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Research Article

Phosphorus-containing compounds of alkaline-earth metals as prospective antimicrobial composites for packaging materials

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Abstract

An influence of the qualitative and quantitative composition, phase structure, and the mode of thermal treatment on antimicrobial activity of various phosphorus-containing compounds of some alkaline-earth metals synthesized by co-sedimentation of a metal salt and ammonium hydrophosphate in a solution of ammonium hydroxide has been investigated. The grain size distribution was checked by the laser grain size analysis, which showed that the mean grain size in the materials obtained by the treatment between 400 and 800℃ ranged between 5.48±2.81 and 126.71±3.68 µm. As seen from the sample weight loss analysis, the greatest loss was achieved for the magnesium compounds and became from 3.84±0.13 % (400℃) to 4.13±0.15 % (800℃). Other phosphorus-containing compounds showed weight losses lesser than 3 % (0.36–2.83 %). Antimicrobial activity of the synthesized compounds was tested for different grain sizes against the following reference strains: gram-positive *S. aureus* ATCC 25923, gram-negative *E. coli* ATCC 25922, and yeast-like fungi *C. albicans* ATCC 885-653. Smaller grains showed a greater antimicrobial activity, while all samples proved some retardation in germs proliferation. Possible approaches to the application of the synthesized compounds as antibacterial fillers for the food paper packaging materials are discussed.

Keywords

hydroxyapatite, antimicrobial properties, biofilms, *S. aureus, E. coli, C. albicans*

Abbreviations

CFU – colony forming units; UV – ultraviolet.

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Introduction

Despite significant advances in modern medicine and pharmacy, contagious diseases are still very dangerous and cause many problems for humankind. That is why all issues related to antimicrobial protection are very urgent. Composite materials are promising in the abovementioned context because they can disturb the ability of microbes to develop colonies and form so-called biofilms (O'Toole et al. 2000; Flemming et al. 2010; Blackman et al. 2020). On the other hand, such materials are environment and human-organism-friendly. Thus, they can be used as source materials in the manufacturing of medical tools and equipment (Liu et al. 2021; Samrot et al. 2021), food packaging, parts of the food processing machines (Galié et al. 2018; Hage et al. 2021; Vlahova-Vangelova et al. 2022), and in other branches where the risk of contamination by microbial films must be avoided or minimized.

It should be emphasized that proper packaging is one of the key factors in today's food processing technologies and marketing. It's a well-known fact that safe, relevant, and properly designed packaging is a winning solution that attracts more customers' attention to the product and increases its sales. On the other hand, there are several specific requirements set for food product packaging: it should protect the content from mechanical damage, losses, and chemical or microbial contamination during its transportation, storage, and realization according to Commission Regulation (EU) 2022/1616 (2022).

Packaging is a barrier between its content and the surrounding that prevents microbial contamination of the packaged objects. It should be understood that the acceptable level of microbial contamination varies in different fields of activity, and it should be taken into account during research, development, and production of the packaging intended for particular purposes. Besides, there are different sanitary requirements for different types of packaging and different referent germs to be controlled for various packaged items. The ability of microbes to develop biofilms with increased resistance against antimicrobial agents or unfavourable conditions (UV irradiation, extreme temperatures, low oxygen or nutritional components availability) (O'Toole et al. 2000) should be taken into account in the development of specialized medical or pharmaceutical packaging materials.

The formation of biofilms begins with the adhesion of germs to the surface, followed by its gradual colonization (Flemming et al. 2010). Therefore, this process can be prevented or, at least, decelerated by the surface-applied compositions that impair the ability of germs to adhere to the surface. Hydoxylapatites can be mentioned among such materials, and calcium hydoxylapatite is especially interesting in this context because it is one of the best-investigated compounds, which is close to the inorganic substrate of human and animal bones and is highly biocompatible with it (Fiume et al. 2021; Shi et al. 2021). On the other hand, it is not toxic and shows some antimicrobial activity (De Lima et al. 2021; Czechowska et al. 2021; Mirković et al. 2022). The use of natural additives in food products to improve their quality and safety is described by Kolev (2022).

Hydoxylapatites of other alkaline-earth metals are not so well studied. As seen from references (Kobasa et al. 2016; Wanag et al. 2018; Zhang et al. 2019; Khalili et al. 2022), TiO_2-based composites are promising materials for the manufacturing of antimicrobial coatings, ceramics, dyes, packaging materials, etc. Therefore, these compounds can be used to produce antimicrobial items in medicine, pharmacy, food processing industry, and so on.

