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Biogenic hydroxyapatite-based composites modified by magnetite and chitosan: bioresorption in physiological solution and cytotoxicity

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This work is devoted to the investigation of interaction of BHA/magnetite/chitosan composites with a magnetite content of 1, 5, 25, and 50 wt.% with physiological solution and their cytotoxicity. It was established that increasing of magnetite content leads to increasing of composites resorption. Moreover, the use of magnetite obtained by chemical precipitation in amount of 5–50 % allows to achieve resorption rate equal to 2.5–5.3 wt.%/day,which in 3.5–7.5 times higher in comparison with "pure" biogenic hydroxyapatite and 1.2–2 times higher in comparison with composites with magnetite obtained by decomposition in nitrogen media. The process of resorption is also confirmed by change in pH,presence of Ca, P and Fe in physiological solution after experiments *in vitro*, decreasing of particles size and increasing of specific surface area of the composites powders. The results of cytotoxicity study confirmed that BHA/magnetite/chitosan composites have no cytotoxic effect. That is why prepared composites could be promising for use in medicine.

Keywords: hydroxyapatite, magnetite, chitosan, composites, resorption, physiological solution, cytotoxicity

Біогенні композити на основі гідроксиапатиту, модифіковані магнетитом та хітозаном: біорезорбція у фізіологічному розчині та цитотоксичність. *А.Синиця, С.Загородня, О.Сич, Т.Бабутіна, І.Кондратенко*

Робота присвячена дослідженню взаімодії композитів БГА/магнетит/хітозан із вмістом магнетиту 1, 5, 25 та 50 мас.% з фізіологічним розчином та їх цитотоксичності. Встановлено, що збільшення вмісту магнетиту призводить до збільшення резорбції композитів. Крім того, використання магнетиту, отриманого хімічним осадженням, у кількості 5–50 % дозволяє досягти швидкості резорбції 2,5–5,3 мас.%/добу, що в 3,5–7,5 разів вище в порівнянні з "чистим" біогенним гідроксиапатитом і 1,2–2 рази вище в порівнянні з композитами з магнетитом, отриманими розкладанням в середовищі азоту. Процес резорбції також підтверджується зміною pH, наявністю Ca, P і Fe у фізіологічному розчині після експериментів *in vitro*, зменшенням розміру частинок і збільшенням питомої поверхні порошків композитів. Результати дослідження цитотоксичності підтвердили, що композити БГА/магнетит/хітозан не мають цитотоксичної дії. Тому отримані композити можуть бути перспективними для використання в медицині.

1. Introduction

Biogenic hydroxyapatite (BHA) has been used in dentistry, orthopedics and surgery for more than 30 years — already in the 1990s, *in vivo* tests of implants made of hydroxyapatite and biocomposites of hydroxyapatite with organic compounds were carried out [1–3]. Research in recent years has focused on the creation of a number of functional materials based on BHA, modified with various inorganic and organic additives [4–7]. Composites that combine a matrix with BHA, an inorganic component and biopolymers, deserve special attention. Thus, a composite based on BHA modified by magnetite (Fe_3O_4) and chitosan is considered a promising material. Recent studies prove that this material has biocompatibility, antibacterial properties, high hemocompatibility, and at the same time its mechanical characteristics are close to human bones [8–11]. Due to the combination of magnetism properties with nanometer dimensions, as well as the ability to function at the cellular level, magnetite particles are a sufficiently attractive material for biomedical applications. Having a large specific surface area, magnetite particles have a high ability to load medicinal substances, and, if necessary, can be used as carriers of antitumor drugs, genes or biosensors [12–14]. In addition, the presence of magnetic nanoparticles in the composite increases the possibilities of biomedical applications, including medical imaging or hyperthermia-based cancer treatment, for which pure BHA is unsuitable [15, 16].

