Abstract

Colloidal iron oxide nanoparticles (NPs) with surface modification by citrate, starch, dextran and aminopropyl-triethoxy-silane are synthesized using chemical co-precipitation method. The hydrodynamic diameter and colloidal stability of the synthesized NPs are measured by Dynamic Light Scattering (DLS). Surface functionalization was confirmed through FT-IR studies. The spherical shape of the citrate and starch coated iron oxide NPs are confirmed by Atomic Force Microscopy (AFM). How differential surface functionalization and the differential magnetic properties of iron oxide NPs affect biocatalytic functions is illustrated using platelet function as model. The possible role of differential magnetic moment of citrate and starch coated iron oxide NPs in induced varying platelet activity is discussed.

2.1. Introduction

2.1.1. Synthetic routes

The conventional wet chemical method for synthesizing colloidal iron oxide nanoparticles is co-precipitation of ferrous and ferric salts [1,2]. At high pH the first hydroxides are formed through deprotonation. Then oxides are formed via dehydration. The basic principle is schematically illustrated below:
Overall reaction:
\[ \text{Fe}^{+2} + 2\text{Fe}^{+3} + 8\text{OH}^{-} \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O} \]

Step 1: \( \text{Fe}^{+2} + \text{Fe}^{+3} + \text{H}_2\text{O} \)  
Deprotonation

Step 2: \( \text{Fe(OH)}_{x}^{3-} + \text{Fe(OH)}_{y}^{2-} \)  
Dehydration, pH≈9-11

\( \text{Fe}_3\text{O}_4 \)  
(Magnetite)

The mixed iron oxide or ferroso-ferric oxide (\( \text{Fe}_3\text{O}_4 \)) is known as magnetite. The structural composition, shape and size of the nanoparticles depend on molar ratio of the \( \text{Fe}^{+2} \) and \( \text{Fe}^{+3} \) ions, presence of oxygen during synthesis [3], pH of the solution, temperature and stirring rate [4]. In presence of oxygen oxidation of magnetite results in ferric oxide as well as ferric hydroxide [3]. Hence, magnetite nanoparticles synthesis is performed in Nitrogen (inert) environment. There are several other methods for synthesizing magnetite nanoparticles like thermal decomposition [5], electrochemical oxidation [6], laser pyrolysis [7], microemulsion [8], hydrothermal [9], sonochemical synthesis [10], etc.

**2.1.2. Colloidal stabilization**

But the major concern of nanoparticles synthesis is their colloidal stability. For that purpose the surface of the bare nanoparticles are coated (functionalized) with various ligand molecules, which may be inorganic, organic and polymeric.
Nanoparticles get colloidal stability due to formation of electrical double layers by charged ligands [11] or through steric stabilization when uncharged polymer used as ligands [12]. The electrical double layer is made of two layers (see Figure 1). The first one is known as Stern layer and is made of by counter ions (opposite charge with respect to surface charge of bare particles) through strong electrostatic interaction. The second one is known as diffuse layer, which is made of by loosely bound co-ions and counter-ions. Now in between Stern and diffuse layer an intermediate stable layer exists which moves with the particle in gravitational or electrical field. This layer is known as slipping layer and the potential at this layer is known as Zeta Potential (\(\zeta\)), which is very sensitive to the pH of the medium. In Dynamic Light Scattering (DLS) setup \(\zeta\) is measured through measuring the electrophoretic mobility[13] \((U_E)\), which is calculated employing Henry equation:

\[
U_E = \frac{2 \varepsilon \zeta f(ka)}{3\eta}
\]
Where \( \varepsilon \) is dielectric constant, \( \eta \) is the viscosity of the medium and \( f(ka) \) is the Henry function (two values generally used that is either 1 or 1.5).

