

Influence of heat treatment medium on properties of the bovine hydroxyapatite/magnetite systems

Oksana Otychenko¹, Tetyana Babutina¹, Anatolii Perekos²,
Oleksandr Bykov³, Sergiy Korichev³, Oleksandr Koval⁴

¹ Frantsevich Institute for Problems in Materials Science of NAS of Ukraine, Department of Functional Materials for Medical Application, 3, Krzhizhanovsky str., 03142, Kyiv, Ukraine.

² Kurdyumov Institute Of Metal Physics of NAS of Ukraine – Department of Constitution and Properties of Solid Solutions, 36 Academician Vernadsky Boulevard, 03142, Kyiv, Ukraine.

³ Frantsevich Institute for Problems in Materials Science of NAS of Ukraine, Department of Physical Material Science of Refractory Compounds, 3, Krzhizhanovsky str., 03142, Kyiv, Ukraine.

⁴ Frantsevich Institute for Problems in Materials Science of NAS of Ukraine, Department of Physics of Strength and Plasticity of Materials, 3, Krzhizhanovsky str., 03142, Kyiv, Ukraine.

e-mail: oksanaotychenko@gmail.com, babutina@ukr.net, perekos@ukr.net, pavelBykov1@gmail.com, sergey_korichev@ukr.net

ABSTRACT

Technological processes for the synthesis of composite systems based on bovine (rather than synthetic) hydroxyapatite are rarely described. That's why the influence of the heat treatment medium on the properties of the composite system bovine hydroxyapatite/magnetite (BHA/Fe₃O₄) obtained using physico-mechanical and chemical methods were investigated. It was established that the most efficient medium for heat treatment of studied materials is vacuum. Due to XRD analysis data allow us to assert that the most perfect lattice is formed with the use of the chemical method for production and heat treatment in a vacuum (crystallinity 86%), and the least perfect one is formed by the mechanical method for production and heat treatment in a nitrogen medium (71%). The method for adding magnetite has only a minor effect. Investigated conditions of heat treatment of composite systems BHA/Fe₃O₄ promoted obtaining optimal magnetic properties and stable behavior in experiments *in vitro*.

Keywords: bovine hydroxyapatite, magnetite, crystallinity, specific saturation magnetization, *in vitro* experiments.

1. INTRODUCTION

The before carried-out studies of the composition, structure and properties of bone tissue and its recovery mechanism have provided the success in using bioactive calcium phosphate based ceramics. Of the large number of bioceramic materials, two phases are most biocompatible, namely β -Ca₃(PO₄)₂ (β -tricalcium phosphate, in short β -TCP) and hydroxyapatite (HA) Ca₁₀(PO₄)₆(OH)₂ [1, 2]. Of them, HA is more often used as it is the main inorganic component of bone tissue. It is produced in two ways: from native tissues yielding a biological (biogenic, bovine) hydroxyapatite (BHA) and in artificial conditions for a synthetic hydroxyapatite (SHA). The biocompatibility of both materials is at a rather high level, but in BHA, calcium, phosphorus, magnesium and other important trace elements are present in the same ionic forms and ratios as in the natural bone tissue. Moreover, variations in BHA stoichiometry are similar to those in bone apatite (1.3–1.7), which provides a more reactive medium for a balanced running of many physiological and biochemical processes required for the normal formation of bone tissue [3, 4].

The crystalline structure of apatites is stable and perfect, and similar phases are often considered as the most probable end product of many reactions. This explains the large number of apatite groups without impurities and synthetic compounds with various combinations of partial isomorphous substitutions [5, 6]. To provide HA based biomaterials with desired properties and to create multifunctional ceramics, composites are designed. Combination of different substances in a composite gives an effect equivalent to the creation of a new material whose properties qualitatively and quantitatively differ from the properties of each component

[6]. It should be noted that in most of the investigated cases, even a slight change in the concentration of additives leads to significant changes in the physical and chemical properties of substances along with maintaining their overall structure nomination [7]. Iron (Fe^{2+} , Fe^{3+}) [8-10] and its oxides [10, 11] are most often used as an X-ray-sensitive additives. It should also be noted that the biocompatibility of Fe oxides has been confirmed not only in experimental implantation, but also in studies of living organisms, in whose cells iron was found in the form of magnetite [12, 13].

Methods for synthesis of HA and Fe_3O_4 have been described in numerous scientific papers, while synthesis of systems based on them has attracted far less attention [14]. In general, in the material HA/ Fe_3O_4 magnetite acts as a magnetically sensitive component with a reactive surface that allows realization of a chemical design and creation of the required multifunctional nanocomposite architecture, while HA acts as a thin-layer stabilizer [15]. The most commonly used method for synthesis of such composites is the production of individual components through their co-precipitation followed by uniting them using a hydrothermal method [8, 10, 16, 17]. The sol-gel method [18], biomimetic method [19, 20], methods of neutralization [21], ultrasound processing [22], and spray drying [23] are also used. In addition, there is a method that consists in introducing before-prepared magnetite in the form of powder or suspension directly in the course of HA synthesis [24]. The use of mechanical or mechano-chemical methods is less widespread. They include milling of separately synthesized suspensions or powders of magnetite and HA in ball or other mills. In this case, the Fe^{2+} and Fe^{3+} ions replace different positions of calcium in the crystal lattice of HA [25].

