Chapter 5

STUDIES ON FUNCTIONALIZATION OF Fe₃O₄ MNPS WITH CHITOSAN

There is no such thing as a failed experiment, only experiments with unexpected outcomes.

–R. Buckminster Fuller

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Magnetic chitosan nanocomposite for hyperthermia therapy application: Preparation, characterization and in vitro experiments

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5.1. Introduction

Due to better biocompatibility, injectibility, chemical stability over physiological circumstances and substantial accumulation at the diseased site, Fe$_3$O$_4$ nanocrystals have been widely used for biomedical applications. These MNPs can be used either as a diagnostic tool or in therapeutic applications. Their potential as a contrasting agent makes them suitable for diagnosing cancer in magnetic resonance imaging technique, while their heating ability allows them to have therapeutic applications as well like hyperthermia. For the in vivo applications, the inorganic NPs are often capped by a biocompatible agent like biopolymers. The capping agent can either be a surfactant with an organic/inorganic group like oleic acid resulting in oil-soluble NPs or hydrophilic MNPs could be prepared by using polyethylene glycol, citric acid etc. Capping of the MNPs may alter their surface functionality, charge, reactivity and dimensions, making them more chemically stable and suitable for in vivo applications.

Various functional polymers have been used to modify the surface of the magnetic nanoparticles. Such modification can provide magnetic nanoparticles with stability, biocompatibility, and conjugation with ligands. The resulting particles can thus be used for many applications such as biomolecular separations, drug targeting and delivery and magnetic resonance imaging (MRI).
5.2. Chitosan- a biocompatible biopolymer

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. The degree of deacetylation (%DD) can be determined by NMR spectroscopy, and the %DD in commercial chitosans ranges from 60 to 100%. On average, the molecular weight of commercially produced chitosan is between 3800 and 20,000 Daltons. The amino group in chitosan has a pKa value of ~6.5, which leads to a protonation in acidic to neutral solution with a charge density dependent on pH and the %DA-value. This makes chitosan water soluble and a bioadhesive which readily binds to negatively charged surfaces such as mucosal membranes. Chitosan enhances the transport of polar drugs across epithelial surfaces, and is biocompatible and biodegradable.

5.3. Experimental: Functionalization of MNPs with chitosan

Considering biodegradability and toxicity, much attention has been paid to chitosan, since it possesses interesting properties such as biocompatibility, biodegradability, film forming ability, gelation characteristics and bioadhesion. Another advantage of CS is that the amine groups can offer a variety of active sites for further biofunctionalization [1].
Fe₃O₄ NPs were synthesized via alkaline precipitation method [2]. Bare Fe₃O₄ MNPs were used for coating procedure. 1% bare NPs were dispersed in 1% (w/v) CS solution in 2% (v/v) acetic acid. The mixture was ultrasonicated for 30 min [3]. After ultrasonication, the CS-coated Fe₃O₄ NPs (CS- Fe₃O₄) allowed to settle, washed with distilled water 3 times to remove excess CS. These NPs were then separated and dried at 50°C in air.

Fig. 5.1: Flow-chart of the procedure for synthesis of Fe₃O₄ NPs and their surface modification using CS.
5.4. Results and Discussions

5.4.1. Effect of chitosan coating on properties of Fe$_3$O$_4$ MNPs

5.4.1.1. Structural properties

Fig. 5.2 (a) and (b) show the powder XRD patterns for bare and CS-coated Fe$_3$O$_4$ NPs respectively. The main characteristic peaks were obtained with the (hkl) values of (220), (311), (400), (422) and (511). These were then matched with the JCPDS file number 82-1533, which corresponds to Fe$_3$O$_4$ phase. Both the NPs show inverse spinel structure. The crystallite sizes of NPs were calculated from FWHM of the most intense peaks using the Debye-Scherrer formula. The crystallite sizes obtained were 20 nm and 10 nm for bare and CS-coated MNPs respectively.

![XRD patterns](image.png)

**Fig. 5.2:** XRD patterns obtained from (a) bare Fe$_3$O$_4$ and (b) CS-Fe$_3$O$_4$ MNPs.
However, the peaks became broad and intensity decreased after capping of Fe$_3$O$_4$ with CS and hence it can be clearly stated that the particle size decreased after coating procedure. The coating of amorphous CS polymer on crystalline Fe$_3$O$_4$ may induce microstrain which resulted in broadening of peaks in case of coated sample [4-6].