Here the synthesis of iron oxide (mainly magnetite) nanoparticles with different surface functionalization like citrate, starch, APTES and dextran are described. The physical (shape, size, zeta potential, surface modification and magnetic) characterizations were performed using various technique like photon correlation spectroscopy, atomic force microscopy and Fourier transformed-Infra Red (FT-IR) spectroscopy.

2.2. Materials & Methods

2.2.1. Synthesis of citrate coated iron oxide NPs

Colloidal Iron oxide NPs were synthesized through co-precipitation [1] of ferrous and ferric salts. Briefly, Ferric chloride (FeCl\(_3\), 6H\(_2\)O) and ferrous chloride (FeCl\(_2\), 4H\(_2\)O) are taken in 2:1(w/w) ratio and dissolved in 2 N HCl (2 ml solution containing 1gm FeCl\(_3\) and 0.5 gm FeCl\(_2\)) and co-precipitated by one step addition of 1.5 M sodium hydroxide (NaOH) solution (40 ml). Then the black precipitate stirred for half an hour at room temperature (25\(^{\circ}\) C). All the steps were performed in inert nitrogen environment. The formed precipitate was washed several times (6-7 times) by distilled water through magnetic decantation. Citrate functionalized Iron oxide NPs were synthesized by addition of tri-sodium citrate and citric acid mixture (1:2 w/w ratios, 5 ml) in washed precipitate (3 ml) and 4 hours of moderate stirring at 25\(^{\circ}\) C. Then pH was adjusted to ~7.2 by addition of 1.5 M NaOH solution. Finally
suspensions were centrifuged at 10,000 rpm for 12 minutes to remove the larger aggregates.

**2.2.2. Synthesis of starch coated Iron oxide NPs**

Starch-coated synthesis is based on the method, described previously [14], which depends on the Van der Waals reaction between starch and magnetite nanoparticles [15]. Briefly, 6 g starch was dissolved in 40 ml water at 80 °C. 24 ml ferrite solution, contains 0.8 M ferric chloride (FeCl3, 6H2O) and 0.4 M ferrous chloride (FeCl2, 4H2O) in 2 N HCl. The thoroughly dissolved starch was then mixed with the ferrite solution. After proper mixing in a magnetic stirrer, 1 M NaOH was added drop wise until the pH became 11. All steps were performed in an oxygen-free (N2) environment. After 2 h of reaction the final black suspension was neutralized by 0.1 M HCl and centrifuged at 1500 rpm for 15 min to remove the larger aggregates.

**2.2.3. Synthesis of APTES coated nanoparticles**

The bare iron oxide nanoparticles were synthesized using the protocol described in Section 2.2.1. After vigorous washing, 3 ml of bare nanoparticles solution was taken and mixed with 1 ml diluted (25 μL 12 N HCL + 1 ml milli-Q water) HCl solution and stir for 15 mins to break the large clusters. Then freshly prepared 2 ml water-APTES mixture (1:1 v/v) was added. The mixture was stirred for 3 hours at room temperature and for 30 mins at 60 °C.
2.2.4. **Synthesis of Dextran coated nanoparticles**

The dextran coated iron oxide nanoparticles were synthesized using a modified protocol described by Molday [16]. Briefly, 1.5 gm dextran and 230 mg FeCl3 was dissolved in 2 ml de-ionized water. 153 mg FeCl2 was dissolved in 1 ml de-ionized water and store at 4 °C for 10 mins. Then FeCl2 solution was added to the FeCl3-polymer mixture with a syringe at stirring condition in nitrogen environment. After addition cold 8 ml liquor ammonia was added and stirred for 2 hours at 85 °C to remove the excess ammonia.

2.2.5. **DLS study**

Hydrodynamic size and zeta potential of synthesized Iron oxide NPs were measured using DLS (Nano ZS, Malvern, equipped with a 4 mW He-Ne Laser (λ= 632 nm)). Briefly, 10 μL of synthesized nanoparticles was diluted to 1 ml by Milli-Q water and filtered through 0.2 micron membrane filter prior to measurement.

