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Influence of wet chemical processing conditions on structure and properties of magnetic hydroxyapatite nanocomposites

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Received 11 June 2020; Received in revised form 6 August 2020; Accepted 27 September 2020

Abstract

Nanostructured composites containing iron oxide and hydroxyapatite (m-HA) were prepared by two wet chemical methods (single-step and double-step process) having different order of magnetite and HA precipitations. The phase composition, crystal size and lattice constants of the prepared composites were determined by XRD, the functional groups by FTIR and the structure, morphology and elemental composition by TEM. The magnetic susceptibility values and the colour indexes of each composite were also determined. Biocompatibility testing was performed in simulated body fluid. The results show that the composites produced by different (single-step and double-step) co-precipitating methods show different chemical and phase composition, average particle size, and therefore different superparamagnetic properties and bioactivities, with possibilities for different applications.

Keywords: magnetic properties, nanocomposites, apatite, biomedical applications

I. Introduction

Magnetic hydroxyapatite-based nanocomposites are currently the most promising and the most studied biomaterials having various possibilities for biomedical and bioengineering applications (such as cancer treatment, drug delivery, magnetic resonance imaging contrast agents, etc.) [1–3]. Many elements (e.g. Fe, Mn, Ni, Co) have the potential to be used in magnetic hydroxyapatite nanocomposites with varying levels of bioactivity and magnetization. Among them Fe is the most important and iron oxide nanoparticles are by far the most studied magnetic nanoparticles because of their interesting superparamagnetic behaviour [4]. However, the direct application of superparamagnetic iron oxide nanoparticles is not possible due to biofouling and aggregation processes into the bloodstream and consequent rapid segregation by macrophages. This aggregation decreases the intrinsic superparamagnetic behaviour [5]. Therefore, modification of the surface of

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iron oxide particles with different types of coatings (polymers or ceramics) is required. The disadvantage of biocompatible polymers is that they can disappear under harsh *in vivo* physiological conditions [5–8]. On the other hand, the inorganic hydroxyapatite (HA) is frequently used due to its well-known biocompatibility, bioactivity, lack of toxicity and its variable solubility in body fluids [9,10]. Thus, the combination of iron oxide and HA could be useful for bone cancer therapy because HA has bone bonding ability [11].

The iron oxide magnetic nanoparticles are generally fabricated in the form of core-shell-like structure, in which the central part contains magnetic phase (magnetic, maghemite) covered by HA for improving bio-compatibility, reducing cytotoxicity and avoiding particle aggregation [12,13]. These structures restrict the growth of iron oxide particles, prevent the release of iron ions and provide the stability of the nanoparticles in solutions, but also help the binding of different ligands to the surface, which is favourable for drug delivery [5,14–16]. The HA-based composites usually contain iron oxide (Fe₃O₄) particles with size below 20 nm uniformly embedded in submicron HA crystals. These

nanoparticles are classified as superparamagnetic due to its linear-type hysteresis loop [11,17] and can be rapidly magnetized when exposed to a magnetic field; when the magnetic field is removed they are rapidly demagnetized without a remnant or residual magnetization. This feature is necessary to avoid coagulation and agglomeration of nanoparticles causing possible embolization of capillary vessels.

Iron oxide/hydroxyapatite nanocomposites (m-HA) are mainly prepared by wet chemical methods by using one or two-step process. In the two-step wet chemical method, as reported by Mondal *et al.* [12], hydroxyapatite is synthesized in the first step and after that the precipitation of iron oxide nanoparticles is performed. Thus, the m-HA composites are prepared by mixing the two suspensions under appropriate conditions. On the other hand, in one-step method, proposed by Ansar *et al.* [6], the precursors required for magnetite formation are reacting first in an acidic medium, and then $Ca(NO_3)_2 \cdot 4H_2O$ is added for HA formation and $(NH_4)_2HPO_4$ for adjusting the appropriate pH.