Taking into account the above information, the purpose of this work was to synthesize a composite system based on BHA and magnetite using described in the previous work two versions of the condensation physicochemical method [26] followed by heat treatment (thermolysis) in vacuum, hydrocarbon or nitrogen media and to establish the influence of the medium on the properties of the systems obtained.

2. MATERIALS AND METHODS

2.1 Synthesis of composites

As a starting material, pure BHA (bone tissue of cattle) "Osteopaty keramichny"[®] ("Poputnyy Viter", Ukraine) was used. Doping BHA with ferromagnetic additives was performed using two procedures of the condensation physicochemical method described in [26]. The chemical method for preparation of a doped powder material consisted in introducing BHA directly in the process of obtaining an iron oxalate precipitate. First of all, the following reagents are prepared:

- 2.3 mg of iron sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and 6.4 mL of distilled water;
- 2.8 mL of isopropanol ($\text{C}_3\text{H}_8\text{O}$) with purity 99,8%;
- 1.05 mg of oxalic acid ($\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$) and 7 mL of distilled water;
- 0.56 g of sucrose ($\text{C}_{12}\text{H}_{22}\text{O}_{11}$) and distilled water in the amount required for the complete dissolution of the polysaccharide;
- suspension from 70 g of BHA and distilled water.

The solutions are cooled to 5–10 °C and poured out drop by drop in the above sequence under intense mixing for 2.5 h. The resulting suspension settles for 3–4 h. Then intense mixing and subsequent settling are repeated 3–4 times. An important condition for the production of iron oxalate nanoparticles is to strictly follow the above sequence of introductions of the reaction components. In this stage, the structure and specific properties of the particles are formed. Their uniform distribution in the material is an important factor, which is achieved by thoroughly mixing. It is worth mentioning that the formation of crystallization centers depends on the rate of mixing the solutions [27].

After filtration, the resulting BHA/iron oxalate material is dried in the air. In order to obtain the BHA/magnetite composite systems, the synthesized materials are subjected to heat treatment (low temperature thermolysis) in vacuum, hydrocarbon or nitrogen media for 2 h at $T = 500$ °C (below the Curie point T_c for magnetite equal to 572 °C).

The method consists in deposition of ready iron oxalate onto the surface of the initial BHA particles in the form of hydrosuspension (Figure 1). The number of reagents was chosen based on the final amount of iron not more than 2 mass%.

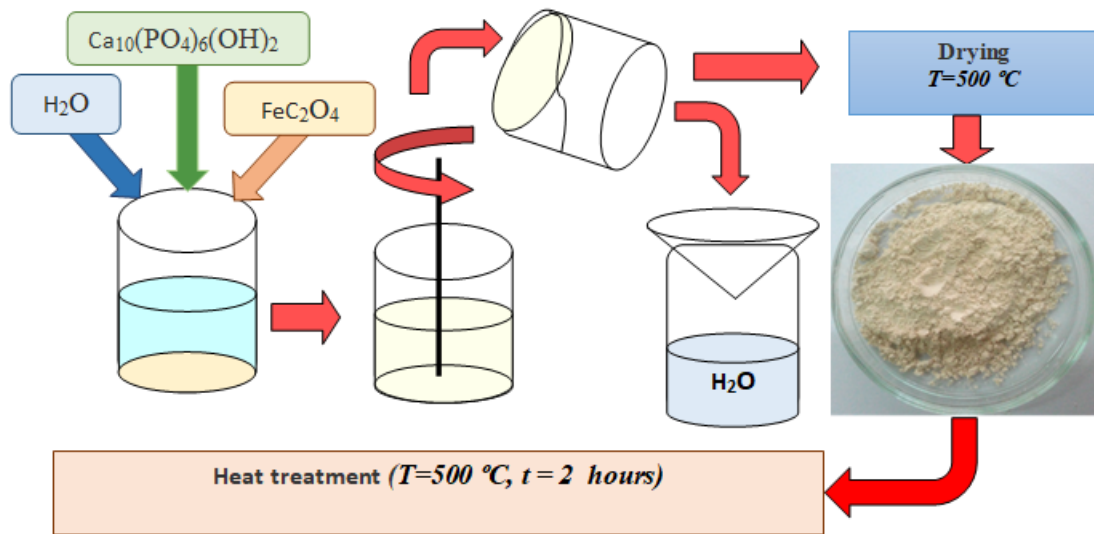


Figure 1: Technological scheme of sample preparation by the physico-mechanical method.