2.2.6. **Atomic Force Microscopy**

Freshly cleaved micas were silanized prior to sample preparation. 3-aminopropyl tri-ethoxysilane (APTES) was used as silanizing agent. Freshly cleaved micas were incubated in 0.8% APTES in anhydrous toluene for ~4 h. They were then washed five times with ethanol, with sonication for 10 min for each washing, to remove the APTES clusters. Then the micas were incubated in a diluted solution of starch and the citrate coated iron oxide nanoparticles for half an hour. Finally, the mica pieces were dried and analyzed by Atomic Force Microscopy (AFM, Veeco multi mode NanoScope IIIa) using tapping mode with a tip model RTESPA
equipped with 1–10 Ω-cm phosphorous (n)-doped Si at a scanning rate of 1 Hz utilizing a phase data type and a resonant frequency of 314.5 kHz.

### 2.2.7. Fourier Transformed Infra Red (FT-IR) spectroscopic studies

Different Iron oxide NPs surface conjugations were confirmed by FT-IR spectroscopy (Thermo, NICOLET 6700). Samples were prepared through formation of Potassium Bromide (KBr) pellet. Briefly the colloidal nanoparticles were lyophilized to get dried powder. Then 2-3 mg powder and ~100 mg solid KBr were mixed thoroughly in mortar pestle. Finally pellets are formed through application of ~12 ton pressure in a hydraulic unit (Pci, India).

### 2.3. Results and Discussions

#### 2.3.1. Hydrodynamic size, morphology and colloidal stability of synthesized nanoparticles

The hydrodynamic diameter and zeta potential of the synthesized different Iron oxide NPs are given in Table 1. Figure 3 and 4 shows the hydrodynamic size distribution of the synthesized iron oxide nanoparticles. Atomic Force micrographs (see Figure 5) show that the synthesized starch and citrate coated iron oxide NPs are spherical in shape having average diameter of 20-30 nm.

The Citrate and APTES coated Iron oxide NPs show higher colloidal stability due to formation of well defined double layer at the surface. On the other hand polymer (starch and dextran) coated Iron oxide NPs show lower value of zeta
potential, but they form stable colloidal suspensions mainly due to steric hindrance, as the used polymers are uncharged [12].

**Table 1: hydrodynamic diameter and zeta potential of synthesized Iron oxide NPs**

<table>
<thead>
<tr>
<th>Iron oxide NPs</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential (mV) at pH 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate coated</td>
<td>~30</td>
<td>-48</td>
</tr>
<tr>
<td>Starch coated</td>
<td>~65</td>
<td>+02</td>
</tr>
<tr>
<td>APTES coated</td>
<td>~50</td>
<td>+40</td>
</tr>
<tr>
<td>Dextran coated</td>
<td>~70</td>
<td>-06</td>
</tr>
</tbody>
</table>

**Figure 3: Hydrodynamic size distribution of citrate (a) and starch (b) coated iron oxide nanoparticles.**
2.3.2. Surface conjugation analysis

Figure 4 shows the FT-IR spectrum in absorbance mode for citrate and starch coated iron oxide NPs.
Assignment of Bonds:

Bare Iron Oxide Nanoparticles:

<table>
<thead>
<tr>
<th>IR absorption Peak</th>
<th>Vibrational assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>568 cm⁻¹</td>
<td>Fe-O stretching vibration at the tetrahedral site.</td>
</tr>
</tbody>
</table>

Lyophilized Citrate Buffer:

<table>
<thead>
<tr>
<th>IR absorption Peak</th>
<th>Vibrational assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1080-1300 cm⁻¹</td>
<td>C-O vibration of alcohols and carboxylic acid.</td>
</tr>
<tr>
<td>1397 cm⁻¹</td>
<td>Symmetric stretching vibration of –COOH.</td>
</tr>
<tr>
<td>1586 cm⁻¹</td>
<td>Asymmetric stretching vibration of -COOH.</td>
</tr>
</tbody>
</table>

Soluble Starch:

The IR absorption peaks at 1082 and 1157 cm⁻¹ are the evidence of presence of starch moiety.