However, the toxicity of materials containing iron nanoparticles is an important issue due to a number of factors, including high reactivity and nonspecific interactions with biological objects determined by the shape, size, and structure of the particles [17–19]. When magnetite nanoparticles are used *in vivo*, the human body is able to convert them into iron ions and use the released iron for biological processes, for example, the formation of erythrocytes [20, 21]. At the same time, the particles should not be subjected to an unexpected release of iron, which will lead to an increase in its amount and cause an overload of the body with iron [22].

Magnetite particles can be surface-functionalized using a variety of chemical modifications, including biocompatible molecules such as natural and synthetic polymers. In addition, it was established that the cytotoxicity of Fe_3O_4 particles depends on the presence of a surface coating [23]. Chitosan is produced from chitin, which is the second most abundant biopolymer after cellulose. It can be found in crabs, shrimps, lobsters, corals, jellyfish, butterflies, ladybugs and mushrooms. Chitosan demonstrates excellent biocompatibility, is almost non-toxic to humans and animals, has high bioactivity, biodegradability, selective permeability, polyelectrolyte action, antimicrobial activity, chelating and absorption capacity [24, 25].

In our previous work [5], composites based on biogenic hydroxyapatite, magnetite, and chitosan were obtained, and their phase composition and structure were investigated. However, it was necessary to study in detail the issue of cytotoxicity and behavior of these materials in a model environment as necessary criteria for predicting behavior in a living organism with the prospect of application in medicine.

Therefore, the purpose of this work was to study the behavior of composites based on biogenic hydroxyapatite, magnetite (Fe_3O_4) and chitosan with different ratios of components in physiological solution (experiments *in vitro*), as well as to determine the cytotoxicity of materials in the presenceof living cells.

2. Materials and methods

Powder composites based on biogenic hydroxyapatite modified by magnetite (1, 5, 25 and 50 wt.%) and chitosan were obtained according to the technology described in our previous work [5]. Magnetite added to composition of materials was obtained by two methods: thermal decomposition of iron oxalates in nitrogen media and chemical precipitation from iron chlorides [14].

Experiments *in vitro* (resorption rate) of composite were conducted in physiological solution (saline) — 0.9 % NaCl aqueous solution, which is an isotonic solution of body fluids and is one of the most often used to dissolve various drugs and injections. Previously, the samples were dried in drying cabinet at 50–100°C for 2–8 hours, weighed by an analytical balance "OHAUS Pioneer PA214C" (OHAUS Corporation, China) with an accuracy of 0.0001 g and immersed in saline with the ratio of solid phase:liquid phase=1:50. The constant temperature of 36.5±0.5° was maintained by TS-1/80 SPU thermostat (Smolenskoe SKTB SPU OAO, Russian Federation). After 48 hours the samples were thoroughly washed with distilled water, dried and weighed. The resorption rate of composite materials was deter-

mined as a specific mass loss during the experimental time. The chemical composition of physiological solution after investigations was analyzed by energy dispersive X-ray fluorescence elemental analysis (Expert 3L Analyzer, INAM, Ukraine). The pH of solution was also monitored using a portable pH meter "Checker HI 98127" (HannaInstruments, USA).

Before and after interaction of composites with saline, the phase composition, specific surface area, density, and microstructure were monitored. XRD patterns of composites powders were collected on an DRON-3 X-ray diffractometer (Bourevestnik, Russia) using Cu–Kα radiation with λ = 1.54178 A. The microstructure was observed by scanning electron microscopy (SEM) with Tescan Mira 3 LMU microscope (Tescan, Czech Republic). Specific surface area was measured by analysis of the BET isotherm method using a Gemini 2360 instrument by Micromeritics according to ISO 9277:2010. The density was measured using a helium pycnometer (AccuPyc II 1340, Micromeritics) at 24±2°C, according to ISO 12154:2014. Before the density and SSA measurements were carried out, composite powders were dried at 50°C for 15 h in vacuum (VacPrep 061 Sample Degas System by Micromeritics).