Magnetic bioactive nanoparticles can also be obtained by direct incorporation of magnetic ions into the HA structure [18]. Thus, transition metals might successfully replace calcium in the HA lattice. A wide range of substitution in the crystal structure of HA is possible, which allows tailoring material properties [3]. Usually, divalent transition metals, such as: Mn, Fe and Co, are used which cause magnetization decrease in the order of Mn-HA > Fe-HA > Co-HA composites. The changes in the crystallinity and lattice parameters of HA refer to the size of substituting ion. The replacement of Ca²⁺ with iron ions resulted in apatite nanoparticles with much lower crystallinity. According to Panseri et al. [19] the Fe^{2+} and Fe^{3+} ions can occupy different Ca²⁺ positions in the HA lattice, so they are not situated in interstitial cell positions. An increase of the lattice parameter a (from 9.4218(5) to 9.4557(1) A) and a decrease of the lattice parameter c (from 6.8813(3) to 6.8785(1)Å) in the case of Ca substitution with iron ion having a lower radius were found by Rietveld analysis [19]. The replacement of Ca^{2+} with Fe^{2+} or Fe^{3+} ions in the HA structure has two opposite effects on the crystal lattice. Shrinkage of the lattice is caused due to the smaller ionic radius of Fe^{2+} with respect to Ca^{2+} . In contrast, although Fe³⁺ has an ionic radius smaller than those of both Ca^{2+} and Fe^{2+} , an increase in the size of cell parameters of HA doped with Fe³⁺ ions was observed. This expansion cannot be explained by the simple replacement of Ca^{2+} with Fe^{3+} . In this case it was supposed that the substitution involves the coinsertion of iron with carbonate and interstitial oxygen ions. The Fe³⁺ ions are preferentially positioned in areas with low crystal order [1]. Further investigation showed that the Fe³⁺ ions (similarly to Cu⁺) formally substitute hydrogen of the [OH]⁻ groups located in the hexagonal channels of HA crystal structure [20,21].

In this paper a modified two-step and one-step meth-

ods were used and the effect of the processing parameters on the structure and properties was investigated.

II. Materials and methods

2.1. Synthesis of magnetic HA nanocomposites

Iron (II) chloride tetrahydrate (FeCl₂ · 4 H₂O, 99%), calcium nitrate tetrahydrate (Ca(NO₃)₂ · 4 H₂O, 99%), hydrochloric acid (37%) and ammonium hydroxide (30%) were purchased from Sigma-Aldrich. Diammonium hydrogen phosphate ((NH₄)₂HPO₄, 99%) and iron (III) chloride (FeCl₃, 99%) were purchased from Reanal. Starting materials were used without further purification.

Nanocomposites containing HA and magnetic iron oxide (in amount of 5, 10, 15, 20, 25, 30, 35 and 40 wt.%) were prepared by two different wet chemical methods: i) single-step (m-HA/SS) [6] and ii) double-step method (m-HA/DS) [22]. In the single-step method the synthesis of HA particles was carried out by coprecipitation from solution prepared by iron salts and calcium-phosphate precursors in alkaline medium. The amounts of components required for the formation of hydroxyapatite with Ca/P ratio of 1.67 were calculated according to the following equation:

$$5 \operatorname{Ca}(\operatorname{NO}_3)_2 \cdot 4 \operatorname{H}_2 \operatorname{O} + 3 (\operatorname{NH}_4)_2 \operatorname{HPO}_4 + \operatorname{NH}_4(\operatorname{OH}) \longrightarrow \operatorname{Ca}_5(\operatorname{PO}_4)_3(\operatorname{OH}) + 10 \operatorname{NH}_4 \operatorname{NO}_3 + 3 \operatorname{H}_2 \operatorname{O}$$
(1)

The iron-salt solution was prepared in an acidic medium of HCl using $FeCl_2 \cdot 4H_2O$ and $FeCl_3$ in the molar ratio of 1:2. The reaction may be written as follows:

$$\operatorname{Fe}^{2+} + 2\operatorname{Fe}^{3+} + 8\operatorname{OH}^{-} \longrightarrow \operatorname{Fe}_{3}\operatorname{O}_{4} + 4\operatorname{H}_{2}\operatorname{O}$$
 (2)

