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Synthesis, Characterization and *in vitro* Evaluation of Ferrous Phosphate Nanoparticles Loaded Iron for Bioavailability Studies

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In the present study, the ferrous phosphate nanoparticles for bioavailability applications in the size of 70-110 nm were prepared by controlled hydrolysis method. The particle size is controlled by the variation of molar ratio, altering the metal salt concentration. The synthesized particles were characterized by XRF and FTIR spectroscopic analysis, to observe the elemental content of the particles. The morphological characteristics were studied using SEM and AFM, which shows the sponge like structure during aggregation and spherical shaped particles when disintegrated, respectively. The size distributions in range of 70-110 nm were observed using centrifugal particle size analyzer (CPS). BET analysis and zeta potential analysis showed the maximum surface area of 220 m^2/g and positive charge of +6.2 mV, respectively for the stoichiometric reaction synthesis. Low particle size and high surface area are the advantageous factor of the particles for the bioavailability analysis, obtained by stoichiometric concentration. Furthermore, the high *in vitro* solubility of these particles were observed at pH 5 relative to pH 2. The dissolute Fe ions in HCl solution were evaluated by using ferrozine indicator colour complex absorbance at 562 nm. These, experimental data reveals that the significant properties of size and surface chemistry, present with the particles are capable of producing substantial improvement in the bioavailability linked to the direct uptake of the nanoparticle.

Key Words: Bioavailability, Ferrous phosphate nanoparticles, Stoichiometry, Solubility.

INTRODUCTION

In recent years, the increasing knowledge on the molecular mechanisms underlying mineral uptake and absorption in the body, has paved way for more targeted approach to improve the nutritional values. There is a confirming need for reliable methods to assess the bioavailability of ferrous compounds especially in countries where the fortification is extensively practiced. Several approaches have been used to estimate the bioavailability of Fe sources. Water soluble iron compounds used for the enrichment were found to cause adverse organoleptic changes during the interaction with other ingredients[1]. Poorly soluble iron compounds although more stable without producing any adverse effects, they tend to have low bioavailability[2]. A variety of physio-chemical methods were used to evaluate the bioavailability of Fe powders by measuring particle size, solubility and reactive surface area[3,4]. To overcome these issues, the particle size of the iron compounds were targeted to be the determinant factor, where the reduction in particle size lead to high reactive surface area, thereby produce high absorption capacity. Considering these means, nanostructured iron compounds are trending to be of a great interest now, when compared with their bulk counterpart due to their large surface area, high rate of reactivity and the possibility of enhancing environmental friendly reactions[5]. They have superior characters in non toxicity, chemical stability, durability and low costs. However, the success of a Fe fortification depends largely on the careful choice of the Fe compound[6]. Furthermore, direct nanoparticle uptake is controlled by the size and surface chemistry of the nanoparticle system. The use of this direct nanoparticle uptake, in particular for soluble but poorly absorbed ingredients, is one of the areas that needs to be explored in the future, as well as the potential side effects of these nanoparticle carriers[7].

Ferrous phosphate is a poorly soluble iron compound, with high biological impact and unknown absorption values because of its bulk size. The scientific opinion of the European food society authority indicates the importance of ferrous phosphate compounds as a food additive and its unrevealed nature of absorption and acidic solubility[8].

This paper reports on the synthesis of $FePO_4$ nanoparticles by a controlled hydrolysis reaction. Furthermore, the dependence of particle diameter and properties on the molar ratio of ferrous and phosphate ions contained in the metal salt solution is discussed, with the aim of establishing a procedure for the facile formation of ferrous phosphate nanoparticles.

EXPERIMENTAL

Synthesis of ferrous phosphate nanoparticles: The ferrous phosphate nanoparticles were synthesized by controlled hydrolysis method. To the aqueous solution of 0.5 M sodium phosphate, 0.5 M ferrous sulphate solution were added drop wise to obtain the stochiometric concentration (sample A) of the precursors. Besides this, the synthesis procedure was followed, altering the concentration of the Fe salt (0.25 M (B), 0.75 M (C)) to understand the effect of molar ratio and to obtain an appropriate Fe content in the synthesised particles. The bluish grey solution developed was kept at room temperature under vigorous stirring. The pH of the solution was maintained at 6.2 throughout the process at room temperature. After stirring for 40 min, the particles were allowed to settle down, vaccum filtered, washed with ethanol and secondly with deionized water to remove the impurities. The final product was dried at 80 ºC and subjected for characterization. All the chemicals and reagents used were of analytical grade.

Elemental analysis by XRF spectroscopy: The qualitative and quantitative elemental analysis of the prepared samples were performed using X-ray fluorescence spectroscopy (XRF: EDX-720, Shimadzu, Japan) with the 10 mm of selected sample area diameter, the analyte percentage of elements were screened from Na to Sr.