<table>
<thead>
<tr>
<th>IR absorption Peak</th>
<th>Vibrational assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1082 cm⁻¹</td>
<td>C-CH deformational vibration.</td>
</tr>
<tr>
<td>1162 cm⁻¹</td>
<td>Glycosidic bond.</td>
</tr>
<tr>
<td>927, 859 and 764 cm⁻¹</td>
<td>These three peaks confirm the presence of α-configuration of the glycosidic linkage typical for starch.</td>
</tr>
<tr>
<td>709 cm⁻¹</td>
<td>C-H bending vibration</td>
</tr>
<tr>
<td>983 cm⁻¹</td>
<td>May be due to H bonded O-H vibration.</td>
</tr>
<tr>
<td>1463 cm⁻¹</td>
<td>May be due to C-H/O-H bending vibration.</td>
</tr>
</tbody>
</table>
Figure 4: FT-IR spectrum of citrate (a) and Dextran (b) coated iron oxide nanoparticles. (a): red: bare nanoparticles, blue: citrate and green: citrate coated nanoparticles. (b): cyan: bare nanoparticles, red: starch and pink: starch coated nanoparticles.

**FT-IR analysis:**

Presence of peaks at 568 cm\(^{-1}\) is the evidence of presence of nano iron oxide [17]. In bulk iron oxide these two peaks found at shorter wave number and they
shifted to higher wave number with reduction in size (ultra fine particle with proper surface modification). Absence of the peak at 800 and 900 cm\(^{-1}\) confirmed then absence of Geothite (\(\alpha\)FeOOH), which could be formed during synthesis if pH is not properly maintained.

**Conjugation of Citrate moiety (from citrate buffer) with iron oxide nanoparticles:**

Shifting of absorption peak at 1397 and 1586 cm\(^{-1}\) directly confirms the participation of \(–\text{COOH}\) group in surface modification. The alcohol and carboxylic acid group specific spectra (1080-1300 cm\(^{-1}\)) also changed upon conjugation, which also indicated that \(–\text{COOH}\) as well as \(\text{O-H}\) may involve in conjugation. Shifting of some other (841 and 1078 cm\(^{-1}\)) unassigned peaks further confirmed the conjugation. Retention of some (910 cm\(^{-1}\), 950 cm\(^{-1}\), etc.) citrate specific bands and iron oxide nanoparticles specific bands (595 cm\(^{-1}\)) confirms the presence of citrate and nano iron oxide in the system (iron oxide nanoparticles in the Citrate buffer).

**Conjugation with Starch:**

All the peaks specific for starch moiety (1082 and 1157 cm\(^{-1}\)) and for glycosidic bond (764, 859 and 927 cm\(^{-1}\)) are present upon conjugation, which confirms the presence of starch in the starch coated iron oxide nanoparticles (SIONs) [18].

Two bands (983 and 1463 cm\(^{-1}\)), which are related to C-OH vibration has vanished upon conjugation, which further indicated that the starch conjugated with nanoparticles using the \(–\text{OH}\) group. Apart from direct contribution of \(–\text{OH}\) group,
shifting of some other unassigned bands also confirmed the conjugation. But, the nano iron oxide specific peaks overlapped with two unassigned starch specific bands. This may have happened due to presence of thick polymeric coating of starch. Because, any change in local mass changes the spring constant of chemical bond. As a result the IR absorption peak could be shifted to lower wave number. The ligand specific (citrate and starch) ‘C-O’ vibrations also blue shifted upon surface conjugation. Hence the main sites of conjugation are Fe-O and C-O moieties.

2.3.3. Purpose of synthesizing different iron Oxide NPs

In this chapter I provide the details of synthesis protocol with emphasis on the physical characterization of citrate and starch coated iron oxide NPs.

