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Folic acid conjugation of magnetite nanoparticles using pulsed electrohydraulic discharges

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Abstract: The sonochemical coprecipitation reaction with moderate ultrasound irradiation in low vacuum environment was used to obtain aqueous colloidal suspensions of iron oxide nanoparticles (IONPs). Synthesized magnetite nanoparticles were conjugated directly by Folic Acid using electrohydraulic discharges as a processing technique before modification of the surface of the nanoparticles. Electrohydraulic discharges were applied in two operational modes with high and low power pulsed direct currents between the electrodes. The physical and chemical properties of the obtained samples were studied using X-Ray Powder Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Dynamic Light Scattering (DLS), and Small Angle X-Ray Scattering (SAXS). The investigation has proved an inverse cubic spinel structure of magnetite with Folic Acid attachment to the magnetite surface (mean crystallite diameter in the samples $D = 27-29 \pm 2$ nm by XRD and SAXS). It was found that the processing with electrohydraulic discharges increases the colloidal stability of the Folic acid-magnetite nanoparticle dispersions.

Keywords: iron oxide nanoparticles; sonochemical coprecipitation; pulsed arc discharge; surface functionalization

INTRODUCTION

The high-voltage pulsed discharge (HVPD) into the liquid medium is an efficient method used in plasma science and technology. The interaction of non-equilibrium plasma (where the temperature of ions is much less than the temperature of electrons $T_e > T_{ion}$ depending on the energy and discharge type) with a liquid medium finds many important applications in the area of mechanical processing, fracturing technology in mining,1 environmental remediation,2 production of nanomaterials and health care.3-5 During pulsed electrohydraulic

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discharges (PEHD) the released electrical energy is converted to mechanical energy. Moreover, different physical and chemical phenomena occur during discharge such as heat emission, shock wave formation, ultraviolet/visible radiation, the formation of chemically active species (O\(^{\cdot}\), H\(^{\cdot}\), OH\(^{\cdot}\), HO\(_2^{\cdot}\)), ions (O\(^{-}\), H\(^{+}\), H\(_2\)O\(^{+}\)), molecular species (O\(_2\), O\(_3\), H\(_2\), H\(_2\)O\(_2\)) which either recombine to form stable by-products or return to a lower energetic state emitting in ultraviolet (UV) range.\(^6,8\)

In recent years, among the variety of discharge types, the underwater spark discharges were intensively used in the low-cost production of nanoparticles (NPs) in solution. Although many experimental setups for nanoparticle production have been reported exploring the variation of discharging type,\(^3\) parameters (voltage, current), electrode material and configuration, etc., there are very few reports about the processing of chemically synthesized nanomaterial’s surface by high-voltage pulsed arc electrohydraulic discharge for deagglomeration,\(^9\) and further modification of bare nanoparticle’s surface by bioactive molecules for medical application.

Nowadays, the use of iron oxide nanoparticles (IONPs) in diagnostics and therapy is one of the modern strategies in nanomedicine. Magnetic nanocolloids containing IONPs of magnetite (Fe\(_3\)O\(_4\)) and maghemite (\(\gamma\)-Fe\(_2\)O\(_3\)) are promising and popular biomedical materials due to their unique magnetic properties. Magnetite exhibits antibacterial activity due to the reactive oxygen species (ROS) generated by Fe\(_3\)O\(_4\) NPs, they are hydrophilic and biocompatible. They can be encapsulated with a suitable coating substance for biomedical applications.\(^10-12\)

The most common and cost-effective method for obtaining IONPs is chemical co-precipitation. Although this method is relatively simple, it is necessary to develop an effective strategy to improve the precise control of the main properties of the NPs for bioapplications such as size,\(^13\) shape, stability, reproducibility, and dispersibility of NPs in desired solvents. The bare iron oxide NPs have high chemical activity and are easily oxidized in the air (especially magnetite).\(^14\) Therefore, providing proper surface coating and developing effective protection strategies to keep the stability of magnetic iron oxide NPs is an important direction of current research.

The method of conjugating folic acid as a specific targeting molecule to magnetite particle is the key to the successful application of magnetic nanoparticles (MNP) in targeted drug delivery for cancer treatment. Numerous attempts have been made to develop therapeutic drug delivery systems to synthesize folic acid-linked nanoparticle components using surface activation of IONPs with functional molecules such as chitosan,\(^15\) polyethylene glycol,\(^16,17\) polyethyleneimine (PEI),\(^18\) carbodiimide,\(^19\) carboxymethyl dextran,\(^20\) serum folate-binding protein,\(^21\) which increase their specific uptake by the tumor and inhibit cell growth in microbes and cancer. FA are forms of a water-soluble B\(_9\)
vitamin which is a stable, non-immunogenic and low-cost apart from protein-targeting molecules such as antibodies.