The work aimed to investigate the influence of the phosphorus-containing compounds of alkaline-earth metals on the microbial biofilms formed by some referent strains of germs and to check the possible applicability of the compounds as components of the antibacterial food packaging materials.

Materials and Methods

Synthesis and study of phase composition. Studied compounds were synthesized by the "wet method" – sedimentation of the corresponding salts from ammonium-alkaline solutions similar to the synthesis of calcium hydoxylapatite described by Narasaraju and Phebe (1996) according to the reaction:

$$10\ Me(NO_3)_2 + 6\ (NH_4)_2HPO_4 + 8\ NH_4OH =$$

$$= Me_{10}(PO_4)_6(OH)_2 + 20\ NH_4NO_3 + 6\ H_2O$$

where Me: Ca, Mg, Sr or Ba.

Reagents containing 99.99% of the base substance were used for synthesis.

After drying at 105℃ the powder surfaces were analyzed with X-ray Photoelectron Spectroscopy (XPS) also referred to as Electron Spectroscopy for Chemical Analysis (ESCA). XPS is an elemental analysis technique which is capable of detecting all elements except for H and He and has a nominal detection limit of ~0.1 atom %. Spectral interferences may prohibit the detection of some elements in relatively low concentrations. Samples were measured at a 90° Take-Off-Angle yielding a sampling depth of ~10 nm. The analysis area was ~500 µm in diameter. Analyses were performed with a monochromatic Al kα x-ray source.

Thermal treatment and the grain size analysis of the samples. The samples were thermally treated at 400, 600, and 800℃ for 3 h until a stable weight was reached. The grain size analysis was done in the water suspensions using the laser diffractometer PSA 1190 by Anton Paar. Up to 2 bulk ml of a sample were added to the mixture to achieve the obscuration range between 5 to 30 %. Then the mixture was exposed to ultrasound for 1 min, and the measurements were taken at constant stirring.

Preparation and study of antimicrobial activity. The microbial strains used in this investigation were gram-positive *S. aureus* ATCC 25923, gram-negative *E. coli* ATCC 25922, and the yeast-like fungi *C. albicans* ATCC 885-653, part of the microbial collection of the Microbiology and Virology Department at Bukovinian State Medical University, Chernivtsi, Ukraine.

A melted and cooled to 50℃ meat-peptone agar (MPA) or Sabouraud agar (for bacteria or yeast-like fungi, respectively) was mixed with the solutions under investigation in a ratio of 9:1. After an agar gets solidified, a suspension sample of the 24 h broth culture of the referent germs (a 0.02 ml sample consisting of 10^5 alive bacterial cells or 10^3 fungal *Candida* individuals per 1 ml) was applied on the surface of an agar in such a way to ensure a uniform distribution of the sample on the agar surface. Then a Petri dish with this mixture was placed in the thermostat and kept at 37℃ for 24 h (for the bacteria) or at 24℃ for 72 h (for the fungi). Finally, the number of germs' colonies grown on the agar surface was counted. The lesser that number, the stronger the anti-adhesion and anti-germ properties of the composite agent added to the nutritional medium. Thus evaluated the ability of the compositions to disturb the formation of the germ's biofilms.

Statistical analysis of the obtained data. The experimental investigations were repeated three times, and then the descriptive analysis of the data was performed with a confidence probability of 0.95.

Results and Discussion

Phase composition of the synthesized compounds. It is known that the adsorption, antibacterial and catalytic parameters of TiO_2 can vary depending on its chemical composition, structure, porosity, dispersibility and preliminary thermal and/ or chemical treatment (Diichuk et al. 2018; Mazurkevich et al. 2001; 2002). Therefore, it is possible to govern the catalytic and antibacterial activity of TiO_2 by changes in the above characteristics that can be realized through thermal or chemical modification of the material. This way either highly active catalysts and bactericide compositions or low active passive materials can be obtained. Both solutions seem promising in the context of construction and development the effective fillers for modern packaging.

In this context, we determined the phase composition of the synthesized phosphorus-containing compounds (Table 1) and calculated their weight losses after different modes of thermal treatment (Fig. 1).

