was stirred using the magnetic stirrer at high RPM to dissolve the solutions before the reaction at room temperature. The reducing agent was stirred at 500rpm using the magnetic stirrer continuously. The precipitating agent was poured into the burette and was added dropwise into the precursor forming a precipitate of a brownish color. This reaction is termed a precipitation reaction. The mixture was then stirred continuously for 3-4 hours vigorously at high RPM (600-700). The resulting solution was allowed to settle down as the precipitate. The precipitate was removed and allowed to dry in the hot air oven at 80^{O}C overnight. The dried sample was taken in a silica the crucible and incinerated at different temperaturesas follows

– ferric nitrate at 300℃ for 2 hours, ferrous sulphate at 250℃ for 24 hours, and iron chloride nanoparticles at 250℃ for 7 hours. The resultant samples as a finely powdered form of iron oxide

nanoparticles were stored at room temperature for further studies.

Characterization of synthesized nanostructures

FT-IR of Nanostructures

Fourier transform infrared spectroscopic studies were performed using Bruker FT-IR instrument. FT-IR absorption bands correlate to functional groups located in the molecules. The spectrum is measured as wave number typically extending from 4000 cm^{-1} to 600 cm^{-1}. It works on the principle of the emission source of IR which is recorded, resulting in the spectrum from the IR source. The sample spectrum to the background spectrum fraction is proportional to the emission spectrum of the sample. Natural vibration frequencies of different chemical and functional groups are detected in the samples. Each organic functional groups and metal-oxygen bonds possess a characteristic frequency due to vibrations in the infrared regions.

AES of iron oxide nanostructures

Elemental analysis was carried out using a microwave plasma atomic emission spectrometer, agilent 4200. It is a quantitative technique used to detect the presence of metal and metalloids in the given sample, very reliable and simple to handle and can also quantitate metals. This technique uses the principle of radiation absorption at a particular frequency to generate free atoms in the atomizer at a specific frequency. The atom absorbs the photons and is excited to a greater energy level, hence, the amount of absorption is directly proportional to the concentration of analyte present in the samples. The concentration is measured by constructing a calibration curve with a known standard concentration. The radiation source used is a hollow cathode lamp. Atomization is the process in which the particles are converted into the individual molecule and further into atoms at high temperatures.

FE-SEM of iron oxide nanostructures

Field emission scanning electron microscopy of nanostructures utilizing FEI Nova NanoSEM 450. Microscopic images were helpful to assess the size of the nanostructures. Energy dispersive spectroscopy Bruker XFlash 6130 was used to identify elemental composition.

Cell culture

PC-12 rat pheochromocytoma cells from National Centre for Cell Sciences, Pune were cultured in F12K (pH 7.4) which was enriched with 1% antibiotics (streptomycin, penicillin) and 5 % FBS. Cells were cultured at 37 degrees in 5 % CO_2 and 95 % humidified air. Nanostructures were dispersed in the culture media before their use and allowed to stabilize for 10 min to prevent agglomeration. Various concentrations of iron oxide nanostructures were prepared using culture media.

Viability of Cells

PC-12 cells were grown in HAMS F12K along with 1% antibiotics (penicillin and streptomycin), 5% (V/V) FBS, and cultivated at 37 degrees with 5% CO_2 and 95% humidified air. The cells (5×10^4 per well) were allowed to multiply in 96-well plates, and the viability of cells in iron oxide nanostructures was determined by the MTT assay. After exposing the cells to different doses (0.1, 0.5, 1, 5,10, and 50 µg/mL) of iron oxide nanostructures and time points 24, 48, 72, and 96 hours, 100 µL of MTT in PBS from 5mg of MTT in 1mL of PBS was supplemented to each sample for 4 hours. The supernatants were removed and 100 µL of DMSO was added to each well. A Microplate reader was used to measure the absorbance at 570 nm (Fardanesh *et al*., 2019).

Results

Characterization of Iron oxide nanostructures by FT-IR

The FT-IR studies of iron oxide nanostructures showed the presence of structures at nanometer level (Figure 1). FTIR spectrum exhibits various well-defined peaks. Peaks at 643.16 and 530.50 cm^{-1} confirms iron - oxygen (Fe-O) bond and the synthesized nanostructures.