The Selected Area Electron Diffraction (SAED) patterns for bare and coated NPs are shown in Fig. 5.3 (a) and (b) respectively. It shows bright ring patterns indicating polycrystalline nature of the MNPs, as indicated by XRD patterns. The ring pattern corresponds to (220), (311), (400), (422) and (511) planes which can be clearly seen in XRD results.

**Fig. 5.3:** Selected Area Electron Diffraction (SAED) patterns of (a) bare Fe$_3$O$_4$ and (b) CS- Fe$_3$O$_4$ MNPs.
TGA provides a quantitative evidence of the coating of CS on NPs. In this experiment, the MNPs are heated to 600 °C under flowing N₂ and changes in mass loss of organic material are recorded. This is an extremely valuable technique for surface characterization of NPs. The information one can retrieve from TG measurements is mutual. TG analysis allows us to determine the bonding strength of the ligand to the NPs surface and its thermal stability [7]. Typically, ligands that are bound more strongly desorb at higher temperatures.

**Fig. 5.4:** Thermogravimetric spectra of (a) bare Fe₃O₄ and (b) CS-Fe₃O₄ MNPs in nitrogen with a scanning rate of 10 °C min⁻¹ up to 600 °C.

The weight losses of the bare Fe₃O₄, CS-Fe₃O₄ MNPs were measured and shown in Fig. 5.4 (a) and (b) respectively. Since the TG was performed under N₂ atmosphere, the oxidation of coated and uncoated MNPs surface was greatly
reduced. The weight loss of Fe$_3$O$_4$ NPs (~2%) occurred up to temperature 100 °C was ascribed to the evaporation of water. For the CS- Fe$_3$O$_4$ MNPs, after a gradual loss of water molecules in the polymer matrix, a great weight loss started after 200 °C at which the decomposition of CS occurred (Fig. 5.4-b). After the CS was decomposed completely, the residual substance mostly was magnetic particles. The percentage of CS in the CS- Fe$_3$O$_4$ MNPs was revealed in the TGA curve was about 21.3 wt %.

To demonstrate the successful attachment of CS onto the surface of Fe$_3$O$_4$ MNPs, NPs were investigated by FTIR spectra in the range of 450 to 4000 cm$^{-1}$. Fig. 5.5 (a), (b) and (c) show FTIR spectra of bare Fe$_3$O$_4$ and CS-Fe$_3$O$_4$ MNPs along with CS respectively. The dip around 3376 cm$^{-1}$ observed in all the three samples correspond to –OH group. The dip around 3376 cm$^{-1}$ is assigned to stretching (ν) vibrations and dip at 1620 cm$^{-1}$ is assigned to bending (d) vibrations of -OH group due to adsorbed water on the surface of MNPs. The dip at 560 cm$^{-1}$ relates to M-O vibration, which is Fe-O bond in case of bare Fe$_3$O$_4$ [8]. In the IR spectrum of CS, the characteristic absorption dip appeared at 1621 cm$^{-1}$ which can be assigned to N-H bending vibration [9] i.e. characteristic dip of primary amine(-NH$_2$) [10]. The dips at 1421 cm$^{-1}$ and 1378 cm$^{-1}$ correspond to C-H bending, dips at 1147 cm$^{-1}$, 1096 cm$^{-1}$ and 1020 cm$^{-1}$ relate to C-O stretching of primary alcoholic group in CS and dips at 2847 cm$^{-1}$ and 2923 cm$^{-1}$ appear as C-H stretching in CS.
In the spectrum of CS- Fe\textsubscript{3}O\textsubscript{4} NPs, compared with the spectrum of CS, the characteristic dip at 1621 cm\textsuperscript{-1} is shifted to 1631 cm\textsuperscript{-1} due to amide absorption and found to be relatively larger. The increase in absorption intensity at 1631 cm\textsuperscript{-1} indicated that the hydrogen of primary amino group (-NH\textsubscript{2}) in CS form strong hydrogen bonding with the oxygen in Fe\textsubscript{3}O\textsubscript{4}[11]. The other dips corresponding to C-H bending, C-H stretching and C-O stretching in primary alcoholic group also appeared in the spectrum like that of in the CS. And a new sharp dip at 583 cm\textsuperscript{-1} relates to Fe-O vibration. These results indicate that CS was successfully coated on the Fe\textsubscript{3}O\textsubscript{4} MNPs without change in phase.