FTIR analysis: The chemical bond linkages of the samples were studied by FTIR spectroscopy. Fourier transform IR spectra were recorded for the prepared powders in order to study the presence of functional groups by forming a powder pellet with KBr in the region 4000-400 cm^{-1} (spectrum 100, Perkin-Elmer, USA).

Scanning electron microscopy studies: To find out the structural features of nanopowders, different methods were followed for SEM sample preparation. First, the samples were taken in a small amount as such in a metallic stub. Secondly, the samples were resuspended in ethanol and a drop of it was spread on a cover slip and dried. The samples on both the cover slip and in the metallic stub were coated with gold in a sputter coating unit for few minutes. Then the coated samples were observed in a LEC-435-VP (LEO Electron microscopy Ltd., Cambridge, UK).

Atomic force microscopy studies: The shape and surface morphology of the synthesised powders were observed using atomic force microscopy (Nanosurf Easyscan 2.0, Zurich). To do this, the samples were dispersed and coated in a glass substrate using spin coater unit (Milman Spin coater, India). The images were collected in contact mode using a Si tip with a 450 µm × 50 µm cantilever, with a force constant and resonant frequency of 13 KHz and 0.2 N/m, respectively at 256 points/line and the scan rate were set to 1 s/line.

Particle size distribution analysis: The disc centrifuge measures the particle size distributions using sedimentation, a well known and reliable method of particle size analysis. Particles settle in a fluid under a gravitational field according to stoke's law. The samples were analyzed using CPS disc centrifuge particle size analyser (CPS Instruments, Inc. Florida, USA) operated at a speed of 24,000 rpm for 40 min and a sample volume of 350 µL. All the analyses were run against a known calibration standard and the particle size in the solution were observed by size distribution graph.

Surface area analysis (BET method): BET is a technique that is used to measure the surface area and pore volume of a sample. Before subjecting to the surface area analysis (Quantochrome, Nova 12000, USA) samples were degassed with N_2 gas at 150 ºC for 3 h prior the determination of surface area. The analyses were performed under liquid nitrogen at 77 K.

Zeta potential analysis: The zeta potential of the nanopowder samples were determined according to laser Doppler electrophoretic mobility theory using the Zetasizer 2000 (Malvern Instruments Ltd, UK) at 25 ºC. The concentration of the sample was diluted to 0.1 % (v/v) by pH 7.0 PBS (0.05 M).

***In vitro* solubility analysis:** The *in vitro* solubility of the Fe compounds were tested following swain *et al.*[9]. The sample containing 20 mg of Fe compounds were added to 150 mL aqueous solution of 0.1 and 0.01 M/L HCl, corresponding to the pH of 2 and 5, respectively and mixed at 150 rpm with stirrer at 37 ºC. The dissolved Fe content was assessed after 30, 45, 60, 75, 90, 105 and 120 min in a 1.5 mL solution aliquot. The solutions were added with 0.5 mL of ferrozine indicator (Merck) and incubated for 15 min, for Fe binding interactions[10,11]. The colour developed by the indicator was read at 562 nm using UV visible spectrophotometer (Elico, SL 207, India).

RESULTS AND DISCUSSION

The iron salt and phosphate salt chemical interactions resulted in the formation of ferrous phosphate nanoparticle powders. The eminent features of the synthesised particles were subjected to characterization.

Spectroscopic studies: X-Ray fluorescence spectroscopy results reveal the quantitative and qualitative elemental content of the ferrous phosphate nanoparticles. The X-rays emitted from the ionizing atom have energies, characteristic of the element involved and the intensity is proportional to the concentration of an element. The major elemental content as expected was found to be Fe and P, with some trace elements such as Mn, S and Ti. The variations in the Fe to P percentage were observed (Table-1), for the different mode of synthesis and these changes are in relation with the molar ratio of the synthesis precursors. The stoichiometric concentration synthesis in this study has created much interest since it showed the maximum Fe content, resulting beneficial for the Fe bioavailability study.

TABLE-1
PERCENTAGE OF Fe CONTENT, PARTICLE SIZE DISTRIBUTION, SPECIFIC SURFACE AREA AND ZETA POTENTIAL VALUES OF THE PARTICLES PRESENT IN DIFFERENT METHOD OF SYNTHESIS, ALTERED BY THE MOLAR RATIO OF THE METAL SALT

Sample	Fe content (%)	Particle size range (nm)	SSA* (m^2/g)	Zeta potential value (mV)
A	72.7	70-130	220	+ 6.8
B	65.4	110-160	104	+ 8.2
C	59.2	180-220	87	+ 13.8

*Specific surface area analysed by BET method.