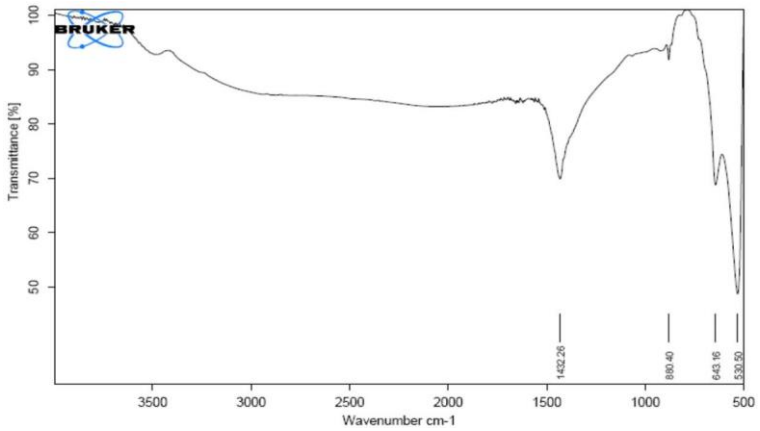


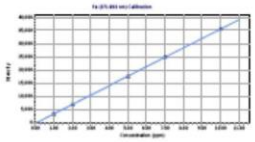
Figure 1: FT-IR spectra of iron oxide nanostructures

Quantitation of iron in nanostructures using atomic absorption spectroscopy

The presence of iron and its concentration is confirmed by atomic absorption spectroscopy. The absorptionn wavelength of the Fe is 371.993 nm. Concentrations were measured at this wavelength. 0.1g of samples were diluted with 50 ml of aqua regia independently before analysis. Standards from 1 ppm to 10 ppm were used to generate a calibration curve (Table 1). Based on the calibration curve, the concentration of samples of different batches F2, FC-01, and FC- 02 were found to be 1.79 ppm, 5.38 ppm, and 9 ppm respectively (Table 2).

Table 1: Calibration Cure for Fe from Atomic Absorption Spectroscopy

Fe (371.993 nm)
Intensity = 3552.58324390 * Concentration - 0.01655351
Correlation coefficient: 0.99998



Standards	Intensity	Method Concentration	Calculated Concentration	% Error
Blank	0.00	0.00	0.00	N/A
Standard 1	3563.94	1.00	1.00	0.32
Standard 2	7074.12	2.00	1.99	0.44
Standard 3	17670.38	5.00	4.97	0.52
Standard 4	25033.50	7.00	7.05	0.67
Standard 5	35629.92	10.00	10.03	0.29

Table 2: Quantitation of iron oxide nanostructures using atomic absorption spectroscopy

Label	Element Label (nm)	Conc	%RSD	Unadjusted Conc	Intensity
Blank	Fe (371.993 nm)	0.00 (ppm)	N/A	0.00 (ppm)	0.00
Standard 1	Fe (371.993 nm)	1.00 (ppm)	N/A	1.00 (ppm)	3563.94
Standard 2	Fe (371.993 nm)	2.00 (ppm)	N/A	2.00 (ppm)	7074.12
Standard 3	Fe (371.993 nm)	5.00 (ppm)	N/A	5.00 (ppm)	17670.38
Standard 4	Fe (371.993 nm)	7.00 (ppm)	N/A	7.00 (ppm)	25033.50
Standard 5	Fe (371.993 nm)	10.00 (ppm)	N/A	10.00 (ppm)	35629.92
191223_F2-10DF	Fe (371.993 nm)	8919.17 (ppm)	0.99	1.79 (ppm)	6365.72
191223_FC-01-20DF	Fe (371.993 nm)	53577.85 (ppm)	0.47	5.38 (ppm)	19127.23
191223_FC-02-20DF	Fe (371.993 nm)	89831.50 (ppm)	0.88	9.00 (ppm)	31986.77

Field Emission Scanning Electron microscopy of Iron Oxide nanostructures

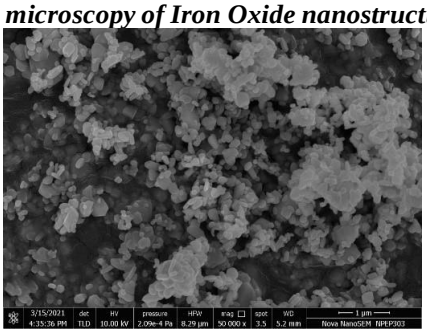


Figure 2: FE-SEM image of iron oxide nanostructures

FE-SEM Nova NANO-SEM NPEP 450 at 200,000x magnification was used to determine the morphology and size of prepared nanoparticles (Figure 2).

The particles werediscovered to be uniform in shape. According to the FESEM analysis, the produced iron oxidenanoparticles exhibited agglomeration,which is a natural property of iron oxide nanoparticles. Image J software was used to determine the particle size distribution, and theaverage particle size was calculated to be 68 nm. Energy dispersive spectroscopy (EDS) was used to determine the purity of the sample, which revealed two distinct peaks for Fe (63.56% by weight) and O (36.44% by weight), confirming the purity of the produced iron oxide nanoparticles.