Moreover, the average diameter of the particles (d_{BET}) was evaluated not only by SEM, but calculated on the basis of SSA and density [26]; assuming that all particles were spherical and identical (1):

$$d_{BET}[nm] = \frac{6000}{SSA[\frac{m^2}{g}] \cdot \rho[\frac{g}{cm^3}]}, \quad (1)$$

where d_{BET} is the average particle diameter calculated using the SSA value, SSA is the specific surface area calculated using the BET isotherm, and ρ is the material density.

Cytotoxicity was studied as cell viability using MTT-assay based mainly on the activity of dehydrogenases in mitochondria, which can convert 3,(4,5-dymetyltriazol-2-yl)-2,5-dyfeniltetrazolium bromide (MTT) to formazan. The conversion of MTT to formazan decreases with cell death and under aninfluence of toxic substances.MTT substrate ("Sigma", USA) was dissolved in sterile phosphate buffer (pH 7.2) at room temperature to a concentration of 5 mg/ml. The composites diluted in the growth media were added (200 μl per well) to 96-well plates with cell monolayer (MDBK — Madin-Darby bovine kidney cells and MDCK — Madin-Darby canine kidney cells were used) and incubated for 48 h at 37°C and 5 of CO_2. After that 20 μl of MTT solution was added to the wells and incubated at 37°C for 2.5–4 hours. Then the growth media was removed and 150 μl of 96 % ethanol was added to dissolve the formazan crystals. The optical density of solutions was determined by a Multiscan FC spectrophotometer (ThermoFisherScientific, USA) at wavelength of 538 nm. Percentage of cell viability was determined according to quantity of formazan that was synthetized in the experimental samples and compared with the control ones (2):

$$Cellviability[\%] = \frac{A}{B} \cdot 100, \quad (2)$$

where A is the average value of the optical density for experimental samples, and B is the average value of the optical density for control samples.

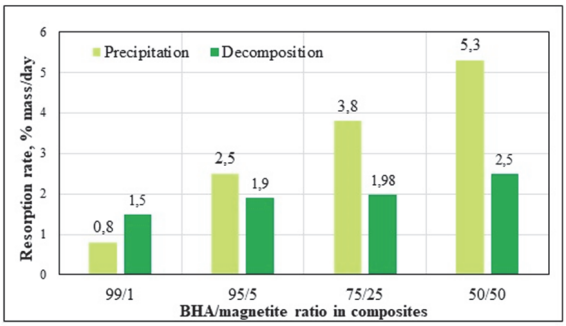

Fig. 1. Resorption rate of BHA/magnetite/chitosan composites in physiological solution

3. Results and discussion

The results of resorption rate investigation of BHA/magnetite/chitosan composites under thermostati cconditions in physiologica lsolution (saline) at 36.5±0.5°C for 2 days are presented in Fig. 1. Comparison of the resorption rate of BHA and composites permits one to conclude that not only the amount of magnetite in composite composition, but also the type of applied magnetite significantly affects the resorption rate of materials. It was shown that increasing amount of magnetite leads to increasing resorption rate of composites, and the use of magnetite obtained by chemical deposition makes it possible to achieve resorption rate equal to 0.8–5.3 wt.%/day, which is 1.2–2 times higher compared to composites with magnetite prepared by thermal decomposition in nitrogen media. At the same time, resorption rate of these composites