In this paper, continuing our previous works, we report a facile method to directly conjugate folic acid (FA) molecule to iron oxide nanoparticles using surface activation with PEHD after their synthesis using in-situ sonochemical coprecipitation in a low vacuum environment.

**EXPERIMENTAL**

**Chemicals**

All chemicals of analytical grade were used without further purification. Chemicals used for the synthesis of magnetite nanoparticles: Ferric chloride hexahydrate (FeCl₃·6H₂O) (≥98 %), ferrous sulfate heptahydrate (FeSO₄·7H₂O), ammonium hydroxide solution (NH₄OH) (25 % of NH₃ basis), and folic acid (C₁₉H₁₉N₇O₆) (water content (by Karl Fischer) - 9 %, purity (HPLC) > 97 %) were purchased from Sigma-Aldrich Co. LLC (Germany).

**Synthesis of bare magnetic nanoparticles**

Bare iron oxide (Fe₃O₄) nanoparticles were prepared by sonochemical coprecipitation with moderate ultrasound irradiation using iron salts’ ratio Fe³⁺/Fe²⁺ = 1.9. First, FeCl₃·6H₂O (9 g) + 333 ml distilled water (DW) (0.1 M solution) was prepared in the jacketed reactor 1 (see Fig. 1) (temperature 45 °C, mixing duration 20 min, vacuum environment), and FeSO₄·7H₂O (4.87 g) + 175 ml DW (0.1 M solution) - in the jacketed ultrasonic reactor 2 (temperature 45 °C, duration 20 min, ultrasonication 30 % of 900 W homogenizer, pulse on 2 s, pulse off 1 s).

After dissolution, the iron salt solutions were passed by a peristaltic pump (P1) into the ultrasonic reactor (reactor 2), followed by further sonication. After that, previously prepared 19 ml NH₄OH (25 %) + 35 ml DW (4 M solution) was added dropwise during 20 min at 45 °C in the middle area of reactor 2 using peristaltic pump P2 (see Fig. 1).

![Fig. 1. The sonochemical coprecipitation reaction of IONPs with moderate ultrasound treatment in low vacuum environment](image-url)
After the formation of the black precipitate, the obtained colloidal suspension was further sonicated for 30 min and gradually cooled down to room temperature. This procedure resulted in 570 ml of black suspension. This fluid was washed several times by an abundant quantity of DW with magnetic separation with permanent magnet to reduce pH to the physiological value (initial pH 12, after washing – pH 7.3) and remove the residues of chemical synthesis. For each sample, we took 100 ml of the suspension for the preparation of bare IONPs (sample: Bare-IONPs), FA-modified IONPs without PEHD processing (sample: IONPs - FA), FA-IONPs after PEHD processing in two operation modes (see description below) called IONPs-HC-FA and IONPs-LC-FA, respectively.

Electrohydraulic processing

Fig. 2 shows the scheme of the electrohydraulic device.

Electrohydraulic processing of 100 ml suspension containing IONPs was performed in a 300 ml volume reactor in low vacuum. The entire fluid was processed at a time for 5 minutes. Brass electrodes were immersed through the suspension in the middle of the discharge reactor.

The high voltage transformer T2 has maximum regulated voltage of 3kV. After that alternating current is transformed to direct current by a diode bridge. The charge accumulated by the capacitor (C1) is transferred through an air gap (Gap 1) which regulates the discharge frequency (by rotating wheel controlled by motor) to the main discharge gap (Gap 2). A digital oscilloscope (Rigol DS1204B) monitors the discharge voltage (voltage transformer: Caltest CT4026) and current (current transformer: Ionphysics CM-01-L).

This device with controlled parameters allows us to process nanofluid in two modes:

1. High current (HC) mode (Fig. 3) – the distance between rods \( d = 0.7 \) mm, discharge current \( A_{\text{max}} = 30 \) A, voltage \( V = 1.3 \) kV, frequency \( f = 2 \) Hz, duration \( t_{\text{max}} = 20 \) ms;

2. Low current (LC) mode – \( d = 2 \) mm, \( A_{\text{max}} = 10 \) A, \( V = 1.6 \) kV, \( f = 2 \) Hz, \( t_{\text{max}} = 20 \) ms.