Table 1. Phase composition of the synthesized compounds after drying at 105℃

Phosphorus-containing compounds of …	Phase composition
Calcium	$Ca_5(PO_4)_3OH$
Strontium	$Sr_5(PO_4)_3OH$
Barium	$Ba_3(PO_4)_2$, $Ba_5(PO_4)_3OH$
Magnesium	$Mg_5(PO_4)_3OH$, $MgHPO_4.3H_2O$

It is known that thermal treatment causes significant changes in the structure, phase composition, and properties of the compounds. Such processes as the formation of pores and increasing defectiveness of the lattice increase the specific surface of a sample and the number of new active centers. The others, like surface caking, lead to the merging of active centers followed by the corresponding decrease in sorption, catalytic activity, and some other properties (Santos et al. 2013; Chen et al. 2022). In this context, it was interesting to study how different modes of thermal treatment affect the antimicrobial activity of the synthesized materials.

Results of the investigation of mass losses at different modes of thermal treatment are given in Fig. 1.

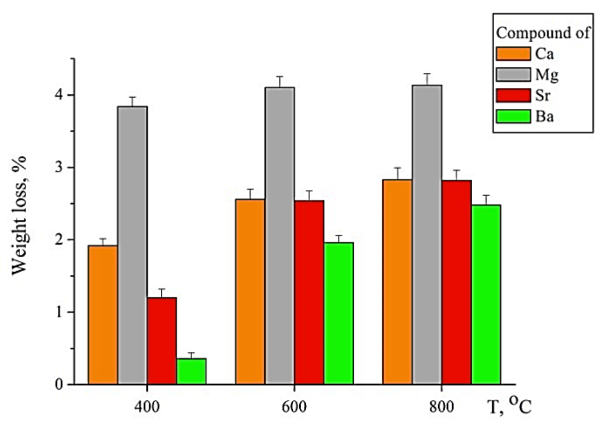

Figure 1. Weight losses of the phosphorus-containing compounds of alkali-earth metals after their thermal treatment at different temperatures

As seen in Fig. 1, the highest weight loss was determined for the thermal treatment of Mg compounds at 400℃ (3.84±0.13 %) and 800℃ (4.13±0.15 %). Such weight losses are probably caused by partial decomposition of the compounds and the loss of certain amount of crystal water, which does not occur at their drying at 105℃. This assumption is based on the phase composition of the synthesized compounds (Table 1). For the other compounds, weight losses were below 3 % (0.36–2.83 %).

An influence of the synthesized compositions on the ability of the test germs to form biofilms. A significant amount of microbes exists in the natural and artificial environment in the form of structured biofilms attached to the surfaces. The formation of biofilms begins from the adhesion of germs on some surfaces, followed by their colonization. Therefore, if the surface is covered with some anti-adhesive composition preventing normal adhesion between the microbes and the surface, the ability of germs to form biofilms would be reduced.

The influence of the thermally treated phosphorus-containing calcium compounds on the number of colonies of various germs is represented in Fig. 2.

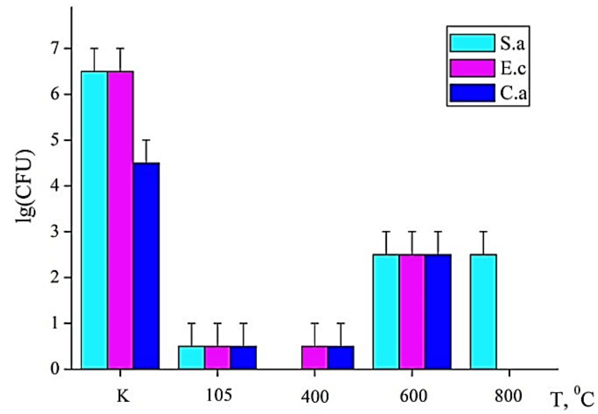

Figure 2. The influence of thermal treatment of the calcium compounds (at 105, 400, 600, 800℃) on their antimicrobial activity against the strains *S. aureus, E. coli,* and *C. albicans*. K – control strain

As seen in Fig. 2, all samples decelerate the proliferation of all tested strains. The highest antimicrobial effectiveness against *S. aureus* is registered for the calcium compounds treated at 400℃, while in the case of *E. coli* and *C. albicans* – for the same compounds treated at 800℃. Other samples also exhibited some antimicrobial activity and decreased the CFU concentration from 10 to 10^3 cfu.mL^{-1}.