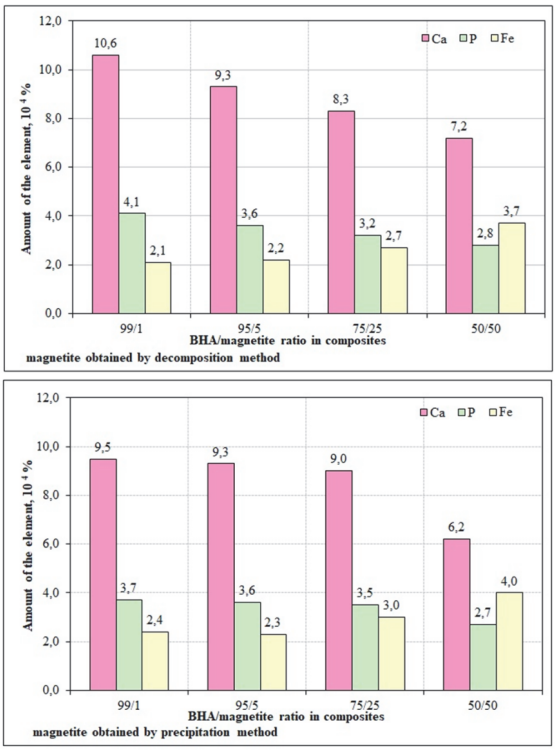

Fig. 2. Results of chemical analysis of physiological solution after interaction with BHA/magnetite/chitosan composites

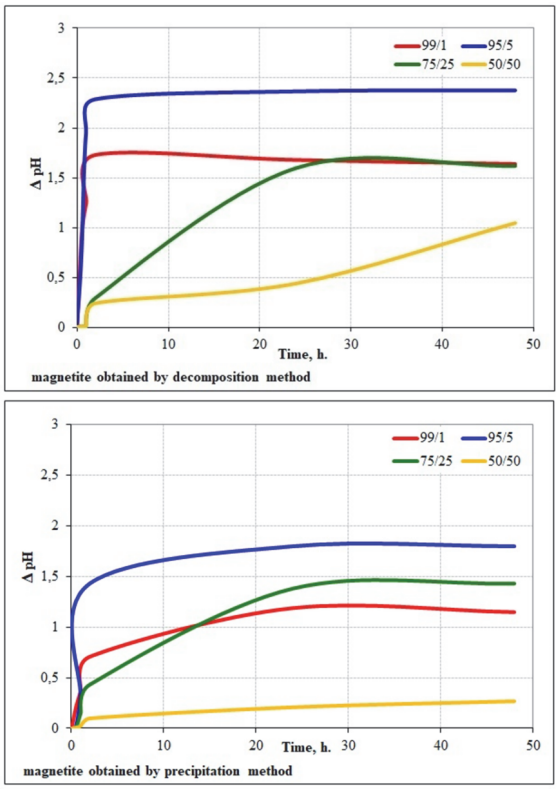

Fig. 3. Change in pH of physiological solution in the presence of BHA/magnetite/chitosan composites

slowly depends on the amount of magnetite. For comparison, the resorption rate of BHA is 0.8 wt.%/day. The obtained results are well correlated and confirmed by the results of energy dispersive X-ray fluorescence elemental analysis of physiological solutions (filtrates) after experiments *in vitro* (Fig. 2). The presence of such chemical elements as calcium, phosphorus and iron was detected in filtrates, which is related to resorption of hydroxyapatite and magnetite. The content of elements in physiological solution is directly related to the composition (BHA/magnetite ration) ofcomposite materials.

The interaction of the physiological solution with BHA/magnetite/chitosan composites can also be demonstrated as a result of change in pH of saline over the time (Fig. 3). The most intense change in pH occurs during the first day followed by stabilization. It could be connected with the fact that hydroxyapatite has a more significant effect on the pH change due to its higher resorption rate as compared to magnetite and chitosan.

The interaction of BHA/magnetite/chitosan composite materials with physiological solution for 2 days also leads to change in morphology of particles. Fig. 4 demonstrates the initia lmicrostructure of powder composites [5] as well as microstructure after exteriments *in vitro* depending on components ratio and method of magnetite synthesis. According to the analysis of photographs obtained by the SEM method, the comparative table of the minimal particle sizes of composites before and after interaction with physiological solution was formed (Table 1). It was established that particle size after interaction with saline is 10–40 % lower than the initial, which can be associated with material resorption. Analogous to the change in pH, the main contribution to resorption is made by hydroxyapatite, although increasing magnetite content leads to increasing resorption of composite materials. It should be noted that the value of particle size obtained based on SSA and density differs from the value obtained based on SEM; this could beassociated with agglomeration of composite particles.