The infra red spectrum of these particles after synthesis presented two regions. The region around 3600 cm^{-1}, corresponded to the hydroxyl groups from the water molecules and the other region, between 1027 and 562 cm^{-1}, corresponds to Fe-O and P-O (Fig. 1), suggesting that the two types of sites exist in the precursors[12].

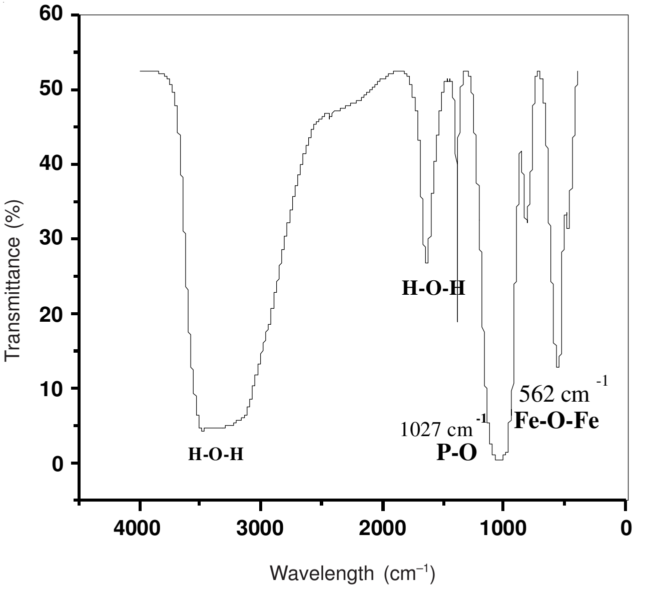

Fig. 1. FTIR analysis of the particles showing regions corresponding to the major elemental content linkages

Morphological studies: The structural features of the synthesised particles were examined using scanning electron microscopy and atomic force microscopy. The SEM micrograph (Fig. 2), showed a sponge like structural arrangement due to the aggregation of the particles, taken immediately after the filtration process of synthesis, in order to observe the bulk nature of the particles. The individual particle morphology were well studied using the AFM image (Fig. 3), showing the spherical shape of the synthesised particles, when disintegrated.

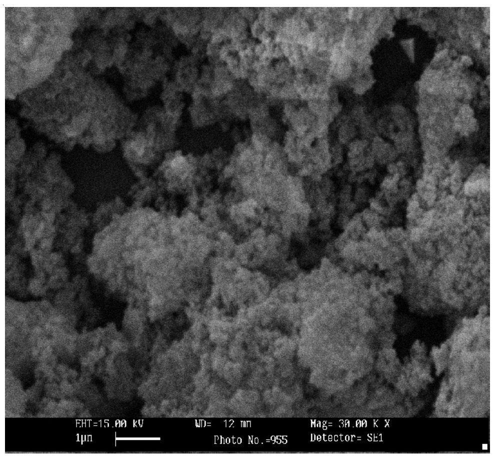

Fig. 2. SEM micrograph of the aggregated particles showing the sponge like structure

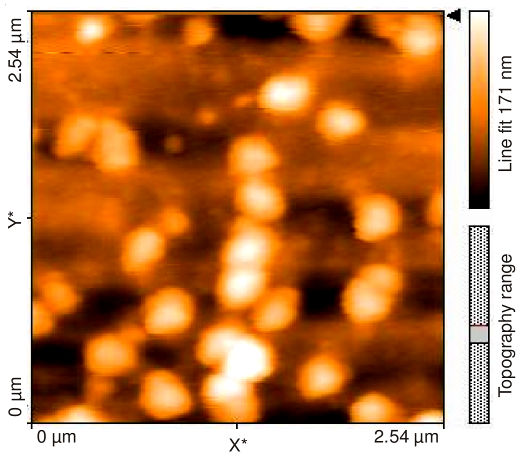

Fig. 3. AFM image presenting the spherical structure of the particles when disintegrated

The size distributions of the particles were observed using the centrifugal particle size analyser. Though the shape and morphology were found to be unique for all three synthesis concentrations, the molar ratio were found to have influenced the size of the particles[13]. The histogram of the sample A (Fig. 4), shows that the diameter of the particles were in the range of 70-110 nm. The size distribution study indicated that altering the concentration of the metal salt, tend to produce changes in the particle size of ferrous phosphate particles. Since, particle size of the compounds alone does not explain the differences in the bioavailability values, the samples were subjected for the analysis of specific surface area.