Subsequently, filtration dewatering and drying of the prepared dispersed system were carried out. After that, the resulting material was thermally decomposed in a protective or a reducing medium under the above mentioned conditions.

2.2 XRD analysis

The phase composition was determined using an X-ray diffractometer "DRON 3.0" under the Co-K α radiation.

2.3 Crystalline structure

The width of the peak (002) at half its height (broadening FWHM) represents the size of the crystallites (area of coherent scattering) in the direction of the c axis (D_c) of the BHA crystalline lattice, whereas the peak (300) is related to the crystallite size in the direction of the a axis (D_a). Calculation of the sizes was performed using the Scherrer formula (1):

$$D = \frac{k \cdot \lambda}{\beta \cdot \cos \theta}, \quad (1)$$

where D is the size of crystallites, Å; k is the Scherrer constant (0.89), rad; λ is the wave length of X-ray radiation (1.7903 Å); θ is the diffraction angle; $\beta = \sqrt{B^2 - b^2}$, rad (B is the line broadening at half the peak intensity, while b is the instrument-caused broadening).

Crystallinity (X_C) of HA was calculated by the formula (2):

$$X_C = 1 - \frac{I_{112/300}}{I_{300}}, \quad (2)$$

where I_{300} is the intensity of the diffraction peak (300); $I_{112/300}$ is the intensity of the space between the (112) and (300) diffraction peaks [28].

2.4 Chemical analysis

The sample mass was measured on an analytical balance "OHAUS Pioneer PA214C" ("OHAUS Corporation", China) with an accuracy of 0.0001 g. The iron, calcium and phosphorus contents in the powders were determined by the photocalorimetric method [29] using an FEK-56M device (Russia):

- the iron content in the powder materials was determined by the reaction of formation a yellow complex compound after chemical interaction of the sample and sulfosalicylic acid ($C_7H_6O_6S$) in ammonia (NH_3) medium [29];
- the total amount of phosphorus in solutions and powder materials was determined by method based on the formation of yellow phosphorus-molybdenum heteropolyacid with its subsequent restoration in hydrochloric medium by Fe^{2+} ions in the presence of hydroxylamine to formation of the complex compound of molybdenum blue [29];
- the calcium content of powders and filtration solutions was determined by the method with arsenazo I, where Ca^{2+} ions react with arsenazo I or neotorine in the basic medium ($pH = 12-13$) to form the complex compound of red-violet color [29].

The total amount of carbon was determined with help of an "AN-7529" express analyzer (Belarus).

2.5 Specific surface area

The specific surface area of the powder biomaterials was determined by the thermal nitrogen desorption (BET method) using a sorbtometer "EMS-61" (Estonia).

2.6 Morphological analysis

The morphology of the particles of composites was studied using a scanning electron microscope (SEM) "JEOL Superprobe 733" in secondary electron imaging (SEI) mode.

2.7 Magnetic properties

The specific saturation magnetization was measured using a ballistic magnetometer at room temperature in magnetic fields up to 1 T (10 kE) [30].

2.8 Experiments *in vitro*

The investigation of specific mass loss was carried out in experiments *in vitro* by placing powders in an isotonic physiological 0.9% NaCl ("Arterium", Ukraine) solution under the thermostatic conditions at a temperature similar to that of healthy human body (36.5-37.0 °C) for 2, 5 and 7 days. The solubility rate, as a characteristic of the specific mass loss, was determined by the formula (3):

$$Q_R = \frac{\Delta m}{m_{ob} \cdot t} \cdot 100, \quad (3)$$

where Δm is the change in sample mass during the exposure to the model medium, g; m_{ob} is the initial mass of the sample, g; and t is the time of the sample exposure to the model medium, days.

3. RESULTS AND DISCUSSION

3.1 Chemical analysis

The technological process of manufacturing any material includes a stage of control over the chemical composition. The data on the contents of iron and carbon in the investigated materials (Figure 2), as well as the values of the specific surface area are listed in Table 1.

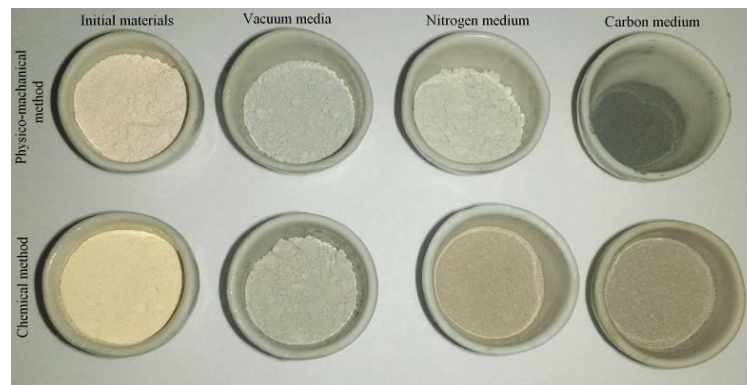


Figure 2: Initial materials and the composite after low temperature thermolysis in different media.