Starch and citrate coated NPs are used to study their differential role as anti-platelet agent. Citrate coated NPs is also used as a template to synthesize core-shell NPs (see Chapter III) and to study the differential magnetic environment of human serum (see Part-2 of Chapter V). The citrate coated NPs is also used to study the differential effect on subcellular flow of live HeLa cells (see Chapter VII).

The APTES and dextran coated NPs are synthesized for comparison of magnetic moment of synthesized magnetic nanoparticles only, using a proposed optical technique discussed in Chapter IV.

2.3.4. The unexplained reversal of pro-aggregatory to anti-aggregatory response induced by iron oxide NPs-possible role of magnetic interaction
Apart from being a Magnetic Resonance Imaging (MRI) contrast agent iron oxide NPs may be used as diagnostic probe. Such dual role makes iron oxide NPs an ideal thrombotic agent. We have shown [18] that citrate coated iron oxide NPs can potentially inhibit the agonist induced platelet aggregation. Platelet hyper aggregation cause thrombotic disorder such as Acute Coronary Syndrome (ACS). Generally metallic nanoparticles show pro-aggregatory effect [19-21], that is enhancement of aggregation. We have also found pro-aggregatory effect of citrate coated gold NPs [18]. The summary of the observed results is given below in tabular form.

<table>
<thead>
<tr>
<th>Platelet rich plasma with</th>
<th>Platelet aggregation</th>
<th>Magnetic moment (emu/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only agonist</td>
<td>aggregation</td>
<td>---</td>
</tr>
<tr>
<td>Agonist + citrate coated iron oxide NPs</td>
<td>No aggregation</td>
<td>3.58</td>
</tr>
<tr>
<td>Agonist + starch coated iron oxide NPs</td>
<td>Aggregation like only agonist</td>
<td>0.19</td>
</tr>
<tr>
<td>Agonist + citrate coated gold NPs</td>
<td>hyper-aggregation</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Now there may be a role of differential magnetic property of the NPs. Recently, I have found magnetism in citrate coated gold NPs (see Chapter III). I have also observed that the magnetization of iron oxide NPs reduces upon polymeric coating like dextran, (see Section 4.3.6 of Chapter IV) and starch [15]. Citrate coated iron oxide NPs shows highest magnetic moment (see section 3.3.1 of Chapter III and section 4.3.6 of Chapter IV). Platelet aggregation studies were performed at 37 °C and at that temperature range the magnetic moment of citrate coated NPs is
higher than the polymer coated NPs and citrate coated gold NPs. For gold NPs anti-ferromagnetic exchange is found at higher field strength (see Section 3.3.5 of Chapter III). This differential magnetism of NPs may be a reason of the observed NPs specific response towards platelet aggregation. We may say this is the differential response of platelet itself towards different magnetic exposure. Higher magnetic moment results in anti-platelet activity, lower magnetic moment results in neutral response and negative magnetic moment results in pro-aggregatory effect. Recently one group [22] has shown the differential platelet aggregation in presence of inbuilt magnetic field, required for stirring, within aggregometer. Hence, platelet function may be altered in presence of magnetic field.

In following Chapters I have shown the effects of magnetic field on small molecules, proteins and live cells. In Part-2 of Chapter V, differential magnetic environment of human serum has also been addressed by using citrate coated iron oxide NPs (local magnetic field) and external static magnetic field.

2.4. Conclusions

Iron Oxide nanoparticles have been successfully synthesized using chemical co-precipitation of ferrous and ferric salts. AFM images confirm that the synthesized nanoparticles are spherical in shape. Surface modification by two different ligands (citrate and starch) has been confirmed by FT-IR studies. Different iron oxide NPs are synthesized to use in the studies of following Chapters. Here the
probable functional role of magnetic moment of citrate and starch coated iron oxide NPs on platelet activity has been suggested.

### 2.5. References