Iron(III) and manganese(II) substituted hydroxyapatite nanoparticles: characterization and cytotoxicity analysis

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Abstract. Calcium hydroxyapatite (HA) is the main inorganic component of natural bones and can bond to bone directly in vivo. Thus HA is widely used as coating material on bone implants due to its good osteoconductivity and osteoinductivity. Metal ions doped HA have been used as catalyst or absorbents since the ion exchange method has introduced new properties in HA which are inherent to the metal ions. For example, Mn²⁺ ions have the potential to increase cell adhesion while Fe³⁺ ions have magnetic properties. Here, Fe(III) substituted hydroxyapatite (Fe-HA) and Mn(II) substituted hydroxyapatite (Mn-HA) were produced by wet chemical method coupled with ion exchange mechanism. Compared with pure HA, the colour of both Fe-HA and Mn-HA nanoparticles changed from white to brown and pink respectively. The intensity of the colours increased with increasing substitution concentrations. XRD patterns showed that all samples were single phased HA while the FTIR spectra revealed all samples possessed the characteristic phosphate and hydroxyl adsorption bands of HA. However, undesired adsorption bands of carbonate substitution (B-type carbonated HA) and H₂O were also detected, which was reasonable since the wet chemical method was used in the synthesis of these nanoparticles. FESEM images showed all samples were elongated spheroids with small size distribution and of around 70 nm, regardless of metal ion substitution concentrations. EDX spectra showed the presence of Fe and Mn and ICP-AES results revealed all metal ion substituted HA were non-stoichiometric (Ca/P atomic ratio deviates from 1.67). Fe-HA nanoparticles were paramagnetic and the magnetic susceptibility increased with the increase of Fe content. Based on the extraction assay for cytotoxicity test, both Fe-HA and Mn-HA displayed non-cytotoxicity to osteoblast.

Keywords: Hydroxyapatite, nanoparticles, ion-exchange, paramagnetic, cytotoxicity.

1. Introduction
Population ageing is a global phenomenon. It is expected that by the year 2050, the number of people aged 60 years and above will be the first time in history to exceed the number of young people (under 15 years of age). Osteoporosis is common among older people because of the reduction in bone density, leading to increased risk of bone fracture, which requires prosthesis implantations (usually metallic implants) for total recovery. However, due to the bioinert property of metallic implants, the slow osseointegration process tends to cause failure of implantations. This problem is more serious for older persons who have lower proliferation and differentiation rates of bone cells [1].

Hydroxyapatite (HA, Ca₁₀(PO₄)₆(OH)₂) attracts great interest due to its good osteoconductivity and
osteoinductivity. However, pure HA implants feature inferior mechanical characteristics, which limit its use to low load bearing applications [2]. Therefore, a better alternative will be to use composite structures of thin HA layer coated metallic implants, since these implants possess both good mechanical strengths of metal and bioactivity of HA. HA coated metallic implants promote osseointegration that enhances tight-fit fixation and reduces motion damage to surrounding tissues [3-5]. Hence, the adhesion of osteoblast to HA coating is a crucial step for subsequent osteoblast functions. Mn$^{2+}$ ions increase ligand binding affinity of integrin and activate cell adhesion [6]. His-Chin Wu et al. [7] reported that iron (Fe$^{3+}$) substituted HA nanoparticles were superparamagnetic and showed good biocompatibility. This work aims to synthesize Mn$^{2+}$ and Fe$^{3+}$ doped HA and investigates their bioactivity compared to pure HA.

Wet chemical method coupled with ion exchange mechanism was used to produce pure HA, Mn$^{2+}$ substituted HA (Mn-HA) and Fe$^{3+}$ substituted HA (Fe-HA). All samples were characterized by X-ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Field Emission Scanning Electron Microscopy (FESEM) coupled with Energy Dispersive X-ray Spectroscopy (EDX) and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES). The magnetic property of Fe-HA was tested using a Vibrating Sample Magnetometer (VSM). Cytotoxicity tests for all samples were performed on osteoblast cells by extraction method according to ISO 10993 part 5 and 12.