The development of a spark discharge in time occurs by successive “germination” of streamers in the interelectrode gap (Fig. 2, GAP2) in which two types of ions (\( H^+ \) and \( OH^- \)) play the main role. A negative charge accumulated between the electrodes from \( OH^- \) ions dissolve into the liquid, which easily gives off its electrons to the growing streamer channel and form hydrated electrons (\( e_{\text{aq}} \)). The hydrated electrons have high reduction potential and induce \( H^+ \) reduction at the anode with \( H_2O \) and \( OH^- \) generation trough electrolysis of water at the cathode respectively contributing to pH value increase (Fig. 4).\(^{2,7,8} \)
Fig. 3. PEHD in HC mode. a) HC discharge set in 2.5 s; b) - one discharge in HC mode

c → c_{aq} + H_{2}O_{aq} → H_{aq} + OH_{aq}; c_{aq} + H_{aq}^{+} → H_{aq}

Discharges in water enhance the deprotonation reactions of suspended magnetic nanoparticles as their surface presents amphoteric behaviour:

≡MOH + OH⁻ =MO + H₂O

Where M is the metal on the surface.

Fig. 4. Comparison of change in pH during electrohydraulic discharge and ultrasonic processing. TW – tap water, DW – distilled water

Accumulation of the superficial charge on the surface of nanoparticles (see Fig. 2) is due to the specific adsorbing ions (in our case Fe ions, etching metals during discharge between brass electrodes) during pulsed discharge into water. If pH is greater than characteristic point of zero charge (concentrations of positive and negative sites are equal), the discharge develops a negative charge on the nanoparticle surface.

When the streamer passes through the liquid (in this case, through water), the liquid ions, discharging onto a growing streamer as a “pull-out” electrode, form a thin gas cavity on its surface which separates the already formed branch of the streamer from the surrounding liquid. The insulating cavity is formed by atomic and molecular oxygen and hydrogen,
gaseous hydrogen peroxide, as well as electrically neutral free radicals H, OH, existing in water vapor. Fluid molecules gaining acceleration from the discharge channel, move from discharge zone in all directions, forming a cavitation zone causing the first (main) shock wave. Then the cavity closes at high speed, creating a second spark discharge at given pulse parameters. A sharp increase in channel temperature and sharp pressure jump induces additional decomposition/deagglomeration products in the liquid inside the channel.

Modification by folic acid

For direct modification (sample IONPs-FA), we took 100 ml washed precipitate sonicated during 10 min by 30 % energy (900 W, pulse – 2 s - on, 1 s - off). For 100 ml of suspension, we took 0.178 g of FA. Previously, FA in 10 ml DW was stirred at 30 °C for 15 min and added by the peristaltic pump to the magnetic fluid by sonication with 14 % power during 20 min at 30 °C (Fig. 1). After a day, we washed by decanting and separation by a permanent magnet, increased pH to 7.4 by adding NH₄OH (initial pH was 5.2) drops diluted in DW, and finally, centrifuged at 4000 rpm during 10 min. The same procedure was applied to the PEHD processed samples. For obtaining IONPs-HC-FA and IONPs-LC-FA samples initial 2×100 ml of washed and sonicated suspension was processed separately by HC and LC - mode for 5 minutes each in a vacuum environment and modified directly by FA (as described for sample IONPs-FA).

RESULTS AND DISCUSSION

X-ray diffraction (XRD)

The phase purity and crystal structure of the synthesized materials were identified via XRD analysis (Fig. 5) using a DRON 3M X-ray diffractometer, operating with Cu Kα radiation (λ = 1.541 Å) filtered by a nickel foil, voltage 40 kV, current 20 mA, and scanning speed 2° per min. The diffraction peaks at 2θ values of 30.56, 35.86, 43.46, 54.01, 57.38 and 63.00° are assigned to the crystal planes of (220), (311), (400), (422), (511), and (440), respectively. All peaks match well the characteristic peaks of the magnetite (Fe₃O₄) (JCPDS file No. 19-0629).

Fig. 5. XRD patterns of synthesized bare – IONPs, bare-IONPs conjugated directly by FA, bare-IONPs processed in HC and LC mode and conjugated by FA after processing (IONPs-LC-FA and IONPs-LC-FA, respectively)
The X-ray diffraction pattern showed that the materials were crystalline, and no impurity peaks were observed. The average crystallite diameter \((D = 28\pm2\ \text{nm})\) was calculated using the Scherrer equation from the FWHM of the (311) peak at \(2\theta = 35.86^\circ\). The average value of the lattice parameter was found to be \(a = 8.37\ \text{Å}\). Although presence of maghemite cannot be excluded by the diffraction data, the black color of the suspensions and the large lattice parameter indicate the majority of magnetite phase in the prepared nanoparticles.