**Fig. 5.5:** FTIR spectra of (a) bare Fe\textsubscript{3}O\textsubscript{4}, (b) CS- Fe\textsubscript{3}O\textsubscript{4} MNPs and (c) CS.
The EDAX spectra were used for a quantitative elemental analysis of bare and CS-coated Fe₃O₄ NPs, which are shown in Fig. 5.6 (a) and (b) respectively. The corresponding peaks in bare NPs were due to Fe and O only, while CS-coated NPs showed additional peaks corresponding to C and N as expected. Both the spectra did not show any additional impurity peak implying purity of the samples.

![EDAX spectra](image)

**Fig. 5.6:** EDAX spectra of bare (a) Fe₃O₄ and (b) CS-Fe₃O₄ MNPs.

### 5.4.1.2. Morphological properties

SEM images of Fe₃O₄ and CS-Fe₃O₄ are shown in Fig. 5.7 (a) and (b) respectively. The bare particles showed a high degree of agglomeration due to dipole – dipole interaction. Both the MNPs were spherical in shape. It was observed that after coating, the particles showed less degree of agglomeration as compared to bare MNPs due to presence of non-magnetic CS layer at the surface of magnetic core NPs since the extent of dipolar coupling is related to the distance...
between particles [12]. Although surface modified NPs were distinct, they formed small clusters mainly due to magnetic nature of the NPs.

![SEM images of (a) bare Fe$_3$O$_4$ and (b) CS- Fe$_3$O$_4$ MNPs.](image)

**Fig. 5.7:** SEM images of (a) bare Fe$_3$O$_4$ and (b) CS- Fe$_3$O$_4$ MNPs.

The size and shapes of the MNPs before and after surface modification were observed using TEM. The TEM images of Fe$_3$O$_4$ and CS- Fe$_3$O$_4$ are shown in Fig. 5.8 (a) and (b) respectively. Bare Fe$_3$O$_4$ NPs were highly agglomerated with particle size 21.8 ± 5.3 nm while CS coated NPs were well dispersed with particle size 15.1 ± 5.0 nm. The particle size obtained using FeCl$_2$ only is comparable with earlier reports (8-20 nm) in which Fe$_3$O$_4$ NPs were synthesized using common co-precipitation method [13-15]. The particle size is reported to be decreased after coating procedure in earlier reports [16]. After surface modification, the particles maintained their original spherical shapes without any deformation or growth. The particles obtained were with less degree of aggregation and were well dispersed.
5.4.1.3. Magnetic properties

Generally Fe$_3$O$_4$ NPs showed superparamagnetic behavior below size less than 20 nm which is characterized by zero coercivity and zero remanence [17-19]. The magnetic characterization of both the MNPs was recorded using SQUID at 300 K as well as at 100 K with maximum applied field up to ±20,000 Oe. $M$-$H$ curves of bare Fe$_3$O$_4$ and CS- Fe$_3$O$_4$ MNPs at 100 K and 300 K are shown in Fig. 5.10 (a) and (b) respectively. The Saturation Magnetization ($M_s$), Coercivity ($C_e$) and Remenance ($M_r$) values calculated from the $M$-$H$ curves for both bare and CS-coated MNPs are given in Table I. The graphs clearly show superparamagnetic nature of both the NPs at 300 K as $C_e$ and $M_r$ values were very negligible. Earlier reports where Fe$_3$O$_4$ NPs were synthesized using co-precipitation method also exhibited superparamagnetic behavior [14, 15]. This can be ascribed to the small size of NPs which were smaller than the superparamagnetic critical size (25 nm)
The values of Ce and Mr decreased with increase in temperature showing transition from ferromagnetic to superparamagnetic nature. The Ms increased with decrease in temperature due to overcoming of magnetic anisotropic energy over thermal effect.

![MH curves of bare Fe₃O₄, CS-Fe₃O₄ MNPs at (a) 100K and (b) 300K](image)

**Fig. 5.10**: MH curves of bare Fe₃O₄, CS-Fe₃O₄ MNPs at (a) 100K and (b) 300K, insets show M-H at low fields.

Ms values of bare NPs were observed to be 55.74 and 51.68 emu/g for 100 and 300 K respectively which were small compared to that of theoretical value of bulk Fe₃O₄ (Ms = 92 emu/g). Ms has been reported to decrease as the particles size of Fe₃O₄ decreases below 30 or 20 nm, due to finite size effect [17]. Ms of the MNPs was also smaller to those in reported ones which were synthesized by a
typical co-precipitation method [15]. Coating process did not affect much the magnetization of bare Fe₃O₄ NPs.