The required amount of $Ca(NO_3)_2 \cdot 4H_2O$ (Eq. 1) was dissolved in distilled water and the iron salt solution was added during stirring and heated up to 70 °C. Since the formation of magnetite requires alkaline medium, the pH of the solution was slowly increased to 11 by adding NH₄(OH) solution. The suspension was stirred for 1 h at 70 °C, then the solution of $(NH_4)_2HPO_4$ was added in order to form hydroxyapatite. After the reaction was completed, the temperature was increased to 80 °C and continuously stirred for 1 h to eliminate excess ammonia. The suspension was aged for 24 h and then the formed precipitate was washed three times with distilled water and filtered. Finally the samples were dried at 80 °C and ground for further investigations (Fig. 1) [6].

In the double-step method the HA suspension with Ca/P ratio = 1.67 was previously precipitated by mixing Ca(NO₃)₂ · 4 H₂O and (NH₄)₂HPO₄ solutions [21]. This 12.2 wt.% hydroxyapatite containing suspension was stirred and heated to 70 °C. Then the acidic solution of FeCl₂ · 4 H₂O and FeCl₃ with the ratio of Fe³⁺ : Fe²⁺ = 2 : 1 was added to the HA suspension and the pH = 11 was adjusted by addition of NH₄(OH) solution.



Figure 1. Different synthesis routes for preparation of m-HA nanocomposites: a) one-step and b) two-step method

The reactant mixture was continuously stirred for 1 h at 70 °C followed by ageing for 24 h. The samples were washed three times with distilled water, filtered, dried and then dispersed for further investigations (Fig. 1). In the sample notation m-HA/SS and m-HA/DS denote the synthesis method and number indicates magnetite content in the m-HA nanocomposites (5, 10, 15, 20, 25, 30, 35, and 40 wt.%).

2.2. Testing methods

The phase composition and crystal structure of the magnetic nanocomposites were investigated by Philips PW3710 type X-ray diffractometer, performed on powders within a 2θ range of $2-70^{\circ}$ by CuK_{α} ($\lambda = 1.5405$ Å) radiation. The quantitative phase composition of some samples was determined using the Rietveld analysis, where addition of 10 wt.% ZnO was used as a standard.

The crystallite size of the nanoparticles was determined using the Scherrer equation:

$$D = \frac{K \cdot \lambda}{B \cdot \cos \theta} \tag{3}$$

where *D* is the average crystallite diameter, *K* is the shape factor, *B* is the broadening of the full width at half maximum of the diffraction peak measured in radians, λ is the wavelength (1.5405 Å) of the X-ray radiation and θ is the diffraction angle [13]. The full width at half maximum was measured at the reflections 002 and 311 of HA and magnetite, respectively.

The differential thermal (DTA) and thermogravimetric (TGA) analysis were carried out by Q-1500D MOM type derivatograph in the temperature range of 20-1000 °C with a 10 °C/min heating rate. Fourier-transform infrared (FT-IR) spectroscopic measurements were carried out using a PerkinElmer Spectrum TWO type spectrometer in ATR mode without additional sample manipulation. The spectra were recorded at a resolution of 4 cm^{-1} .

Transmission electron microscopy analyses were performed using a Talos F200X G2 instrument (Thermo Fisher), operated at 80 kV accelerating voltage equipped with a field-emission gun and a four-detector Super X energy-dispersive X-ray spectrometer. STEM highangle annular dark field (HAADF) images were collected both for characterisation and for determining elemental composition by energy-dispersive X-ray spectrometry (EDS). Samples for TEM were prepared by depositing a drop of aqueous suspension of the nanocomposite particles on copper grids covered by lacey carbon amorphous support film.

Magnetic properties were studied by magnetic susceptibility measurements by an Agilent 4284A impedance analyser in the frequency range of f = 60 Hz to 1 MHz. The susceptibility was measured with an error of ± 0.01 . The data were extrapolated to 0 Hz in order to obtain the static initial susceptibility (χ_0).