**Fourier-transform infrared spectroscopy (FTIR)**

In order to determine the functional groups on the surface of the obtained IONPs, FTIR analysis was performed using an Agilent Cary 670 (Mid-IR Spectral range: 5000–400 cm\(^{-1}\)) on the powder samples after drying in vacuum. The absorption bands of synthesized iron oxide-NPs (Bare-IONPs) were observed at 3447, 1630, 895, 798, and 578 cm\(^{-1}\) (Fig. 6). The peak located at the 578 cm\(^{-1}\) is associated with the stretching vibration mode of Fe–O characteristic for the magnetite NPs, while the absorption bands at 3447 cm\(^{-1}\) indicated the O–H stretching vibration, because MNPs’ surfaces are covered with OH groups, as a result of the chemical co-precipitation. The H–O–H deformation peaks at 1630 cm\(^{-1}\) (for bare magnetite and other samples also) prove the presence of water adsorbed on the surface of the nanoparticle.\(^{27,28}\) Comparing the spectrum of bare IONP with Folic acid-conjugated IONP it can be seen that several peaks appeared in the range of 1600-1100 cm\(^{-1}\) for the conjugated samples (Fig. 6c, d, e). These bands are attributed to carboxylic group (C=O) group vibrations of folic acid in the FA-conjugated samples (especially, Fig. 6c), indicating FA attachment to the NP surface.

![FTIR spectra of FA (a), Bare – IONPs (b), IONPs – FA (c), IONPs - LC – FA (d), IONPs - HC – FA (e) samples](image.jpg)
Dynamic light scattering (DLS) and zeta potential

In order to determine the hydrodynamic size ($D_{\text{hyd}}$) distribution profile of primary nanoparticles and their aggregates in suspension, we used Anton Paar Litesizer™ 500 equipped with 658 nm laser, in back scattering geometry, thermostated at 25 °C. The refractive indices (RI) for the investigated particles (Fe₃O₄ phase) was set to $n = 2.3636$ and $n = 1.3310$ was set for the solvent (distilled water). For the assessment of the stability of the colloidal dispersions and thus the strength of electrostatic repulsion between similarly charged particles, the zeta potential ($\zeta$) of the aqueous suspensions was measured using the same instrument with a scattering angle of 173° at 25 °C and adjusted voltage 200 V. As expected, bare uncovered IONPs possess high hydrodynamic diameter and smaller magnitude of the zeta potential because of the stronger agglomeration. All FA-conjugated samples have the same diameters, although IONPs-FA have less zeta potential (19.3) because of partial, insufficient covering.

Small-angle X-ray scattering (SAXS)

The angular distribution of X-rays scattered by the samples injected into quartz capillary at very small (< 10°) angles and at 25 °C was performed on a SAXSpoint 2.0 instrument (Anton-Paar GmbH) equipped with a MicroSource, Primux 100 copper X-ray generator ($\lambda = 0.154$ nm) and an Eiger R 1M Tilt 2D detector. In case of isotropic scattering, the measured intensities are regrouped by radial averaging and presented as the intensity $I$ in function of momentum transfer or length of the scattering vector $q$:

$$q = \frac{4\pi \sin(\phi)}{\lambda}$$

where $\phi$ – is the scattering angle and $\lambda$ - is the wavelength of the X-ray radiation. In order to get information of scattering of the particles only, background subtraction was performed by software ATSAS (Fig. 7).29,30

Fig. 7. Experimental image data by a 2D detector (insets) and the circularly averaged scattering curves before and after subtraction of the background scattering. (sample – IONPs-HC-FA diluted in DW) and buffer material (capillary with DW)
One of the most important parameters that provides an estimate of the overall size of a particle (the average electron density-weighted squared distance to center-of-mass in a particle) is the radius of gyration, $R_g$ (see Fig. 8), which describes the average size of the particles:\(^{31}\)

$$I(q) = I(0)e^{-\frac{R_g^2 q^2}{3}}$$

(2)

where $I(0)$ is forward scattered intensity. The linearity of the Guinier plot ($\ln I$ against $q^2$) is a sensitive indicator of the quality of the experimental data. Deviation from the straight line can indicate aggregation, or a large polydispersity of the scattering particles.

For evaluating the pair distance distribution function $p(r)$ (probable distribution of distances between electrons) (Fig. 9) we used GNOM, an indirect Fourier transform program for small-angle scattering data analysis.\(^{32,33}\)

Using the output data of GNOM we performed an \textit{ab initio} shape determination using the bead-modeling program DAMMIF.\(^{32}\) Starting from an arbitrary initial model DAMMIF utilizes simulated annealing to construct a compact interconnected cluster of small spherical beads yielding a calculated scattering curve matching the experimental data. In Fig.10, as expected, the reconstructed particle shapes resemble aggregates of nearly spherical particles. The IONPs-FA clusters have more irregular shapes, which can be related to the effect of the FA coating on the particle agglomeration process.