The chemical analysis findings presented in Table 1 demonstrate the similarity of materials after low temperature thermolysis in a nitrogen medium and vacuum concerning the contents of both iron and carbon.

Table 1: Some physical and chemical characteristics of the samples studied.

MATERIAL (METHOD OF PRODUCTION)	MEDIUM OF HEAT TREATMENT, 500 °C, 2 h	SPECIFIC SURFACE AREA, m ² /g	CONTENT OF ELEMENTS, mass%	
			Fe	C
BHA	-	5.60	0.08	0.03
BHA/Fe ₃ O ₄ (phys.)	Vacuum (1·10 ⁻¹ mm Hg)	7.38	1.07	0.51
BHA/Fe ₃ O ₄ (chem.)	Vacuum (1·10 ⁻¹ mm Hg)	8.82	1.01	0.62
BHA/Fe ₃ O ₄ (phys.)	Nitrogen	6.28	0.98	0.57
BHA/Fe ₃ O ₄ (chem.)	Nitrogen	7.46	0.94	0.57
BHA/Fe ₃ O ₄ (phys.)	Hydrocarbon	5.23	1.04	0.68
BHA/Fe ₃ O ₄ (chem.)	Hydrocarbon	6.03	0.96	0.77

According to the experimental studies *in vivo* by I.V. GURIN *et al.* [31], it is evident that the specified amount of carbon will not lead to a significant dystrophic-atrophic process in the case of implantation in an organism, since even purely carbonic materials do not cause cell infiltration both near the implant and throughout the visibility zone.

3.2 XRD analysis

According to the XRD data, powders obtained using the chemical method (Figure 3a) and then heat-treated in a vacuum contain some phases of the Fe–O system. In this process, magnetite is reduced to iron, as indicated by the peak at $2\theta \sim 51.8^\circ$. However, the magnetite (Fe₃O₄) subjected to slight oxidation remains in the material, which is confirmed by the presence of hematite (Fe₂O₃) traces on the XRD pattern. In comparison with other iron oxides, the test powder contains more wustite (FeO) characterized by various Fe/O ratios.

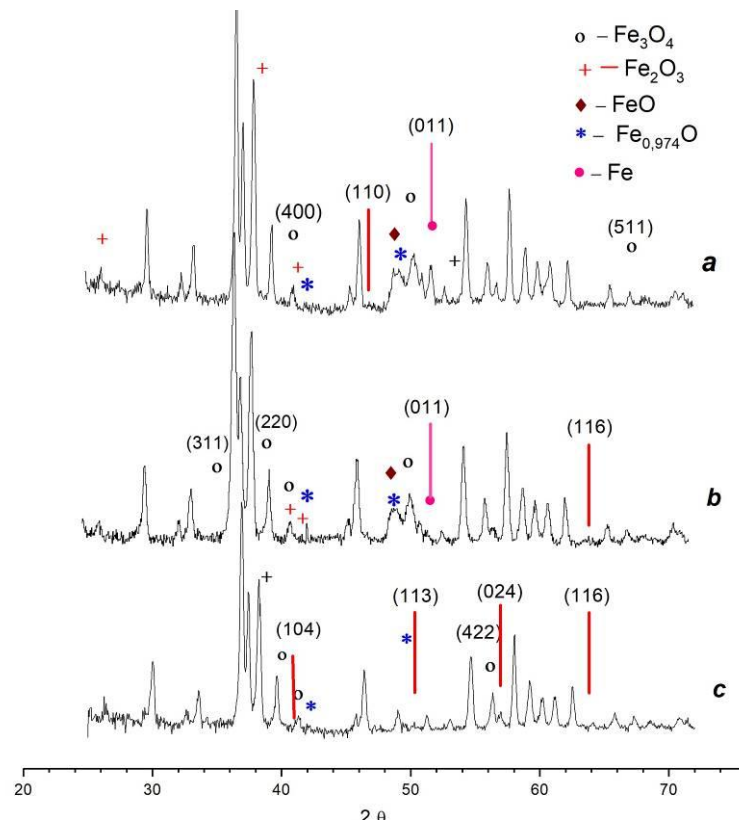


Figure 3: Fragments of the XRD patterns recorded from powdered samples obtained by the chemical method after heat treatment in (a) vacuum, (b) nitrogen and (c) hydrocarbon media (with designation of the main peaks of the $Fe - O$ system phases).

The phase composition of the powder BHA/ Fe_3O_4 obtained by the chemical method and subjected to thermolysis in a nitrogen medium, is qualitatively similar to that of the powders after heat treatment in a vacuum. However, its XRD pattern (Figure 3b) is characterized by a significant decrease in the iron peak intensity and a slightly higher magnetite (Fe_3O_4) oxidation.