The results for the SSA of initial BHA/magnetite/chitosan composites and after interaction with physiological solution for 2 days are also presented in Table 1. As it was shown earlier, magnetite obtained by chemical precipitation from iron chlorides for 5 minutes has an order of magnitude

Table 1. Specific surface area, density and particle size of BHA/magnetite/chitosan composites before and after interaction with physiological solution

BHA/ magnetite ration in composites	Particle size, nm				SSA, m^2/g		Skeleton density, g/cm^3	
	SEM (min)		BET					
	starting	after saline	starting	after saline	starting	after saline	starting	after saline
magnetite-chemical precipitation (5 min)								
99/1	50	30	376	273	5.2	7.3	3.07±0.02	3.01±0.01
95/5	40	30	194	167	10.0	11.6	3.10±0.03	3.09±0.01
75/25	37	26	58	44	33.8	42.7	3.08±0.06	3.17±0.01
50/50	35	24	42	27	46.6	65.3	3.08±0.05	3.38±0.01
magnetite-thermal decomposition (N_2)								
99/1	100	70	411	381	4.8	5.2	3.04±0.02	3.03±0.0195/5
95/5	90	55	304	264	6.3	7.3	3.13±0.02	3.11±0.01
75/25	50	40	150	110	13.1	17.2	3.05±0.03	3.18±0.01
50/50	45	40	106	86	17.0	20.3	3.33±0.03	3.43±0.01

Table 2. Crystal structure parameters of BHA/magnetite/chitosancomposites before and after interaction with physiological solution

BHA/magnetite ration in composites	Size of the crystallites, Å				Crystal cell volume V, $Å^3$	
	Da		Dc			
	starting	after saline	starting	after saline	starting	after saline
Standart HA 09-432	9.418	–	6.884	–	528.80	–
BHA [28]	9.411	–	6.878	–	527.54	–
magnetite-chemical precipitation (5 min)						
99/1	9.451	9.453	6.895	6.885	533.34	532.80
95/5	9.407	9.436	6.909	6.886	529.46	530.96
75/25	9.406	9.450	6.857	6.892	525.42	533.00
50/50	9.405	9.461	6.874	6.893	526.48	534.32
magnetite-thermal decomposition (N_2)						
99/1	9.454	9.427	6.889	6.885	533.22	529.87
95/5	9.393	9.450	6.860	6.892	524.21	533.00
75/25	9.394	9.470	6.851	6.884	523.55	534.64
50/50	9.410	9.457	6.866	6.886	526.54	533.32

higher specific surface area (141 m^2/g) , in comparison with magnetite obtained by the method of thermal decomposition from iron oxalates (25 m^2/g) [23]. Therefore, increasing SSA with increasing content of magnetite in composition of BHA/magnetite/chitosan composites is regular. Moreover, increasing SSA after interaction with saline is associated with the resorption process and influencing hydroxyapatite on it. For comparison, the specific surface area of BHA and chitosan used to obtain composites is 4.5 and 0.8 m^2/g, respectively [27].

Unfortunately, no unambiguous correlation beetween density and composite composition before and after interaction with saline has been established. Thus, for composites with 1–5 wt. % of magnetite the values of the density were within standart deviation. At the same time, it is possible to note increasing of density of composites after interaction with physiological solution compared to the initial when the amount of