The colour of the magnetic nanocomposites was characterised by the values of the CIELAB colour space measured by a Konica Minolta CM-3600d spectrophotometer.

In order to investigate the bioactivity of the composites the samples were immersed in SBF for 7 and 26 days. The SBF solution was prepared according to wellknown Kukobo recipe [23], which is the closest to the ionic concentrations of the human blood plasma. The chemical composition of the body fluids obtained after immersion were measured by X-ray spectroscopy using a Philips Minipal 4 spectrometer.



Figure 2. X-ray diffraction patterns of m-HA/SS (a) and m-HA/DS (b) composites with different magnetite contents

Table 1. Phase composition of the samples determined by Rietveld analysis

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Swatam	Phase composition [wt.%]			
System	HA	Magnetite		
m-HA/SS5	82.8	8.3		
m-HA/DS5	94.6	0.2		
m-HA/SS15	70.7	21.4		
m-HA/DS15	85.4	9.62		
m-HA/SS30	62.6	29.6		
m-HA/DS30	72.7	15.2		

III. Results and discussion

3.1. X-ray diffraction analysis

The X-ray diffraction patterns of the samples prepared by two different methods and with different iron oxide contents (Fig. 2) show that the synthesized m-HA samples do not contain any crystalline phase beside hydroxyapatite (JCPDS number 9-0432) and magnetite (JCPDS number 019-0629) (Fig. 2). Comparison of the peaks shows higher peak intensities of magnetite phase for the m-HA/SS samples prepared by the single-step method. Using a preformed HA suspension as starting material in the double-step method resulted in composite containing a well crystallized hydroxyapatite phase. The Rietveld analysis of the samples containing different amounts of iron oxide confirmed that by the doublestep synthesis nearly all of HA is well crystallized, but only partial crystallization of HA (87-90%) was determined for the single-step process (Table 1). At the same time the iron oxide is well crystallized in these samples (m-HA/SS), while in the m-HA/DS samples produced by double-step method a large portion of iron oxide is poorly crystallized. This means that in the m-HA/SS samples prepared by the single-step process, the majority of poorly crystallized phase is HA, while in the samples obtained by the double-step method a significant part of iron oxide is in an imperfectly crystallized form.

The cell parameters of the pure HA and m-HA powders were determined by the Rietveld analysis and pre-

Table 2. Cell	parameters	of HA	and	m-HA	particles

System	Lattice constant [Å]			
	а	С		
HA	9.413	6.886		
m-HA/SS5	9.494	6.859		
m-HA/SS15	9.479	6.847		
m-HA/SS25	9.497	6.881		
m-HA/SS30	9.527	6.870		
m-HA/SS35	9.511	6.898		
m-HA/SS40	9.506	6.903		
m-HA/DS5	9.436	6.889		
m-HA/DS15	9.425	6.881		
m-HA/DS25	9.441	6.895		
m-HA/DS30	9.442	6.894		
m-HA/DS35	9.446	6.898		
m-HA/DS40	9.449	6.898		



Figure 3. Change of the hydroxyapatite *a* lattice constant in the magnetic nanocomposites prepared by different methods and iron oxide content

sented in Table 2 and Fig. 3. The results (i.e. evidence of the lattice expansion and the elemental composition

Hydroxyapatite			Magnetite				
	2θ	Peak width [°]	<i>D</i> [nm]		2θ	Peak width [°]	<i>D</i> [nm]
HA	25.567	0.284	53.8	М	35.465	0.734	67.5
m-HA/SS5	25.842	0.583	27.3	m-HA/SS5	35.373	1.100	22.8
m-HA/SS15	25.768	0.543	31.5	m-HA/SS15	35.320	1.124	21.4
m-HA/SS30	25.782	0.583	27.3	m-HA/SS30	35.399	0.987	33.0
m-HA/DS5	25.685	0.284	54.6	m-HA/DS5	35.316	0.823	56.0
m-HA/DS15	25.692	0.273	56.9	m-HA/DS15	35.402	0.953	38.1
m-HA/DS30	25.754	0.307	51.1	m-HA/DS30	35.501	0.889	53.8

 Table 3. Average crystallite size (D) of HA and magnetite in the composites prepared by different methods and iron oxide content

of the separated hydroxyapatite particles) confirm that in the samples synthesized by the single-step method part of Fe^{3+} ions is incorporated into the structure of apatite (in the middle of the hexagonal channel [21]) by an ion exchange process. A deeper knowledge about the incorporation mechanisms, the specific positioning of Fe^{2+}/Fe^{3+} ions and determination of the magnetic behaviour would greatly contribute to develop new biomaterials with tailored magnetic and biological properties [1].

