Assessing the structural, morphological and magnetic properties of polymer-coated magnesium-doped cobalt ferrite (CoFe$_2$O$_4$) nanoparticles for biomedical application

SR Mokhosi$^1$, W Mdlalose$^2$, S Mngadi$^1$, M Singh$^1$ and T Moyo$^2$

$^1$Discipline of Biochemistry, $^2$Discipline of Physics, University of KwaZulu-Natal, Westville campus, P/Bag X54001, Durban 4000, South Africa

Email: Mokhosis@ukzn.ac.za

Abstract. In this study, we have functionalised cobalt ferrite (CoFe$_2$O$_4$) nanoparticles (NPs) by doping with a natural bio-mineral magnesium (Mg) and coating with three polymers to enhance biocompatibility and feasibility for therapeutic applications. The glycol-thermal method was employed to synthesise CoFe$_2$O$_4$ and Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ NPs. The latter NPs were functionalised with chitosan (CHI), poly-ethylene glycol (PEG) and poly-vinyl alcohol (PVA) to produce CHI-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$, PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ and PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$. The structure and morphology of NPs were characterized using transmission electron microscopy (TEM), high resolution TEM (HR-TEM), X-ray diffraction (XRD), Fourier transform infra-red (FTIR) spectroscopy and nanoparticle tracking analysis (NTA). Magnetic measurements were carried out using a vibrating sample magnetometer (VSM). XRD patterns confirmed inverse cubic spinel phase structure typical of ferrite NPs. NPs exhibited spherical shape with average size diameters of ranging between 8 nm and 11 nm. Coating increased these average size diameters up to 13 nm. Zeta potential measurements indicated low colloidal stability of the NPs which improved considerably with PEG and PVA coating. FTIR confirmed surface modifications seen in additional peaks characterised by amine and carbonyl groups for chitosan and PEG/PVA, respectively. CoFe$_2$O$_4$ NPs exhibited high saturation magnetisations of 73.861 emu/g. This value decreased with magnesium-doping and polymer-coating due to shielding effect. In vitro cytotoxicity analysis demonstrated significant tolerability of coated Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ NPs at concentrations of 800 μg/ml in cervical cancer (HeLa) cell lines. Conclusively, these polymer-coated ferrites present feasible nanocarriers in magneto-targeted drug delivery.

Keywords: cobalt ferrites; metal-doping; chitosan; PEG, PVA, biocompatible; cytotoxicity

1. Introduction

Magnetic nanoparticles (NPs) are receiving increased attention in a diverse range of fields such as physics, medicine and biology owing to their many unique properties. Iron oxide based magnetic NPs, including magnetites (Fe$_3$O$_4$) and maghemites (γ-Fe$_2$O$_3$) have been most commonly explored in biomedical applications as contrast agents for MRI, cell labelling, thermal ablation therapy and magneto-targeted drug delivery [1-5].

There are several critical features that are important in the use of magnetic NPs for biotherapeutic applications. One aspect to consider in synthesis is the size of the NPs. With a decrease to a critical diameter range below 15 – 20 nm, the multi-domain structure transforms to a single domain, thus conferring superparamagnetism [5]. Superparamagnetic NPs are reported to be the most suitable candidates for biomedical application. This is attributed to their high saturation magnetisation and negligible remanence and coercivity [5, 6]. However, bare NPs present a myriad of challenges in vivo which include cellular uptake, colloidal stability and RES clearance. Advantageously, when sizes are below 100 nm, the large surface-to-volume ratio ensures relatively easy surface modifications [4, 5]. This feature in turn permits for a more precise design and engineering approach. Conjugation with bioactive molecules is thus commonly employed to improve biocompatibility, enhance circulation in the blood stream, enable for optical detectability, and target specificity in therapeutic delivery and most importantly, present no toxicity in the body [1-5].
Spinel ferrites (\(MFe_2O_4\)) have in recent years, emerged as attractive NPs within the field of material synthesis and engineering. This is attributed to their facile synthesis, good chemical stability and magnetic tunability [5-8]. Despite this, they remain under-researched in biomedicine. Cobalt ferrites (CoFe_2O_4) are considered hard magnetic materials and have demonstrated great potential as candidates for bio-application [6]. They can be synthesised using a variety of methods such as co-precipitation, sol-gel, ball milling, micro-emulsion, solvo-thermal and thermal decomposition. As with all the spinel ferrites, CoFe_2O_4 allows for incorporation of various other metal ions, such as magnesium, manganese and zinc into its lattice to give rise to new and interesting materials. This could play an important role in enhancing their physical and magnetic properties [5-6]. Magnesium is one of the most abundant intracellular minerals in the body. It is thus, hypothesised that doping CoFe_2O_4 with this cation may present favourable advantages for cellular uptake.