2. Experiment

2.1. Synthesis of Pure Hydroxyapatite and Metal Ion Substituted Hydroxyapatite

Ca(OH)$_2$ (Greenrich Chemical Enterprise, 96.0 wt.% pure with less than 4% CaCO$_3$), H$_3$PO$_4$ (Merck), MnCl$_2$.4H$_2$O (Riedel-de Haën) and FeCl$_3$.6H$_2$O (Sigma-Aldrich) were used as sources of Ca, P, Mn and Fe. Pure HA was produced using the following chemical reaction:

$$10\text{Ca(OH)}_2 + 6\text{H}_3\text{PO}_4 \rightarrow \text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2 \downarrow + 18\text{H}_2\text{O}.$$ 

3.71g Ca(OH)$_2$ was suspended in 250 ml DI water (Mili-Q unit from Milipore). The suspension temperature was kept at 98.5°C and stirred at 500 rpm for 30 minutes. 2 ml 85 wt.% H$_3$PO$_4$ was diluted into 250 ml DI water and added into the Ca(OH)$_2$ suspension at a rate of 4 ml/min until the pH of mixture decreased to the desired values. Ion exchange was used for Mn-HA and Fe-HA. After the addition of H$_3$PO$_4$ was stopped, metal salt solution (different molar quantity according to the different exchange ratio $X_{M}/X_{Ca}$) was added to the mixture. The pH of the mixture was kept at desired values with NH$_4$OH. The mixture was left stirring at 500 rpm and aged at 98.5°C for 2 hours. After 24 hours at room temperature, the supernatant was replaced by DI water three times to wash away non-reacted substances. The samples were finally dried at 100°C for 12 h and grounded to powder for further characterization.

2.2. XRD Characterization

Bruker D8 Advance X-ray diffractometer with Cu-Ka radiation was used for crystal phase analysis. The diffractometer was operated at 40 kV and 40 mA. Data was collected at step size of 0.04 degree and counting time of 15 seconds per step in a 2θ range of 20-80°.

2.3. FTIR Characterization

FTIR (PerkinElmer, Spectrum GX) characterization was carried out under classic KBr pellet technique. The samples were diluted by KBr at ratio 1/300 (by weight). The FTIR spectrum was the average of five scans with resolution of 1.0 cm$^{-1}$ in the range of 400-4000 cm$^{-1}$.

2.4. FESEM and EDX Characterization

FESEM (JEOL JSM-6700F) was used to study the morphology of samples. To get the best view under FESEM, samples were slightly pressed into pellets at 0.5 ton-load. The elements present in each sample were also characterized by EDX, which is coupled with FESEM.
2.5. ICP-AES Analysis
The element concentrations in each sample were quantified by ICP-AES (Prodigy-Teledyne Instruments Leeman Lab). ICP-AES was calibrated by standard solutions for elements interested and samples were prepared by dissolving 40 mg powder in 250 ml 1 wt.% HNO₃.

2.6. Magnetic property test
The magnetic property of Fe³⁺ doped HA was studied at room temperature in the range of magnetic field 0-10 kOe using VSM (Lake Shore 7404).

2.7. Cytotoxicity Test
Cytotoxicity test was based on ISO 10993-5 and ISO 10993-12. Human osteoblast cell line hFOB 1.19 ATCC was used. Specimens were prepared by pressing 120 mg powder in a 13 mm die (Specac®, PT. No. 3006 BDH) at 1 ton-load. The pellets were autoclaved and placed into 35 mm petri dishes. 2.12 ml DMEM/F12 medium for each pellet was added and incubated at 37°C for 24 hours in an incubator. The extraction medium may contain leachable substances from samples. Before the extraction, cells were seeded into 24-well plates at a density of 2×10⁴ cell/ml and incubated for one day. The medium in each well was then replaced by the extraction medium (4 replicates for each composition) and incubated for another 24 hours. The cells cultured with DMEM/F12 medium acted as the control. MTT method was used to measure the metabolic activity of living cells. After the cells were incubated with extraction medium for 24 hours, the medium was replaced with 0.5 ml MTT solution (1 mg/ml) and incubated for another 2 hours. 0.5 ml Dimethyl Sulfoxide (DMSO) was then added and incubated for another 2 hours to dissolve the precipitate. The absorbance was measured by the Multiwell Scanning Spectrophotometer (ELISA reader) at 490 nm.

3. Results and discussion
Pure HA, Mn-HA and Fe-HA nanoparticles were successfully synthesized by wet chemical method. Pure HA was white, while the Mn-HA nanoparticles were pink and the Fe-HA nanoparticles were brown. As substitution concentration increased, the colour intensity increased accordingly.