Cytotoxicity assay for Iron Oxide nanostructures

The highest concentration used was 50 µg/ml whereas the lowest concentration was 0.1µg/ml. The time-dependent decline in the viability of cells wasobserved at a large number of nanostructures (50 µg/ml). In the case of low concentrations ranging from 0.1 - 5 µg/ml, nanostructures do not affect the cell viability till 72 hours (Figure 3). Only at 96 hours, there was a major difference in the cell viability. Nanostructure with a concentration of 10 µg/ml exhibits intermediate cell viability at 96 hours and deviates for low concentration from 72hours.

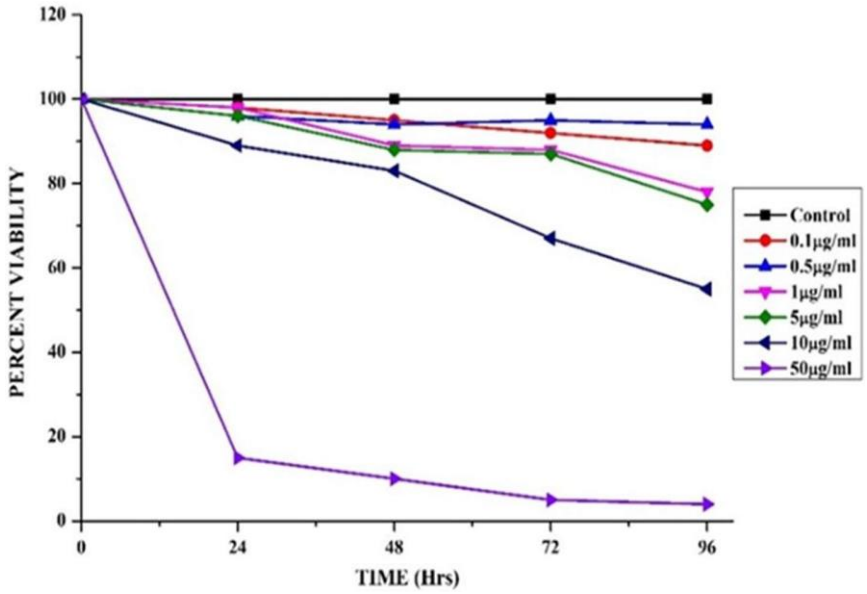


Figure 3: Concentration-dependent cytotoxicity assay for iron oxide nanostructures

Discussion

In this present research work, iron oxide nanostructures using the co-precipitation method were prepared. The synthesized nanostructures were characterized with the aid of analytical techniques viz. Fourier Transform Infrared Spectroscopy (FT-IR), Atomic absorption spectroscopy (AAS), and scanning electron microscopy. The results obtained from the characterization studies have confirmed that pure form of nanostructures was obtained

without any other major contaminants. Drug delivery is one of the important application of nanoparticles, therefore the combination of nanoparticles with organic or inorganic therapeutic mixtures have been proven to be useful (Khan *et al*., 2019). Among the various important and beneficiary effects, one of them is that nanoparticles can cross the blood-brain barrier (BBB) due to their nano-size ranging between 1-100nm (Lockman *et al*., 2002), this characteristic of iron nanoparticles is useful to cross the barrier and can be efficiently used in the treatment of neurodegenerative disorders. Due to magnetic properties and some distinct characteristics, such as nano-size, shape, etc. iron oxide nanoparticles have proven to be effective in treating various diseases including neurodegenerative diseases (Busquets *et al*., 2014). Cell viability test carried out using MTT assay shows the toxicity of the nanoparticles in a time-dependent manner concerning concentration. The synthesized nanoparticle was subjected to toxicity studies using PC-12 cell lines. The present study determines the time and concentration-dependent toxicity of iron oxide nanostructures, concentration up to 5µg/ml doses do not affect the cell viability till 72 hours. Therefore, these concentration ratios can be useful todetermine the dose needed for *in vivo* studies in preclinical studies in animal species and later during clinical trials in humans.

Conclusions

Iron oxide nanoparticles are known for their great use in various fields of science. As a nanomedicine, they have proved efficient against diseases and helpful in the biomedical aspects. Nanoparticles are partly studied for their properties in treating diseases. Extensive studies are needed for accurate results. Iron nanoparticles have been already proven to cross the blood-brain barrier (BBB) which is very important in treating neurodegenerative disorders, more work should be carried out in this research area.In the current work iron oxide nanostructures of uniform size and shape were synthesized and characterized using physicochemical techniques. Synthesized nanostructures were tested with PC-12 cell lines and the therapeutic concentration of iron oxide nanostructures are determined to be 5 ug/ml. The current study on neuronal cell lines will be helpful to develop drug delivery systems for neurodegenerative diseases.

Acknowledgment

The authors thank Symbiosis International (Deemed University) for its facility, funding, and support. The authors thank the Director and Deputy Director of Symbiosis School of Biological Sciences for the discussions. The authors thank National Chemical Laboratory Venture Centre at Pune for FT-IR and AES analysis. Authors than Pune University for FE-SEM studies.

Conflict Of Interest

None declared

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