The average crystallite sizes determined using the Scherrer equation (Table 3) show that the hydroxyapatite crystallite sizes in the composites prepared by different methods change in the range of 27.3–31.5 nm (m-HA/SS samples) and 51.1–56.9 nm (m-HA/DS samples). In addition, the samples prepared by single-step method contain smaller magnetite crystallites (Table 3). Thus, due to smaller crystallite size in the composites prepared by the single-step process, better superparamagnetic properties could be expected in these samples.

3.2. Infrared spectroscopy

To confirm the coordination of iron in magnetic nanocomposites, the samples were also examined by Fourier-transform infrared spectroscopy (Fig. 4). The FTIR-spectrum of the magnetite prepared by co-precipitation shows a strong absorption band at 541.85 cm^{-1} assigned to the vibration of the Fe²⁺–O²⁻ functional groups. The peak at 624.13 cm^{-1} is attributed to the symmetry degeneration on octahedral B-type



Figure 4. FTIR spectra of magnetite, m-HA/SS40 and m-HA/DS40 samples

sites. In the region of 1200–500 cm⁻¹ all m-HA samples display peaks that match the pure HA without any significant shift in peak position. According to the FTIR data the magnetite does not produce any considerable change in the chemical structure of hydroxyapatite, similarly to the results of Ansar *et al.* [6].

3.3. Thermogravimetric analysis

The carbonate content of the samples was measured by thermogravimetric analysis. The CO_3^{2-} content is calculated from the weight loss obtained in the temperature range between 550 and 950 °C [1]. The results of thermal analysis indicate 0.1–0.2 wt.% mass decrease only, which indicates very small amount of carbonate present in the magnetic nanocomposites prepared by different method and iron oxide content.

3.4. TEM analysis

Most of the particles are in the range of 5–50 nm as seen on STEM micrographs (Fig. 5). In the samples prepared by the single-step method the hydroxyapatite particles surrounding the smaller magnetite crystals show flakes and plate-like structure (Fig. 5a), whereas the preformed HA particles used in the double-step process have larger plate-like and nearly isometric structure (Fig. 5b). Examination of the chemical composition of the HA particles shows that the Ca/P atomic ratio of the m-HA/SS and m-HA/DS samples was 1.59 and 1.63, respectively. In addition, the maximum value of iron content in hydroxyapatite particles was 0.65 at.% for the m-HA/SS40 sample. The hydroxyapatite structure in the samples prepared by the double-step method did not show any presence of iron. These results correlated well with the lattice parameters obtained by X-ray analysis (Table 2).

3.5. Magnetic properties

The magnetic properties of the Fe_3O_4/HA nanocomposites were characterized by magnetic susceptibility measurements (Fig. 6). The paramagnetic materials have positive magnetic susceptibility and lose their magnetism after the disappearance of the external magnetic field, so they show no ferromagnetic behaviour. The size of paramagnetic iron oxide particles is in the nanometre range, but the particles having larger size have shown ferromagnetic properties [8]. The curves of



Figure 5. STEM micrographs of: a) m-HA/SS40 and b) m-HA/DS40 samples



Figure 6. The change of magnetic susceptibility of the magnetite (M) and composites prepared by different methods

the samples with the same amount of iron oxide prepared by different precipitation methods show a very small difference of the susceptibility values (the samples with 5 wt.% and 10 wt.% of Fe₃O₄). In the case of the composites with 25 wt.% and 40 wt.% of Fe₃O₄, the difference between the susceptibility values of the m-HA/SS and m-HA/DS samples is much higher, i.e. an order of magnitude. Thus, the m-HA/SS25 sample has better magnetic properties than the m-HA/DS40 sample. It could be concluded that the well crystallised magnetite containing samples prepared by the single-step process have higher magnetic susceptibility.