Therefore, it can be concluded that the treated phosphorus-containing compounds complicate the adhesion of some germs and the formation of biofilms. In the case of the synthesized barium compounds, the highest antimicrobial activity against all three studied germ strains was found for the samples treated at 400℃ (Fig. 3).

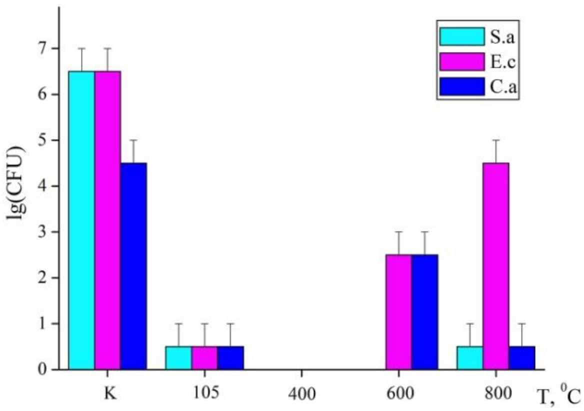

Figure 3. The influence of thermal treatment of the barium compounds (at 105, 400, 600, 800℃) on their antimicrobial activity against the strains *S. aureus, E. coli,* and *C. albicans*. K – control strain

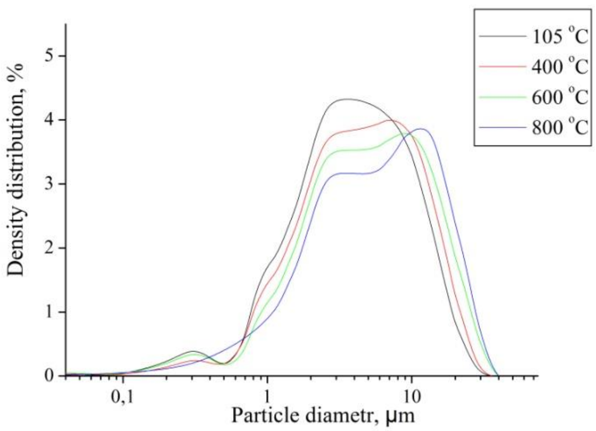

Figure 4. Size distribution of the barium phosphorus-containing compounds for different temperatures of their treatment.

As seen from Fig. 3 and 4, the antimicrobial activity of materials against all microbial strains decreases with an increase in the grain size. Besides, it is seen that the phosphorus-containing materials' activity also depends on their qualitative, quantitative, and phase composition. Previous investigations of this issue (Kobasa et al. 2016; Diichuk et al. 2018) proved that high specific surface area and, correspondingly, small grain size might be the key factors governing the rate of some chemical reactions through the adsorption of reactant molecules on the active adsorption centers. This way, the redox reactions run faster if such materials are present in the reaction mixture. Similar processes can probably intensify the composites' antimicrobial activity and prevent the formation of biofilms on surfaces.

Sufficiently noticeable activity was determined for the samples of Mg dried at 105℃, which decreased the bacterial concentration of *S. aureus*, *E. coli* and *C. albicans* approximately to 10 cfu.mL^{-1}. Other samples exhibited a lower antibacterial activity. Among others, the lowest activity was registered for the compounds of Mg (Fig. 5).

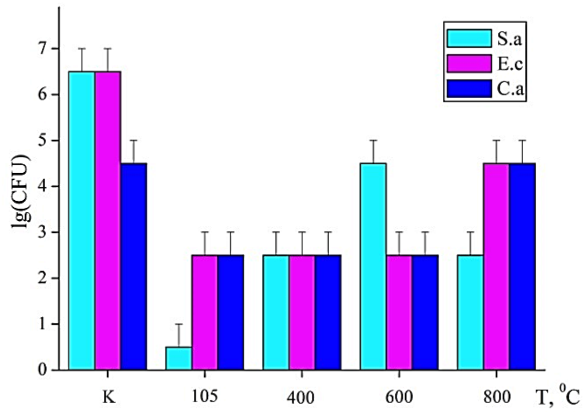

Figure 5. The influence of thermal treatment of the magnesium compounds (at 105, 400, 600, 800℃) on their antimicrobial activity against the strains *S. aureus, E. coli,* and *C. albicans*. K – control strain

A deceleration in the colonies proliferation was registered for all samples of the compounds of Mg with the exception of the sample treated at 800℃ against the strain *C. albicans*, which exhibited the same bacterial concentration as in the control sample. In the case of other compounds of Mg, the CFU concentration was ranged between 10 and 10^5 cfu.mL^{-1}.