3.1. XRD Pattern

Figure 1. XRD patterns of Fe³⁺ doped HA.
Figure 2. XRD patterns of Mn²⁺ doped HA.
Figures 1 and 2 show the XRD patterns of all samples. The peaks present agree well with ICSD file no. 26204 which is hexagonal hydroxyapatite and none of the patterns displayed extra peaks indicating that all samples were single phased HA. Compared with pure HA, all metal ion substituted HA showed comparable peaks without significant shifting of peak positions, regardless of substitution concentrations. This suggested that the ion exchange process did not greatly modify the structure of HA. For Fe-HA nanoparticles, the peak intensities decreased as substitution concentration increased (figure 1), which may be due to the high substituted concentration of Fe (20 wt.%) decreasing the crystallinity. For Mn-HA nanoparticles, the peak intensities did not decrease significantly as substitution concentration increased (figure 2).

3.2. FTIR Spectra
Figures 3 and 4 show the FTIR spectra of Mn-HA and Fe-HA nanoparticles. The detection of PO$_4^{3-}$ ($\nu_1$ to $\nu_4$) and OH$^-$ adsorption bands indicated that all samples possessed fundamental apatite structures [8]. PO$_4^{3-}$($\nu_2$) peak at around 462 – 472 cm$^{-1}$ was relatively harder to observe compared to the other PO$_4^{3-}$ bands in all spectra. In addition, carbonate CO$_3^{2-}$, and water, H$_2$O, adsorption bands were noticeable within all spectra. This was reasonable in wet chemical synthesis which required long maturing time for precipitation of hydroxyapatite. The carbonate was from atmospheric carbon dioxide and/or CaCO$_3$ impurities contained in the reactants. The carbonate groups partially replacing the PO$_4^{3-}$ groups in HA lattice, which was B-type carbonated HA. The presence of hydrogen phosphate, such as HPO$_4^{2-}$ and H$_2$PO$_4^-$ adsorption bands were not visible in all spectra.

3.3. FESEM and EDX Characterization

Figure 3. FTIR spectra of Fe$^{3+}$ doped HA.

Figure 4. FTIR spectra of Mn$^{2+}$ doped HA.

Figure 5. The FESEM image of Fe-HA 20 wt.%. 

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The FESEM image of Fe-HA 20 wt.% is shown in figure 5. This image was representative of pure HA, Mn$^{2+}$ and Fe$^{3+}$ doped HA, where all samples had similar crystal morphology. All samples were of elongated spheroids shape of around 70 nm and with a small size distribution. The elements present in HA phase, such as C, O, P, Ca and Mn or Fe, were detected by EDX for each sample. The representative EDX spectra are shown in figure 6. For both Mn-HA and Fe-HA nanoparticles, no Cl$^-$ ions were detected although MnCl$_2$ and FeCl$_3$ were used for synthesis.

### 3.4. Chemical Analysis

From ICP-AES data, Ca/P, Metal/Ca and (Ca+Metal)/P atomic ratios were tabulated in table 1. For HA (pH = 5) and HA (pH = 5.5), Ca/P atomic ratio was higher than the stoichiometric value of 1.67, which indicated that the synthetic pure HA was non-stoichiometric. This may be due to the carbonate groups partially replacing the phosphate groups in the crystal lattice or the unreacted amorphous Ca(OH)$_2$. For Mn-HA and Fe-HA nanoparticles, Ca/P ratio decreased while M/Ca ratio increased with the increase of initial metal ion concentration, indicating that more Ca ions in the lattice were replaced by the metal ions. Compared with the theoretical value of Ca/P*, ICP-AES result of Ca/P was higher. This was because not all metal ions added in the suspension were involved in the ion exchange process. The result indicated that Fe$^{3+}$ was more active than Mn$^{2+}$ in replacing Ca$^{2+}$ in HA lattice, which was consistent with the ICP-AES result of M/Ca ratio and theoretical value of M/Ca*, as shown in Table 1. For Mn-HA nanoparticles, ICP-AES result of Mn/Ca ratio was lower than the theoretical value, especially for Mn-HA 10 wt.%; while for Fe-HA nanoparticles, ICP-AES result of Fe/Ca ratio was comparable with the theoretical value. This confirmed that the exchange ratio of Mn/Ca was 1/1 while the exchange ratio of Fe/Ca was 2/3 to maintain the charge balance [9]. Thus the (Ca+Fe)/P ratio decreased with the increase of Fe content while for Mn-HA nanoparticles, the (Ca+Mn)/P ratio was comparable.