The scattering curves and subsequent modeling indicate the presence of agglomerates for all the samples.\(^{4,37}\) Small angle scattering data of agglomerated surfactant-stabilized magnetite nanoparticles in aqueous dispersions are often analyzed by using empirical models, such as fractal dependences or Beaucage model.\(^{36-38}\)
Fig. 10. Bead models of bare and FA-conjugated magnetite clusters containing nanoparticles obtained by the DAMMIF. Numbers in nanometers - maximum cluster diameters ($D_{\text{max}}$).

We evaluated the assumption-free size distributions of the particles and agglomerates from the scattering patterns by the Monte-Carlo fitting method using the McSAS software.\textsuperscript{39,40} For the Monte Carlo runs the following parameter values were used: lower and upper $q$ cut-off: 0.05 – 2.9 nm$^{-1}$, respectively; number of repetitions 10; model – sphere, distribution of sphere radii between 3–50 nm. In Fig. 11 the resulting volume-weighted size histograms are shown, with uncertainties on the bars, and the red dashed line indicating the minimum level required for each bin to contribute in a measurable amount to the scattering pattern (i.e. sensitivity limit).

Fig. 11. Particle size distributions obtained using McSAS.
The obtained distributions are nearly similar for all the samples, with some variations in the radius range of 20-60 nm, and most of the particles have radii 13-14 nm which is in good agreement with the single particle or crystallite size obtained from the XRD data.

The analysis results are collected in Table I. The size distribution as given by the MeSAS analysis reveals two populations, the single nanoparticles, with mean diameter of 22 nm, and the agglomerates with a size distribution from 40 to 100 nm in diameter, with different cluster diameters $D_{\text{max}}$ (Fig. 10).

<table>
<thead>
<tr>
<th>Material</th>
<th>$D_{\text{hyd}}$ / nm</th>
<th>$\zeta_{\text{mean}}$ / nm</th>
<th>$\rho$ / mS cm$^{-1}$</th>
<th>$R_g$ / nm$^2$</th>
<th>$D_{\text{max}}$ / nm</th>
<th>$r_{\text{mean}}$ / nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare-IONPs</td>
<td>226.4</td>
<td>21.2</td>
<td>0.019</td>
<td>18.1</td>
<td>58</td>
<td>14</td>
</tr>
<tr>
<td>IONPs-FA</td>
<td>141</td>
<td>-19.3</td>
<td>0.012</td>
<td>16.7</td>
<td>50</td>
<td>13</td>
</tr>
<tr>
<td>IONPs-HC-FA</td>
<td>141.73</td>
<td>-25.6</td>
<td>0.013</td>
<td>15.5</td>
<td>47</td>
<td>13.4</td>
</tr>
<tr>
<td>IONPs-LC-FA</td>
<td>147.27</td>
<td>-24.6</td>
<td>0.038</td>
<td>13.8</td>
<td>50</td>
<td>13.5</td>
</tr>
</tbody>
</table>

a retrieved by PRIMUS; b evaluated by DAMMIF; c calculated by MeSAS

The mechanism of FA conjugation depends on high values of surface charge density of the water-based iron oxide colloids, the pH of the medium, and the ionic strength of the dispersion. In addition, the presence of specific ions (also, the etching particles from brass electrodes in HC-mode consisting of zinc and copper) at the solid-liquid interface can induce the formation of chemical bonds favourable for FA conjugation in addition to the pure Coulombic attraction. This interaction is quite probable due also to the fact that FA interacts with the negative charge of the magnetite surface at increased pH. Comparing dried powder samples of the same mass, the higher intensities of the FA characteristic peaks show that the sample IONPs-HC-FA has better coating compared to the sample IONPs-LC-FA, in which the particles are less stable and may agglomerate and flocculate due to the smaller surface charge.

CONCLUSION

A simple, inexpensive and suitable for upscaling technology of preparation of functionalized iron oxide nanoparticles for biomedical use, using non-thermal plasma discharges in water (electrohydraulic discharges) has been developed. Electrohydraulic discharges assist the modification of MNPs with a bioactive molecule such as Folic Acid, as proved by FTIR analysis. The presence of specific ions, increased pH and superficial charge around the electrohydraulically processed IONPs induce the formation of chemical bonds favorable for FA conjugation in the IONPS-HC-FA dispersions which showed increased zeta potential and stability, compared to the IONPs-LC-FA dispersions and those prepared without electrohydraulic discharges.
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