Chitosan (CHI) is a naturally abundant, hydrophilic, biocompatible and biodegradable polymer. It has repeating units with useful functional groups in its backbone structure including 2 hydroxyl groups and amino group [3, 5]. These characteristics have seen its extensive application in drug delivery, medicine, adhesives, and fuel cells. Poly-ethylene glycol (PEG) and poly-vinyl alcohol (PVA) represent the most studied synthetic hydrophilic polymers. PEG has established itself as an excellent stabiliser of NPs by preventing opsonisation and providing steric hindrance. These polymers have been reported to improve hydrophilicity and solubility of NPs which results in enhanced biocompatibility and biodegradability [1-5].

The aim of this study was to synthesise CoFe_2O_4 NPs using the glycol-thermal method. Furthermore, the influence of magnesium-doping and polymer-coating on structural, morphological and magnetic properties of the CoFe_2O_4 were assessed using various characterisation techniques. In vitro cytotoxicity studies were performed using the Alamar blue assay to evaluate the effect of doping and polymer-coating on viability of cervical cancer (HeLa) cell line.

2. Experimental details

2.1 Synthesis and coating of NPs
The glycol-thermal method was employed to synthesise CoFe_2O_4 and Mg_{0.5}Co_{0.5}Fe_2O_4 NPs. Briefly, stoichiometric measurements of cobalt chloride tetrahydrate (CoCl_2.4H_2O, 98%), iron (III) chloride tetrahydrate (FeCl_3.4H_2O, 98%) and magnesium chloride dehydrate (Cl_2Mg.6H_2O, 99%) were appropriately weighed as reported by Dlamini et al. [9]. Mg_{0.5}Co_{0.5}Fe_2O_4 NPs were further coated with chitosan (from shrimp shells, C_6H_11NO_4)n, ≥75 % deacetylated) using a previously published method by Khalkhali et al. [10] to produce CHI-Mg_{0.5}Co_{0.5}Fe_2O_4 [10]. Briefly, 0.5 grams of chitosan was weighed out and dissolved in a 100 ml of acetic acid in order to prepare the 0.5% chitosan solution maintained at a pH of 4.8. Thereafter, approximately 0.2 grams of Mg_{0.5}Co_{0.5}Fe_2O_4 NPs were weighed and dissolved in the chitosan solution. The resulting mixture was sonicated using a Scientech Ultrasonic bath at 60 °C for an hour. Thereafter, it was stirred mechanically using an IKA RW 20 Digital Dual-Range Mixer System for 18 hours at room temperature. The black homogeneous mixture attained was separated by centrifugation at 3000 rpm for 30 minutes. It was then left to dry at room temperature.

Coating with PEG (Polyethylene glycol, MW: 2000) was carried out as per method by Ehi-Eromosele et al. [11] with modifications. Briefly, 1 gram of dried nanoparticles and 3 grams of PEG in 96 grams of deionised water and complete dissolution of PEG was achieved under vigorous stirring. The final solution had the ratio of NPs: PEG = 1:3. The solution remained stirred for 20 hours at room temperature in order to achieve coating. 3 wt. % PEG-functionalized NPs were then separated with a permanent magnet and washed three times with deionized water. Finally, pure PEG-coated NPs were separated and dried at 60 °C after removal of all the residual PEG with washing. The same protocol was employed for PVA-coating with some modifications. Coating was achieved at 80 °C followed with cooling by switching off the temperature controller for overnight stirring. 3 wt. % PVA-functionalized NPs were then separated with a permanent magnet and washed three times with deionized water.