The XRD patterns of the materials obtained by the chemical method and heat-treated in a hydrocarbon medium (Figure 3c) differs more markedly concerning iron-containing phases from the above described patterns (Figures 3a and 3b). It demonstrates the absence of the lines of iron oxides and pure iron at $2\theta \sim 51^\circ$. Instead, there are observed the HA peaks only. At an angle of $2\theta \sim 38.4^\circ$, an intense peak of hematite is singled out, that is, the magnetite oxidation and transformation of its large amount into hematite (Fe_2O_3) occur. The wustite ($Fe_{0.974}O$) peaks are also fixed. However, part of the magnetite (Fe_3O_4) still remains in the material. In addition, when comparing this pattern with ones in Figs. 3a and 3b, one can see that the HA peaks are shifted toward the bigger angles. This indicates a decrease in the crystalline lattice parameters, presumably due to the replacement of calcium ions with iron ions.

The analysis of diffraction patterns of samples obtained by the physico-mechanical method after heat treatment in different media revealed the similarity of their phase composition to that of materials synthesized by the chemical method, which is confirmed by the fragments of diffraction patterns from powders obtained by different methods after heat treatment in a hydrocarbon medium (Figure 4).

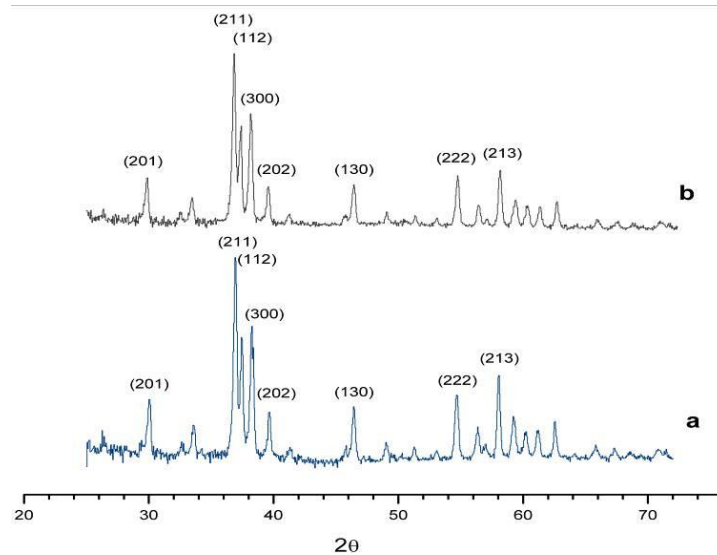


Figure 4: Fragments of the XRD patterns from the samples obtained by (a) chemical and (b) physico-mechanical methods after heat treatment in a hydrocarbon medium (with designation of the main HA peaks).

In other words, the peculiarities of the phase composition of the powders depend predominantly on the medium in which thermolysis takes place. The method for adding magnetite (Fe_3O_4) has only a minor effect.

3.3 Crystalline structure

The crystalline structure of HA can be characterized by the crystallite sizes in the directions of the *a* and *c* axes, the crystallinity H_c , the crystalline lattice parameters, and the crystalline cell volume. The results of calculations performed to characterize the state of the HA crystalline structure are presented in Table 2.

Table 2: Some characteristics of the HA crystalline structure in the powdered samples studied.

MATERIAL (METHOD OF PRODUCTION)	MEDIUM OF HEAT TREATMENT, 500 °C, 2 h	CRYSTALLITE SIZED _c ALONG <i>c</i> AXIS (nm)	CRYSTALLITE SIZED _a ALONG <i>a</i> AXIS (nm)	DEGREE OF CRYSTALLINITY (%)	VOLUME OF CRYSTALLINE CELL (Å ³)
BHA/Fe ₃ O ₄ (chem.)	Hydrocarbon	69.5	39.5	76	533.7
BHA/Fe ₃ O ₄ (phys.-mech.)	Hydrocarbon	64.1	42.2	77	537.8
BHA/Fe ₃ O ₄ (chem.)	Nitrogen	44.6	36.8	79	537.0
BHA/Fe ₃ O ₄ (phys.-mech.)	Nitrogen	61.0	34.5	71	534.3
BHA/Fe ₃ O ₄ (chem.)	Vacuum (1·10 ⁻¹ mm Hg)	45.5	49.5	86	537.5
BHA/Fe ₃ O ₄ (phys.-mech.)	Vacuum (1·10 ⁻¹ mm Hg)	55.1	40.2	81	535.9
BHA initial	-	82.5	49.5	70	532.4

The most vivid change in the state of the HA crystalline lattice is reflected in the change in the degree of HA crystallinity. The above presented data allow us to assert that the most perfect lattice is formed with the use of the chemical method for production and heat treatment in a vacuum (crystallinity 86%), and the least perfect one is formed by the physico-mechanical method for production and heat treatment in a

nitrogen medium (71%).

The elementary cell volume is related to both the presence of vacancies and the size of the ions present in its composition. In the case of replacing calcium ions with iron ions, the lattice parameters are expected to decrease, while the absence of vacancies will increase them.