**Table 5.1:** The Saturation Magnetization (Ms), Coercivity (Ce) and Remanence (Mr) values calculated from the MH curves for both bare and CS-coated Fe₃O₄ MNPs.

<table>
<thead>
<tr>
<th></th>
<th>Fe₃O₄</th>
<th>CS- Fe₃O₄</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100K</td>
<td>300K</td>
</tr>
<tr>
<td>Ms (emu/g)</td>
<td>55.74</td>
<td>51.68</td>
</tr>
<tr>
<td>Mr (emu/g)</td>
<td>13.91</td>
<td>2.23</td>
</tr>
<tr>
<td>Ce (Oe)</td>
<td>160.82</td>
<td>16.85</td>
</tr>
<tr>
<td>Mr/Ms</td>
<td>0.25</td>
<td>0.04</td>
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The Curie temperature is an important magnetic characteristic. It reveals the temperature of transition of the magnetic materials from a ferri/ferro-magnetic to a paramagnetic state. Fig. 5.11 (a) and (b) show the temperature susceptibility plots of Fe₃O₄ NPs and CS-Fe₃O₄ NPs respectively. The plots were used to determine the Curie temperature. Each plot contains two components, viz. heating curve (i) and cooling curve (ii). Heating of the sample was firstly carried out from 77K to 973 K. The lower temperature i.e. 77 K was achieved using liquid nitrogen. After 973 K, temperature is allowed to decrease naturally to obtain the cooling
curve. Finally, the sample comes to the room temperature and the final plot is obtained. The sudden drop in magnetic susceptibility at \( \sim 853 \text{ K} \) in heating curve corresponded to Curie temperature of \( \text{Fe}_3\text{O}_4 \) NPs [20]. The coating of CS did not affect the Curie temperature. The plots showed that alteration started above 853 K, because above that temperature the heating and the cooling curves were not reversible anymore. This changing can be interpreted phase as an oxidation product of the magnetite present [21]. Magnetic susceptibility for bare MNPs started to increase from start, reaches a maximum at about \( \sim 650 \text{ K} \), and then decreased to nearly zero at \( \sim 853 \text{ K} \), which is consistent with the Curie temperature of magnetite. In case of coated MNPs, peak point of magnetic susceptibility shifted to \( \sim 550 \text{ K} \). After that it started to decrease upto \( \sim 700 \text{ K} \) and again rises to \( \sim 840 \text{ K} \) and decreased suddenly to nearly zero at \( \sim 853 \text{ K} \). This change in behavior was may be due to presence of CS in the coated sample.

![Fig. 5.11](image)

**Fig. 5.11**: The susceptibility vs temperature graphs of (a) bare \( \text{Fe}_3\text{O}_4 \) NPs and (b) CS- \( \text{Fe}_3\text{O}_4 \) NPs.
5.4.2. Colloidal stability studies

In order to use these nanoparticles for \textit{in vivo} biomedical applications, they must be in the form of aqueous colloidal suspensions. It is important to study surface chemistry and stability of the particles in water as a function of pH to accomplish this requisite. Electrophoretic mobility data was transformed to zeta potential values, which were related to the surface charge density and depend on the oxide composition, crystalline form, size and surface characteristics. Suspensions of superparamagnetic Fe$_3$O$_4$ nanoparticles have Van der Waals forces and magnetic dipole-dipole interactions generated from residual magnetic moments, which tend to agglomerate the particles. Therefore repulsive forces are required to keep each particle discrete and prevent it from amassing as larger and faster setting agglomerates. Steric hindrance plays an important role in stabilizing suspensions, which is accomplished by the protective shields on the oxide surface produced by molecules or polymers.