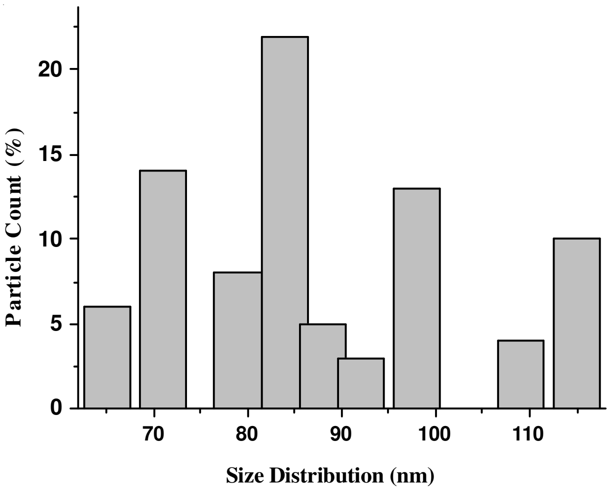

Fig. 4. Histogram showing the size distribution of the sample A analyzed by CPS centrifugal particle size analyser

Surface characteristic studies: The surface area is a suitable parameter that can reflect the solubility of the iron powders. The surface area of the samples were analyzed using the BET surface area analyser. As a general feature, the specific surface area of the particles obtained by different synthesis experiments ranged from 70-220 m^2/g. The sample with low particle size revealed the maximum surface to volume ratio.

Zeta potential is an important and useful indicator of the particle surface charge, which can be used to predict the stability of the synthesised particles. The zeta potential of ferrous phosphate nanoparticles were 6.8, 8.2 and 11.8 mV at pH 7.0 PBS (0.05 M). The particles possessed small positive charges at pH 7.0 indicating that a weak electrostatic repulsive force occurred between the particles. It was found to be beneficial in understanding the stability of the $FePO_4$ nanoparticles.

***In vitro* solubility studies:** Fig. 5 shows the solubility factor of prepared $FePO_4$ nanoparticles. The result of the solubility study indicates that the particles possess low solubility with water. The solubility and the dissolution of Fe ions in the HCl solution increased greatly, as the incubation time prolongs. The ferrozine indicator added to the 1.5 mL aliquot of each sample collected during each 15 min interval, produced light yellow coloured solution as a result of interaction between dissolute Fe ions and pyridyl triazine of ferrozine, in the HCl solution. The OD values of the solution absorbance were found to be directly proportional to the intensity of the colour developed at 562 nm. The solubility rate was observed to be higher at pH 5 relatively to pH 2 of HCl solution. A saturation level of absorbance was observed from the 1 h incubation time in pH 5 solution. The better dissolution in the higher pH were rated to be more advantageous, where it happens to have better absorption in the stomach.

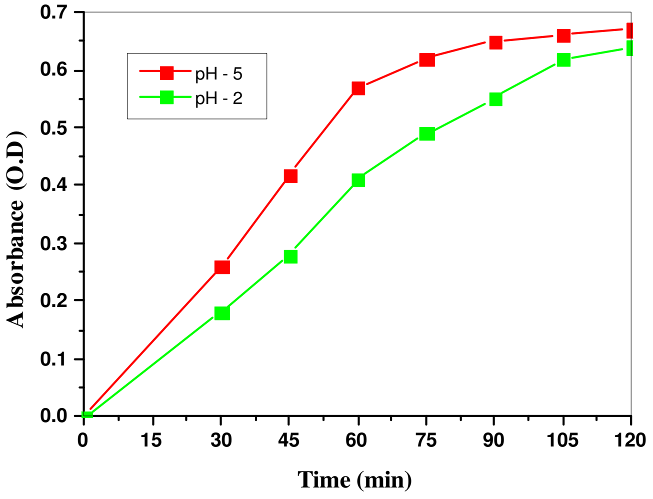

Fig. 5. Graph showing the *in vitro* solubility in HCl solution of sample A. The saturation level of the Fe content dissolute were observed at the 1 h at pH 5

Conclusion

The method of synthesis of $FePO_4$ nanoparticles by controlled hydrolysis method was investigated with a focus on the control of the size and surface properties of the compound. It was clarified that the molar ratio of the metal salt used in the synthesis plays an important role in determining the particle size, surface charge and elemental contents in the obtained product. Low particle size and high surface area are the advantageous factor of the particles for the bioavailability analysis, obtained by stoichiometric concentration. The advantageous characteristics of the particles by the stoichiometric reaction synthesis, makes it favourable to go on further with the *in vitro* studies. The high Fe content with low particle size and high surface to volume ratio favour the increase in absorption capacity of the particle, thereby leads to increase in bioavailability values. The solubility inclined by the pH is considered to be the important molecular property that influences the intestinal absorption and determines bioavailability. The higher *in vitro* solubility of the particles at the higher pH level attributed can be beneficially utilised as an ingredient in both pharmaceuticals and food formulations.

Further studies, on quantification of Fe content dissolute and the relative bioavailability values comparing to the commercial product can able to bring the ferrous phosphate nanoparticles as an effective ingredient, to combat iron deficiency.

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