The volume of the HA crystalline cell in composites obtained by the chemical method is the biggest after heat treatment in a vacuum. At the same time, the corresponding XRD pattern contains a fairly significant peak of iron (Fig. 3a). On the contrary, after treatment in a hydrocarbon medium, the iron peak is absent, and the cell volume is the smallest. It is likely that some iron atoms replace the calcium ions in HA crystals. Treatment in a nitrogen medium gives intermediate values. In the case of using the physico-mechanical method for production, the conditions of interaction of HA with other substances change and a maximum volume is achieved after treatment in a hydrocarbon medium.

The difference between the cell volumes depending on the procedure for introducing magnetite (Fe_3O_4) is the biggest after treatment in a hydrocarbon medium. In this case, more stable results can be obtained after the use of a vacuum medium. The smallest crystallite sizes in the direction of the *a* axis are obtained for a nitrogen medium, where as they in the *c* axis direction are characteristic for a vacuum heat treatment.

3.4 Morphological analysis

The morphological analysis was carried out because surface topology of biomaterials and their porosity are important factors for osteogenesis during bone regeneration. It is mandatory for biomaterials to have pores of 100–200 μm in size, which are required for normal circulation of blood and other physiological fluids around it after implantation [32]. The partical size of such biomaterials varies from 20 nm to 100 μm [33].

According to the SEM data, the initial BHA powder consists of 2–12 μm agglomerates formed by spherical nanoparticles with a diameter of less than 1 μm (~ 280–670 nm) and commensurate pores (Figures 5A and 5E).

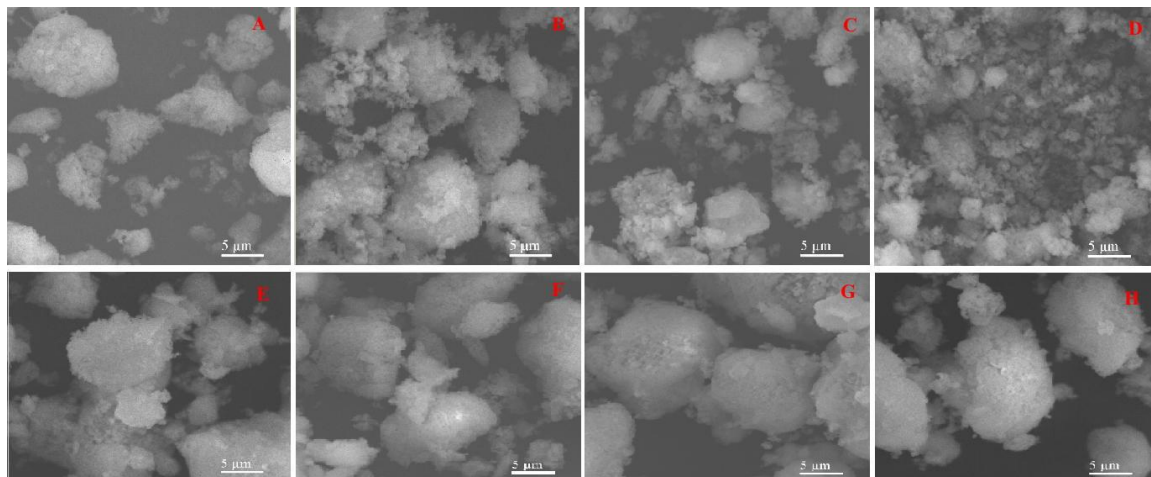


Figure 5: SEM micrographs of the initial BHA (A, E); composite systems BHA/ Fe_3O_4 obtained by the physicochemical method after heat treatment in vacuum (B), nitrogen medium (C), and hydrocarbon medium (D); composite systems BHA/ Fe_3O_4 obtained by the chemical method after heat treatment in vacuum (F), nitrogen medium (G) and hydrocarbon medium (H). Magnification X3000.

As can be seen in Figure 5, the physico-mechanical method makes it possible to obtain a larger variation in the size of agglomerates: from preferably 2.8–5.6 μm for heat treatment in a hydrocarbon medium (Figure 5D) to 6.4–11.5 μm for a vacuum (Figure 5B). Treatment in a nitrogen medium (Figure 5C), where the size of agglomerates is preferably 7.8–8.6 μm and there are many smaller 3–6 μm formations of various shapes, yields intermediate results. However, one can also observe certain similarities, in particular in the sphericity of particles that form large agglomerates and smaller aggregates of irregular shape, which averages 140–700 nm. Large agglomerates after heat treatment in a vacuum contain pores sizing within 140–200 nm and sometimes below 120 nm. After heat treatment in nitrogen and hydrocarbon media, the pore sizes in agglomerates fall in the range 130–250 nm.