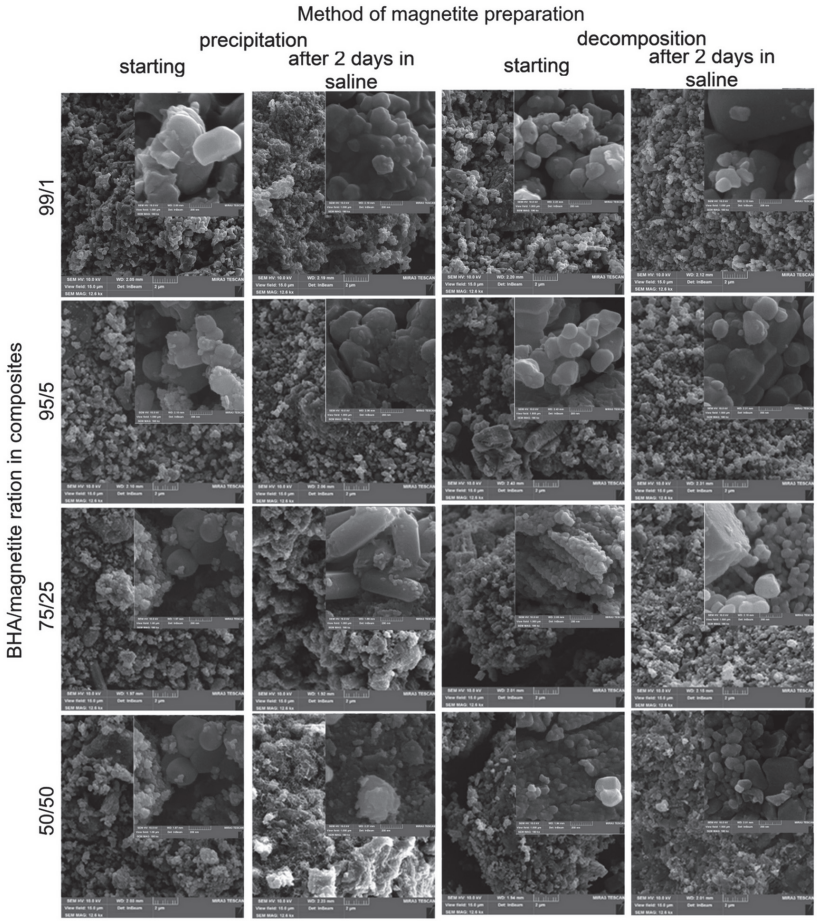

Fig. 4. Microstructure of BHA/magnetite/chitosan composites before and after interaction with physiological solution

magnetite was more than 25 %. For comparison, the density of BHA and chitosan is 3.09±0.01 and 1.49±0.01 g/cm^3 [27], and magnetite obtained by the method of precipitation and thermolysis is 4.22±0.02 and 4.74±0.13 g/cm^3, respectively.

In our previous work [5], the initial phase composition of BHA/magnetite/chitosan composite materials containing 5, 25, and 50 wt. % of magnetite was described in detail. Control of the phase composition before and after interaction with physiological solution showed that, regardless of the type of magnetite used to obtain the composite systems, their phase composition before and after saline were the same. At XRD patterns overlap of the main peaks characteristic of their original components (Fig. 5 and Fig. 6) takes place, namely: chitosan (PDF file No. 39-1894), HA $Ca_5(PO_4)_3(OH)$ (PDF file No. 09-0432), magnetite Fe_3O_4 (PDF file No. 821533) and a small admixture of iron (PDF file No. 01-1262) in the case of using the decomposition method. In addition, the higher the content of magnetite in the composites, the more intense its peaks appear on X-ray patterns.

Based onthe XRD results, the parameters of the BHA crystal lattice in the BHA/magnetite/chitosan system were calculated and listed in Table 2. For comparison, the standard parameters of crystal lattice of hydroxyapatite according to PDF file No. 09-432 and initial BHA were demonstrated. It was found that parameters of crystal structure depend not only on the ratio of components in composite composition, but also on the type of magnetite (method of synthesis). After interaction of powder composites with physiological solution, increasing volume of crystal lattice is observed.

The results of determining the cytotoxic effect of BHA/magnetite/chitosan compositeson MDBK and MDCK cells obtained by MTT-assay are presented in Fig. 6 and Fig. 7, respectively. As can be seen, in the concentration range of 1 mg/ml–1 μg/ml, these substances do not have a cytotoxic effect, since the percentage of cell viability is more