2.2. Characterisations
X-ray powder diffraction patterns were recorded on a Empyrean PANanalytical X-Ray diffractometer using monochromatic CoKα (1.7903 Å) radiation at room temperature in the range of 10 to 80° in the 2θ scale, with a scanning speed of 0.02° per second and a step time of 3 seconds. All the peaks of XRD
patterns were analyzed and indexed using ICD data base. Crystallite size measurements were obtained using the full-width at half maximum (FWHM) of the strongest reflection of the (311) peak, by applying the Scherrer approximation. Transmission electron microscopy (TEM) was carried out under a JEM-1010 Transmission Electron Microscope operated at an accelerated voltage of 100 kV. The MegaView III Soft Imaging Systems (SIS) side-mounted 3 megapixel digital camera was used to document the micrographs. Selection and visualization of samples were performed using SIS iTEM software. Surface morphology of the nanoparticles was investigated using Scanning Electron Microscopy (SEM). Samples were coated with gold using a Q150R Rotary-Pumped Sputter Coater and viewed under a Zeiss Ultra Plus FE-SEM (Field Emission Scanning Electron Microscope) at a magnification of 3500 X. A Perkin Elmer Spectrum 100 FTIR (Fourier Transform Infrared) spectrometer was used for the FTIR analyses in which a Universal Attenuated Total Reflectance (ATR) component was bound to it. The analyses were performed at room temperature and the data needed was obtained by using the Spectrum ® Software. The NPs were loaded onto the ATR crystal for measurements. Stability, zeta potential and hydrodynamic size distributions of the nanoparticles were carried out using a Malvern NanoSight NS500 in distilled water at 25 ℃. Data analyses was performed using the NanoSight NTA 3.2 Software. Magnetization measurements of the NPs were performed at room temperature using the LakeShore Model 735 Vibrating Sample Magnetometer (VSM) in applied fields up 14 kOe.

2.3. Cytotoxicity Studies

Cytotoxicity of HeLa cell lines was investigated using the Alamar Blue assay which measures cell mitochondrial metabolic activity of viable cells. Briefly, cells (2.0 × 10^4 per well) were seeded in 96-well plates containing growth medium and incubated at 37 °C in a 5% CO2 incubator for 24 hours. The medium was replaced with fresh medium and cells were treated with varying concentrations of NPs (100, 200, 400, 800 µg/ml) for 48 hours. The spent medium was then removed, and new growth medium containing 10% Alamar solution was added and cells incubated for 4 hours. Control cells were incubated at same conditions without the NPs. All assays were done in triplicate. The absorbance at 540 nm was detected in a microplate reader and the percentage cell viability was estimated using the equation: % Cell viability = [absorbance of treated cells/absorbance of untreated cells] × 100. Statistical analyses of data were carried out using GraphPad Prism version 5.01 (GraphPad Software Inc., CA, USA). All data were presented as mean ± SD (standard deviation). The significance of the results and differences between control and treatment were evaluated for triplicate sets of data by using one-way analysis of variance (ANOVA). Tukey's-HSD multiple range post hoc test was employed and significant differences at p<0.05, p<0.005 and p<0.001 were represented as follows: *, ** & **** respectively.

3. Results and Discussion

The XRD patterns confirmed successful synthesis of NPs exhibiting single-phase cubic spinel structure typical of ferrites (Figure 1(a)). All peaks were indexed at characteristic 2θ = 30.1°, 35.4°, 43.3°, 53.6°, 57.2° and 62.7° and attributed to (220), (311), (400), (422), (511), and (440) Bragg reflections using the standard Joint Committee on Powder Diffraction for MFe2O4 (JCPDS no. 22-1086) [7]. The full width at half maximum of the (311) XRD peak was used to calculate the crystallite size D of NPs using Scherrer's equation: \[ D = \frac{k \lambda}{\beta \cos \theta} \], where D is the crystallite size, \( \lambda = 1.7903 \, \text{Å} \) is the wavelength of the CoKα and \( \beta \) is the broadening of the diffraction line measured at half maximum intensity (in radians). Polymer-coating of Mg0.5Co0.5Fe2O4 did not alter its crystalline structure as no impurity peaks were detected.