The zeta potential values and hydrodynamic diameters of bare and coated nanoparticle suspensions in water with respect to pH are shown in Fig. 5.9. The zeta potential of coated particles is more positive in the range of acidic pH, as compared to bare indicating that the positive charges on the coated nanoparticles increase with a decrease in pH, may be due to the protonation of free amino groups at low pH. The protonated amino groups provide enough charge to stabilize coated nanoparticles at acidic pH. At pH 6, zeta potential for coated nanoparticles is lower because the isoelectric point of chitosan is 6.3, and the suspension at pH 6 is stabilized by steric stabilization mechanism. At higher pH, zeta potential of bare nanoparticles is more negative than coated. The isoelectric points (pI) for bare and coated nanoparticles were found to be
around 6.7 and 6.4 respectively. The bare nanoparticles possess negative charge at physiological pH which was in agreement with the literature.

![Graph showing Zeta Potential and Hydrodynamic Diameter as a function of pH for Fe₃O₄ and CS-Fe₃O₄ MNPs.]

**Fig. 5.9:** Hydrodynamic diameter and zeta potential as a function of pH for (a) Fe₃O₄ and (b) CS-Fe₃O₄ MNPs dispersed in water.

Dynamic Light Scattering (DLS) measurements were carried out to investigate the hydrodynamic size of bare and coated nanoparticles. The hydrodynamic diameter distribution of unmodified and CS-stabilized Fe₃O₄ nanoparticles at a scattering angle of 90° are shown in Fig. 5.9. The bare nanoparticles showed higher particle size as compared to coated one due to formation of agglomerates by the virtue of dipole–dipole interaction. The particle size of coated nanoparticles was in good agreement with the TEM and zeta potential results, the size was reduced by the repulsive forces acted on the particles due to formation of electrostatic and steric interactions of the coated material.
5.4.3. Cytotoxicity studies

The cytotoxicity study of both, bare and coated nanoparticles was done on L929 and cell line with different concentrations of nanoparticles and the obtained data is shown in Fig. 5.12. The L929 cell line was incubated with nanoparticles for 48 h with the concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mg.mL\(^{-1}\) at 37 °C in 5% CO\(_2\) atmosphere. The relative cell viability (%) compared with control well containing cells without nanoparticles are calculated by the equation: 

\[
\left( \frac{\text{[A]_{tested}}}{\text{[A]_{control}}} \right) \times 100
\]

![Cytotoxicity profiles of MNPs for 48 h on L929 cell line at different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mg.mL\(^{-1}\)).](image)

**Fig. 5.12:** Cytotoxicity profiles of MNPs for 48 h on L929 cell line at different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mg.mL\(^{-1}\)).
Fig. 5.12 shows the cell viability after incubation with different concentrations of both bare and coated Fe$_3$O$_4$ nanoparticles. It can be seen from the graph that the cell viability remained nearly unchanged even after 48 h incubation with both bare and coated MNPs. The results were highly satisfactory in order to use the MNPs for *in vivo* hyperthermia therapy application.

### 5.5. Conclusions

This study demonstrated the effect of CS coating on the surface behavior of Fe$_3$O$_4$ MNPs. The pure phase of Fe$_3$O$_4$ NPs could be obtained using FeCl$_2$ as the sole precursor even at low temperature. Conventional co-precipitation method was more simplified and made cost-effective in this work. A simple ultrasonication method can be used for coating of chitosan on the surface of Fe$_3$O$_4$ MNPs. The probable mechanism of the coating was formation of strong hydrogen bonding between the hydrogen of primary amino group (-NH$_2$) in CS with the oxygen in Fe$_3$O$_4$ NPs. FTIR spectrum of coated MNPs showed larger amide absorption which confirmed coating of CS on Fe$_3$O$_4$ NPs. XRD pattern proved inverse spinel structure of the particles. TEM and SEM images showed that the particles are monodispersed, spherical-shaped having diameter of 21.8 ± 5.3 nm and 15.1 ± 5.0 nm in case of bare and coated particles respectively. SAED and XRD proved polycrystalline nature of particles. SQUID showed that coating procedure did not affect much the magnetization of the Fe$_3$O$_4$ NPs. Both the MNPs were superparamagnetic at room temperature with negligible *Ce* and *Mr*. Cell viability
assay of both the MNPs showed very low cytotoxic effect on L929 cell line even after 48 h incubation period. Thus synthesized MNPs using alkaline precipitation method were suitable for hyperthermia therapy applications owing to their smaller size, superparamagnetic behavior at room temperature, higher magnetization and SAR values and low cytotoxicity.
REFERENCES