The highest activity was found for the Sr compounds (Fig. 5). No colonization by *S. aureus, E. coli* and *C. albicans* was found in the case of Sr compounds treated at 105 and 600℃. Also, no biofilms of *E. coli* and *C. Albicans* developed in the systems consisting of the Sr sample treated at 400℃. Some insignificant colonization was found in the other system, but the CFU concentration was between 10 and 10^3 cfu.mL^{-1}, which is much lower than in the control case.

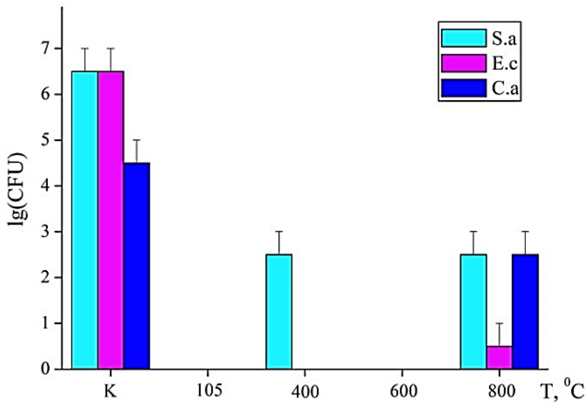

Figure 6. The influence of thermal treatment of the strontium compounds (at 105, 400, 600, 800℃) on their antimicrobial activity against the strains *S. aureus, E. coli,* and *C. albicans*. K – control strain

As seen from the analysis of experimental data, all but one of the 16 samples of the synthesized compounds exhibit some antimicrobial activity against the studied strains. The compound of Mg treated at 800℃ exhibited no antimicrobial activity against the test strain *C. albicans.*

So, it can be seen that the composite materials' synthesis mode affects their antimicrobial activity. Such parameters as pretreatment mode and temperature, admixtures nature and concentration, defectiveness of the material, grain size, and some others may affect the antimicrobial efficiency. The highest efficiency can be achieved at some optimal combination of all these influential characteristics. Then such materials can be considered as components for some practical applications, including antimicrobial food packaging.

Following the above considerations, we prepared some samples of the paper packaging composition consisting of 20 % of the Sr-based composite material thermally treated at 400℃. This choice was based on the highest antimicrobial activity of this class of compounds against the reference germs. Then the antimicrobial activity of the packaging material was determined according to the ISO 27447:2009(E) method. The results are given in Table 2.

Table 2. Sensitivity of germ strains to the paper packaging material with a Sr-based composite treated at 400 °C

Germ strains	Quantity of germs		Percent of remaining germs, %
	Initial	After 4 h	
S. aureus		$5.7 \cdot 10^3$	7.0
E. coli	$8.2 \cdot 10^4$	$4.2 \cdot 10^3$	5.1
C. albicans		$3.3 \cdot 10^3$	4.0

As seen from the data of Table 2, this packaging material efficiently suppresses the proliferation of the reference microorganisms, eliminating more than 90 % of all studied germs (*S. Aureus, E. coli, and C. albicans*) after a 4-hour-long test correspondingly.
An 8-hour-long test ensured the complete disinfection of the samples. In the control experiments with the packaging material not containing the synthesized composite material, no antimicrobial activity was detected. These results give us ground to expect that the synthesized compounds can be applied to the development and production of new non-toxic antimicrobial food packaging materials.

Conclusions

It has been found that all the systems under investigation slow down the proliferation of all tested strains. The compounds of Mg exhibit the lowest antimicrobial activity, while the activity of Sr compounds is the highest. The Ca compounds treated at 400℃ are especially effective against *S. aureus*, and the ones treated at 800℃ – against *E. coli* and *C. albicans*. The highest antimicrobial activity of the Ba compounds was determined for the samples treated at 400℃.

Therefore, it can be concluded that modified hydroxylapatites and other phosphorus-containing compounds of alkali-earth metals can be used as components of antimicrobial compositions for the prevention of the development of biofilms in various food processing technologies, development of non-toxic antimicrobial packaging materials, medicines, and other industrial and household devices.

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