FTIR spectra confirmed the presence of magnesium and polymers as seen in Figure 1(b). The large peaks seen at 534 cm\(^{-1}\) is attributed to the vibration band of tetrahedral Fe-O functional group characteristic of a spinel ferrite. The O-H bending of all NPs was observed at 3305 cm\(^{-1}\). Both PEG and PVA coated NPs displayed additional C-H alkyl stretching vibration at 3072 cm\(^{-1}\) and the C=O stretch was at 1625 cm\(^{-1}\) owing to adsorption of polymers. Other interesting peaks include 1218 cm\(^{-1}\) for C-O-C bending for Mg0.5Co0.5Fe2O4. Intense peaks were observed for CHI-Mg0.5Co0.5Fe2O4 at 1634 and 1619 cm\(^{-1}\) depicting characteristic N-H bending due to amide groups of chitosan. At 1019 cm\(^{-1}\), there is a significant peak due to C-O-C stretching vibrations.
Figure 1. XRD (a) and FTIR (b) spectra of A. CoFe$_2$O$_4$; Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$; C. CHI-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$; D. PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ and E. PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$.

Figure 2. TEM image of A. CoFe$_2$O$_4$; and HR-TEM images of B. Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$; C. CHI-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$; D. PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ and E. PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ respectively.

Figure 3. SEM images A. CoFe$_2$O$_4$; B. Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$; C. CHI-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$; D. PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ and E. PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ respectively.

TEM and HR-TEM micrographs (Figure 2) showed agglomerated NPs with spherical shape. The tendency of the grains to agglomerate is observed in their respective SEM images (Figure 3). This can be related to the higher interaction between magnetic particles [7]. The crystallite sizes obtained from XRD correlated well with those obtained from TEM with a range between ~8 nm and ~13 nm. CoFe$_2$O$_4$ were measured at 8.70 nm, which increased up to 12.96 nm after magnesium-doping and coating with PEG (Table 1). Polymer-coating increased the crystalline sizes of the NPs and interestingly, PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ remained unchanged.

Table 1. Size and stability measurements of nanoparticles using TEM, XRD and NTA.

| Sample               | TEM Sizes (nm) | XRD Crystalline Sizes (nm) | NTA Hydrodynamic Sizes (nm) | Zeta potential ($\zeta$) (mV) |
|----------------------|----------------|-----------------------------|----------------------------|------------------------------|
| CoFe$_2$O$_4$        | 8.70           | 9.51                        | 86 ± 30                    | -1.5 ± 0.3                   |
| Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 10.16        | 10.76                       | 65 ± 60                    | 0.6 ± 1.7                    |
| CHI-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 11.02         | 11.03                       | 129 ± 6                    | -9.7 ± 0.1                   |
| PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 12.96        | 11.06                       | 81 ± 2                     | -33 ± 1                      |
| PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 9.63          | 10.85                       | 97 ± 5                     | -33.6 ± 0.5                  |
Nanoparticle tracking analysis of is a crucial tool in determining size and stability in physiological conditions. The hydrodynamic size range of nanoparticles was observed to be between 64.9 and 129.4 nm (Table 1). CoFe$_2$O$_4$ and Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ both measured broader size distributions compared to the coated NPs which exhibited narrower distributions. This finding demonstrates expected improved stability of the NPs with coating [7]. Agglomeration often results due strong dipolar interactions between the NPs. This was further indicated by the near-zero zeta potential measurements in non-coated viz. CoFe$_2$O$_4$ and Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$. The increase in colloidal stability was considerable with PEG and PVA coated derivatives exhibiting -33 and -33.6 mV, respectively [11]. CHI- Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ exhibited hydrodynamic size of up to 129 nm, with zeta potential of -9.7 mV. The negative charge was unexpected as chitosan is cationic in nature.

![Figure 4. Magnetisation versus magnetic field curves for: (a) uncoated NPs viz. CoFe$_2$O$_4$, Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ and (b) polymer-coated CHI-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$, PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ and PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$.](image)

All NPs were superparamagnetic in nature as seen in VSM hysteresis loops (Figure 4). CoFe$_2$O$_4$ and Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ NPs exhibited high saturation magnetisation ($M_S$) of 73.9 and 66.9 emu/g, respectively (Table 2). This is resultant of shielding effect and was most pronounced with chitosan-coating on Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ as observed by Patil et al. [12]. PEG-coating was found to accentuate $M_S$ of ferrite NPs [11]. Coercivity was observed to be lower in CoFe$_2$O$_4$ (32.0 Oe) compared to Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ (119.9 Oe), revealing near superparamagnetic nature for CoFe$_2$O$_4$ nanoparticles. Similar results were previously reported by Dlamini et al. [9]. In the Stoner-Wohlfarth theory, the coercive fields and saturation magnetisation are related by the equation $H_c = 0.96K/M_S$, where $K$ is the anisotropy constant [13].