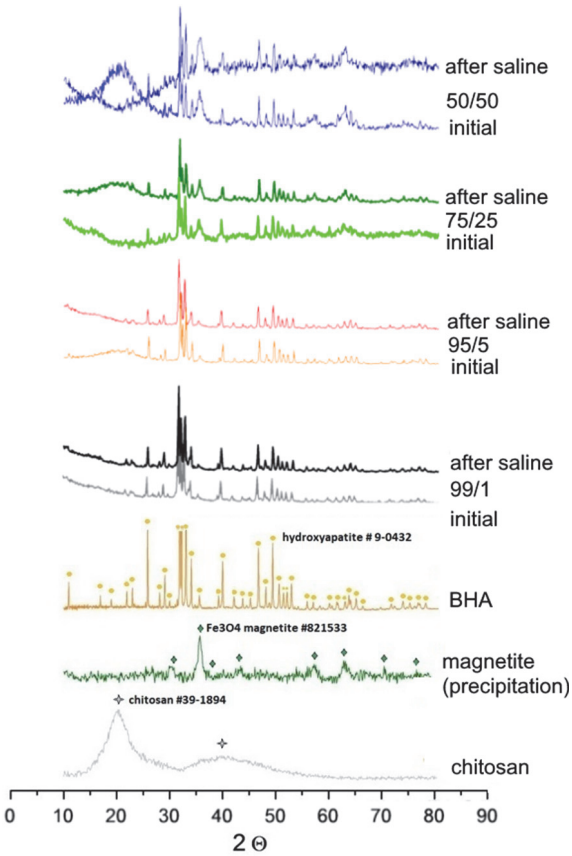

Fig. 5. XRD patterns of BHA/magnetite/chitosan composites (magnetite obtained by chemical precipitation method) before and after interaction with physiological solution

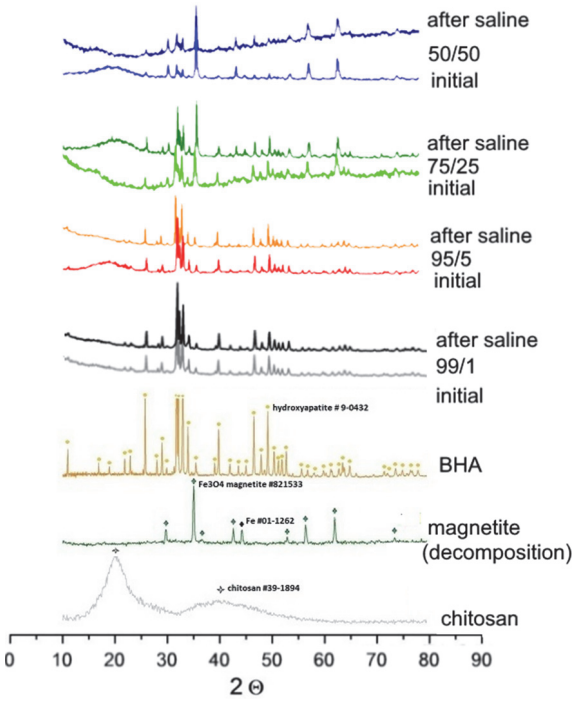

Fig. 6. XRD patterns of BHA/magnetite/chitosan composites (magnetite obtained by thermal decomposition) before and after interaction with physiological solution

than 80 %. Decreasing optical density at concentration of 1 mg/ml for MDBK cells (Fig. 8), i.e. changing of percentage of cell viability from 68 % for a composite with 1 % magnetite obtained by precipitation method to 79 % for a composite with 5 % magnetite obtained by thermal decomposition, is not related with the effect of composites composition, since a monolayer of cells was visible under the microscope.