| Samples       | Ca/P  | Ca/P* | (Ca+M)/P | M/Ca  | M/Ca*  |
|---------------|-------|-------|----------|-------|--------|
| HA (pH = 5.5)| 1.681 | -     | -        | -     | -      |
| Mn-HA 1%     | 1.660 | 1.650 | 1.675    | 0.010 | 0.010  |
| Mn-HA 5%     | 1.620 | 1.584 | 1.681    | 0.041 | 0.050  |
| Mn-HA 10%    | 1.599 | 1.500 | 1.683    | 0.053 | 0.100  |
| HA (pH = 5)  | 1.713 | -     | -        | -     | -      |
| Fe-HA 1%     | 1.661 | 1.642 | 1.678    | 0.010 | 0.010  |
| Fe-HA 5%     | 1.589 | 1.542 | 1.677    | 0.055 | 0.054  |
| Fe-HA 10%    | 1.471 | 1.417 | 1.643    | 0.117 | 0.118  |
| Fe-HA 15%    | 1.334 | 1.292 | 1.591    | 0.193 | 0.194  |
| Fe-HA 20%    | 1.217 | 1.167 | 1.566    | 0.287 | 0.286  |

Ca/P*: theoretical atomic ratio of Ca to P after Ca replaced by all the M ions added at the corresponding ratio. For Mn$^{2+}$ doped HA, the exchange ratio of Mn/Ca was 1:1 and for Fe$^{3+}$ doped HA, the exchange ratio of Fe/Ca was 2/3 to maintain the charge balance.

M/Ca*: theoretical atomic ratio of M to Ca after Ca replaced by all the M ions added at the corresponding ratio.
3.5. Magnetic property of Fe$^{3+}$ substituted HA

The magnetization of the powders versus external magnetic field is shown in figure 7. The magnetization increased linearly with external field, indicating that Fe$^{3+}$ doped HA was paramagnetic. The slope, which represents the magnetic susceptibility ($\chi_g$), increased with Fe concentration. Here, for Fe-HA 10 wt.%, Fe-HA 15 wt.% and Fe-HA 20 wt.%, the $\chi_g$ was $4.4\times10^{-6}$, $7.5\times10^{-6}$, $1.0\times10^{-5}$ emu.g$^{-1}$.Oe$^{-1}$, respectively. However, His-Chin Wu et al. [7] reported that Fe$^{2+}$ substituted HA nanoparticles were superparamagnetic and the highest saturation magnetization ($M_s$) was 20.92 emu/g. This may be due to the different valance of Fe. Here, Fe$^{3+}$ was used instead of Fe$^{2+}$, as reported by His-Chin Wu et al. [7].

3.6. Cytotoxicity analysis

The relative absorbance value for Mn-HA and Fe-HA nanoparticles after MTT test are shown in figures 8 and 9. The colorimetric value presented the quantity of vital osteoblasts. Compared with pure HA, all Mn-HA and Fe-HA nanoparticles had comparable absorbance values, which suggested that all samples were non-cytotoxic. As Mn-HA nanoparticles had slightly higher absorbance values
compared with pure HA, further cell adhesion experiment will be carried out to confirm its increase in bioactivity compared to pure HA.

![Figure 8](image1.png)
**Figure 8.** The relative absorbance value for Mn$^{2+}$ doped HA after MTT test.

![Figure 9](image2.png)
**Figure 9.** The relative absorbance value for Fe$^{3+}$ doped HA after MTT test.

4. Conclusion
HA substituted with manganese (II) and iron (III) were successfully synthesized by wet chemical method coupled ion exchange mechanism. All products were of single-phase and the lattice structure was not affected by the ion exchange process. For Mn-HA nanoparticles, the crystallinity did not change significantly with the increase of Mn content while for Fe-HA nanoparticles, the crystallinity decreased with increase in Fe quantity. All samples were characterized as carbonated HA by FTIR, which was B-type. The metal ion substituted HA nanoparticles were elongated spheroids and uniform in size (70 nm). From the ICP-AES results, it can be concluded that the synthetic pure HA was non-stoichiometric and Fe$^{3+}$ was more active than Mn$^{2+}$ in the exchange of Ca$^{2+}$ ions in HA lattice. To maintain the charge balance for Fe-HA nanoparticles, the vacancies of Ca positions in the lattice increased with the increase of Fe concentration. All powders were tested as non-cytotoxic to osteoblast based on extraction assay. According to the magnetization curves, Fe$^{3+}$ doped HA was paramagnetic and the magnetic susceptibility increased with the increase of Fe content.

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