| Sample          | $M_S$ (emu/g) ±0.1 | $H_c$ (Oe) ±0.5 | $K$ (Oe emu/g) ±38 |
|-----------------|-------------------|-----------------|-------------------|
| CoFe$_2$O$_4$   | 73.9              | 32.0            | 2363±38           |
| Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 66.9        | 119.9           | 8356±37           |
| CHI- Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 50.5         | 77.1            | 4056±28           |
| PEG-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 67.6         | 125.6           | 8844±38           |
| PVA-Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ | 69.9         | 120.7           | 8788±39           |

Values of $K$ are also given in Table 2. The mixed ferrite Mg$_{0.5}$Co$_{0.5}$Fe$_2$O$_4$ has higher anisotropy than CoFe$_2$O$_4$. Interestingly, $K$ was observed to increase in PEG and PVA coated samples. This suggests that PEG and PVA behave as ‘binders thus resulting in enhanced coercive fields and $K$.
Figure 5. Cell viability in HeLa cell line using Alamar blue assay. Values represent mean ± standard deviation (n = 3). Tukey’s-HSD multiple range post hoc test, P<0.05. The asterisk over the bars: *, ** & **** represent the significant differences at p<0.05, 0.005 and 0.001, respectively.

Further, in vitro cytotoxicity studies were conducted to evaluate the effect of polymer-coating on HeLa cell line viabilities (Figure 5). Increasing concentrations resulted in reduced viabilities however, it was found to be statistically insignificant. Notably, a significant difference was observed at concentrations of 800 µg/ml where polymer-coating resulted in improved tolerability compared to the non-coated CoFe₂O₄ NPs. Patil et al. [12] reported non-toxicities of chitosan-coated NPs at concentrations of 2 mg/ml. These results demonstrate the impact of polymers towards enhancing biocompatibility of NPs as reported in various studies [3, 5].

4. Conclusions
CoFe₂O₄ NPs were successfully synthesised by the glycol-thermal method. Doping with a natural biomineral such as magnesium may prove to be a useful tactic to further improve on cellular uptake and consequently biocompatibility. Polymer-coating enhanced colloidal stability and dispersibility of NPs. The coated NPs also exhibited interesting magnetic properties for possible biomedical applications. To this end, the combined effect of doping and polymer-coating led to improved tolerability of these CoFe₂O₄ in HeLa cells at high concentrations, thus presenting potential and suitable candidates for magneto-targeted therapy.

Acknowledgements
The National Research Foundation (NRF), Pretoria, South Africa for the funding through the Thuthuka Grant. The Non-Viral Gene and Drug Delivery Research Lab, Biochemistry (UKZN). Mr Sibusiso Mtshali and Mr Sanele Dlamini for data collection.

References
[1] Gupta A K and Gupta M 2005. Biomaterials. 26 3995
[2] Estelrich J et al. 2015. Int. J. Mol. Sci. 16 8070
[3] Mahmoudi M et al. 2011. Adv. Drug Deliv. Rev. 63 24
[4] Mohammed L et al. 2017. Particuology. 30
[5] Huang et al. 2016. Adv. Funct. Mater.
[6] Jauhar et al. 2016. RSC Adv. 6 97694
[7] Humbe A V et al. 2015. AIP Conf. Proc. 1665 050138-1
[8] Makridis A et al. 2016. Mater. Sci. Eng., C. 63 663
[9] Dlamini W B, Msomi J Z and Moyo T 2015. J. Magn. Magn. Mater. 373 78
[10] Khalkhali M et al. 2015. DARU J. Pharm. Sci. 23 45
[11] Ehi-Eromosele C O, Ita B I and Iweala E E J 2016. Dig J Nanomater Biostruct. 11
[12] Patil et al. 2014. J. Magn. Magn. Mater. 355 22
[13] Hemeda, O M et al. 2014. J. Magn. Magn. Mater. 364 39