4. Conclusions

In summary, the study of interaction of BHA/magnetite/chitosan composites (1, 5, 25 and 50 wt. % of magtetite) with isotonic physiological solution in thermostatic conditions at 36.5±0.5°C has been carried out. It was established that resorption of powder composites occurs, confirmed by change in pH, mass loss, decreasing particle size and increase in their specific surface area. Increasing amount of magnetite in composites leads to increasing resorption rate of materials, i.e. the use of 5–50 wt. % of magnetite obtained by chemical precipitation allows to achieve resorption rate of 2.5–5.3 wt.%/day, which is 3.5–7.5 times higher compared to "pure" biogenic hydroxyapatite and 1.2–2 times higher compared to composites with magnetite prepared by thermal decomposition in nitrogen media. Presence in the composite of magnetite obtained by the chemical precipitation in amount of 1 wt. % does not change the resorption rate of biogenic hydroxyapatite. In addition, after interaction with physiological solution, no change was found in the phase composition of composites, which is represented by the phases of chitosan, hydroxyapatite, and magnetite, regardless of the ratio of components, and in the case of using magnetite obtained by thermolysis, with a small admixture of iron. Moreover, no cytotoxic effect was detected for all composite materials, regardless of the ratio of components and the method of magnetite synthesis.

Thus, the properties of composites make them promising for medical applications.

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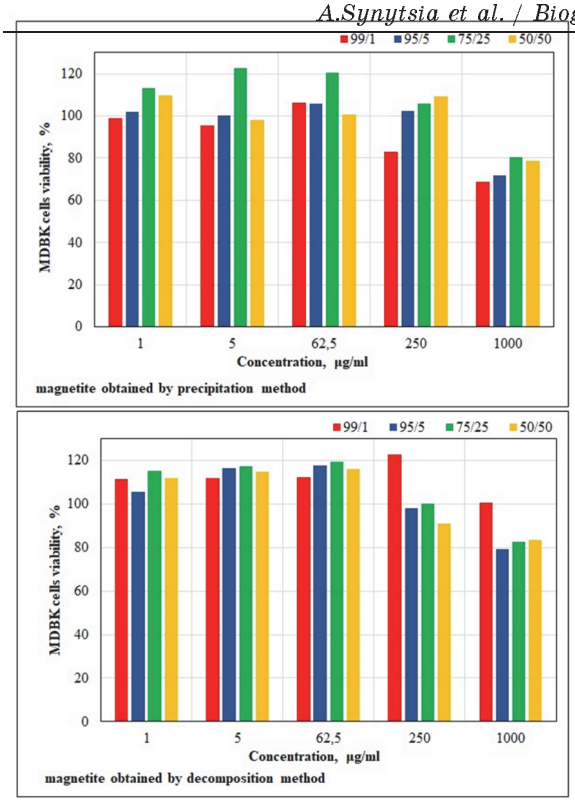

Fig. 7. Cytotoxic effect of BHA/magnetite/chitosan composite on MDBK cells

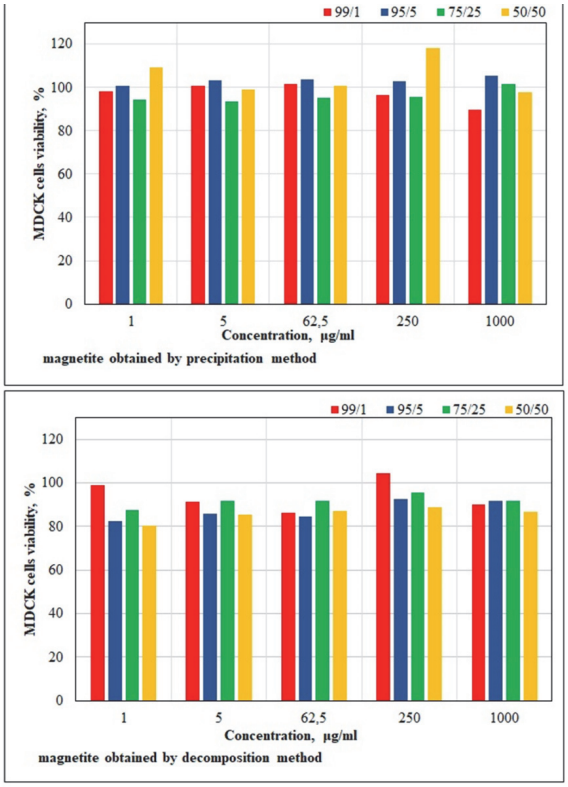

Fig. 8. Cytotoxic effectof BHA/magnetite/chitosan compositeon MDCK cells

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