In the case of using the chemical method to obtain the BHA/ Fe_3O_4 composites, regardless of their treatment medium, the presence of predominantly large agglomerates of particles is characteristic. After thermolysis in a vacuum (Figure 5F), 4.3–16.0 μm formations are observed, which consist of spherical and

rounded 150–420 nm particles and 100–150 nm pores.

Microphotographs of powders after heat treatment in nitrogen (Figure 5G) and hydrocarbon (Figure 5H) media show similar results. They are characterized by the presence of 12–21.5 μm agglomerates formed by particles of predominantly spherical shape with a diameter of most 600–700 nm or less. Noticeable in the structure of such particles are pores sizing within 200–650 nm.

Hence, be reasoned that the chemical method preserved the structure of initial materials regardless of the heat treatment mediums. According to the data on the Figure 5 could argue that the studied materials have a sufficient porosity needed to a drug adsorption and proliferation of biological cells.

3.5 Magnetic properties

The above results are confirmed by the findings for the specific saturation magnetization of the studied materials (Figure 6), which are on the lower limit of the capabilities of the used ballistic magnetometer (0.1 $\text{A m}^2/\text{kg}$) and do not exceed 1 $\text{A m}^2/\text{kg}$, which indicates a low content of magnetite.

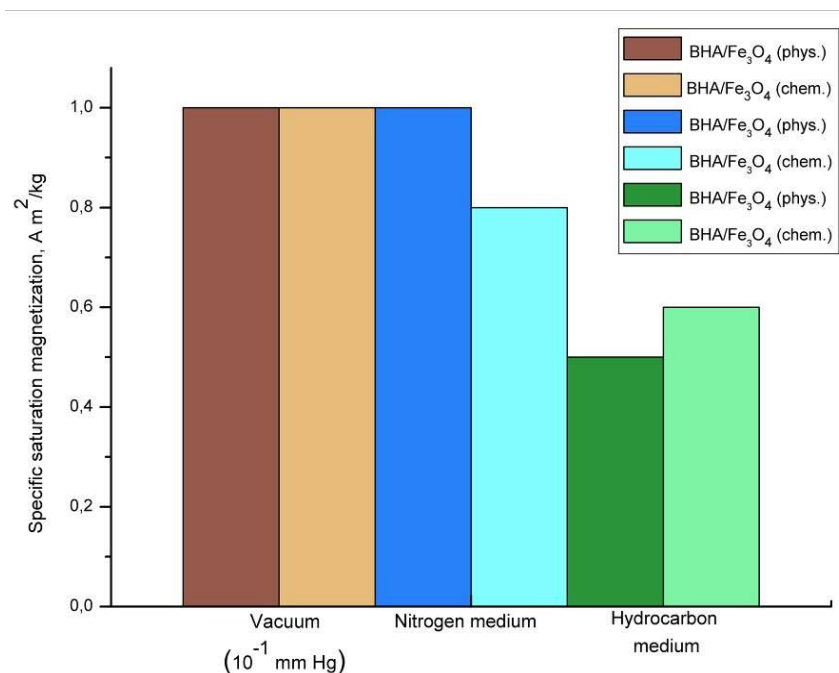


Figure 6: Specific saturation magnetization of the BHA/Fe₃O₄ composites under study.

3.6 Experiments *in vitro*

The probable behavior of BHA and BHA/Fe₃O₄ in an antibiotic solution can be seen *via* determining their bioadsorption in experiments *in vitro* using an analogical dissolvent. For this, the powders were placed in an isotonic physiological 0.9% NaCl (“Arterium”, Ukraine) solution for 2, 5 and 7 days at a temperature equal to that of healthy human body (36.5–37.0 °C). The obtained results are shown in Figure 7. Looking back to the data of Table 1, could argue that the results of bioadsorption are consequence from the difference between the specific surface areas of studied samples: bioadsorption value is directly proportional to the value of specific surface area.

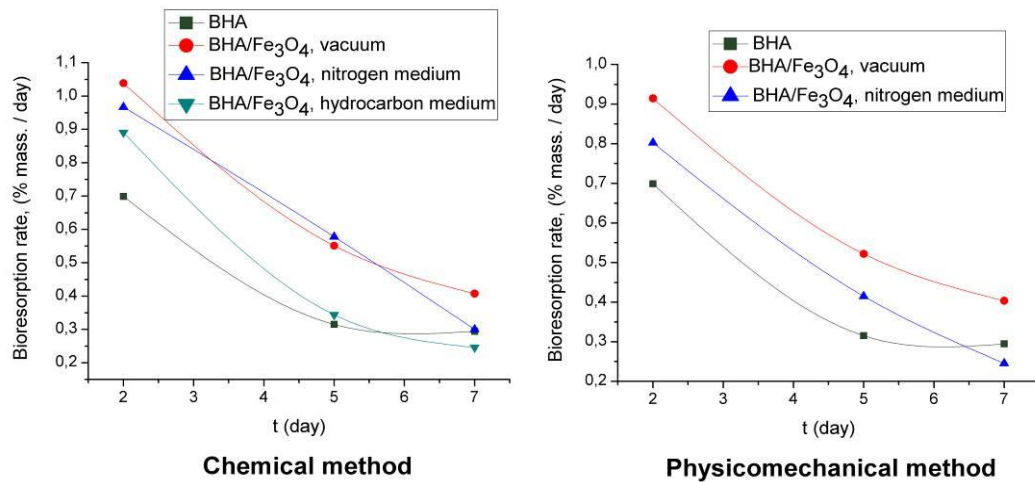


Figure 7: Bioresorption rate of the studied powders (n=10).