3.6. Colour measurements

Figures 7 and 8 show the values of the ΔE and magnetic susceptibility for different m-HA composites. The ΔE value shows the deviation from the colour of the pure magnetite. In the samples prepared by the single-







Colour changes compared to magnetite $(\Delta E^*{}_{ab})$ Magnetic susceptibility (χ')

Figure 8. Correlation of colour changes of the composites with the magnetic susceptibility (measured at f = 10000 Hz) of samples prepared by double-step method

step method, the ΔE value increases with Fe₃O₄ content, having the same trend observed for the magnetic susceptibility. However, in the case of the m-HA/DS



Figure 9. Correlation of brightness factors of the composites with the magnetic susceptibility measured at f = 10000 Hz



Figure 10. Calcium content in SBF after 7 and 26 days immersion for composites prepared by different methods



Figure 11. Phosphorous content in SBF after 7 and 26 days immersion for composites prepared by different methods

samples, the ΔE value of the colour changes decreases. The a^* ($a^* > 0$ red colour content), b^* ($b^* > 0$ yellow colour content) and L^* (the brightness factor) values were also measured at the frequency of f = 10000 Hz (Fig. 9). The results indicate the decrease of L^* brightness factor with the increase of magnetite content in the samples prepared by both methods. This could be an indication that the change of brightness factor can be related to the magnetization at a given frequency.

3.7. Investigation of bioactivity

The in vitro bioactivity of the composites was tested after 7 and 26 days of immersion in the simulated body fluid at 36.5 °C. Bioactivity of the samples was characterized by the measurement of Ca and P contents in SBF after the treatment. The samples with the smallest (5 wt.% - m-HA/SS5, m-HA/DS5) and the highest (40 wt.% - m-HA/SS40, m-HA/DS40) magnetite content were used for the study. During the dissolution test 1 g of the sample was added to 30 ml of SBF and kept for 7 and 26 days at 36.5 ± 0.5 °C in a closed vessel. The Ca and P contents were measured in the filtrates. The samples prepared by the double-step method show higher Ca^{2+} dissolution rate than the single-step samples (Figs. 10 and 11). For all investigated samples the calcium content in SBF observed after 7 days decreased significantly with increasing the treating time to 26 days. This can be explained by the formation of a calcium-phosphate layer on the surface of the particles. After 7 days of SBF treatment, the dissolution of phosphorous from HA is observed in the double-step m-HA/DS samples. In other cases a decrease in the phosphorous content of the SBF (similar to calcium) indicates the involvement of phosphorous in the formation of calcium-phosphates. Based on these results the HA suspension containing the double-step prepared samples have higher solubility despite their better crystallized structure. It is supposed that the large part of amorphous iron oxide next to the HA can inhibit its dissolution. On the other hand, in the m-HA/SS samples prepared by the single-step process the iron ions (~0.65 at.%) positioned in HA structure can accelerate the dissolution.

IV. Conclusions

Nanostructured composites containing iron oxide and hydroxyapatite (m-HA) were prepared by two wet chemical methods (single-step and double-step process) having different order of magnetite and HA precipitations. The magnetite-hydroxyapatite nanocomposites prepared by the single-step method contain wellcrystallized magnetite phase and have higher magnetic susceptibility. In these samples iron ions can be accommodated in the HA structure, which decrease the dissolution rate of HA in simulated body fluid. On the other hand, the samples prepared by double-step method have better crystallinity and contain larger magnetite crystallites. The results of the colour measurement confirm that the magnetic properties can be correlated to brightness factors of a sample.

Acknowledgments: TEM studies were performed at the electron microscopy laboratory of the University of Pannonia, established using grant no. GINOP-2.3.3-15-2016-0009 from the European Structural and Investments Funds and the Hungarian Government.

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