The chemical analysis of the filtrates (Table 3) showed a uniform increase in the content of calcium ions along with a uniform decrease in the content of phosphate ions during the experiment. The maximum concentration of iron ions was fixed in the first two days followed by a sharp decrease. Such results can be attributed to the presence of a significant number of negatively charged ions (Cl^- , OH^-) and compounds of the basic nature, in particular $Ca(OH)_2$, $Fe(OH)_2$ and/or $Fe(OH)_3$ in all filtrates.

Table3: Results of the chemical analysis of filtrates after *in vitro* experiments.

MATERIAL (METHOD OF PRODUCTION)	MEDIUM OF HEAT TREATMENT, 500 °C, 2 h	DURATION OF EXPERIMENT (days)	CONTENT OF ELEMENTS (mg)		
			Ca	P	Fe
BHA/Fe ₃ O ₄ (phys.) [34]	Vacuum (1·10 ⁻¹ mm Hg)	2	0.37	0.019	0.098
		5	0.32	0.013	0.011
		7	0.55	0.015	0.017
BHA/Fe ₃ O ₄ (chem.) [34]	Vacuum (1·10 ⁻¹ mm Hg)	2	0.46	0.019	0.081
		5	0.35	0.006	0.010
		7	0.70	0.018	0.017
BHA/Fe ₃ O ₄ (phys.)	Nitrogen	2	0.31	0.018	0.100
		5	0.32	0.009	0.050
		7	0.46	0.006	0.010
BHA/Fe ₃ O ₄ (chem.)	Nitrogen	2	0.47	0.019	0.089
		5	0.59	0.018	0.043
		7	0.67	0.018	0.014
BHA/Fe ₃ O ₄ (chem.)	Hydrocarbon	2	0.41	0.017	0.073
		5	0.47	0.011	traces
		7	0.52	0.003	traces

Probably, during the experiment the number of these compounds is varying: after the first two days, the solutions contain a minimum amount of $Ca(OH)_2$ and a maximum level of $Fe(OH)_2$, while on the 7th day the amount of $Ca(OH)_2$ increases and the level of $Fe(OH)_2$ becomes minimal. A slight decrease in pH over time for all samples is due to the decrease in the content of phosphate ions and the OH^- groups owing to the formation of insoluble $Ca_3(PO_4)_2$, $Fe(OH)_2$ and/or $Fe(OH)_3$ compounds. In particular, powders obtained by the chemical method and heat treated in a vacuum [34] and nitrogen medium are characterized by a decrease in pH from 8.4 for 2 days to 8.2 for 7 days, while pH of the sample treated in a hydrocarbon medium lowered from 8.3 to 8.1. Powder biomaterials obtained by the physico-mechanical method are characterized by pH decrease from 8.6 for 2 days to 8.4 for 7 days in the case of heat treatment in a vacuum [34] and from 8.6 to

8.3 for a nitrogen medium.

It is worth noting that the results presented in Table 3 are consistent with the data in Figure 7 and the pH values for the filtrates.

4. CONCLUSIONS

The BHA/Fe₃O₄ composites were synthesized using two methods (chemical and physicochemical) combined with subsequent heat treatment in vacuum, nitrogen and hydrocarbon media. The obtained results showed that the most efficient method for obtaining the BHA/iron oxalate material is the chemical method, and an optimal medium for its heat treatment to produce the BHA/Fe₃O₄ composite is a vacuum. Despite the magnetite (Fe₃O₄) dominant oxidation to hematite (Fe₂O₃) and partial reduction to wustite (FeO), these conditions provide a more perfect crystalline structure (crystallinity 86%) compared to the other materials studied. In addition, BHA/Fe₃O₄ (chem.) exhibited the highest values of specific saturation magnetization and a stable behavior in experiments *in vitro*.

5. ACKNOWLEDGMENTS

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ORCID

Oksana Otychenko
Tetyana Babutina
Anatolii Perekos
Oleksandr Bykov
Sergiy Korichev
Oleksandr Koval

<https://orcid.org/0000-0001-9210-1738>
<https://orcid.org/0000-0002-6927-2595>
<https://orcid.org/0000-0002-1669-2342>
<https://orcid.org/0000-0001-6959-3498>
<https://orcid.org/0000-0003-2689-3129>
<https://orcid.org/0000-0003-0315-5190>