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Calcium phosphate nanoparticles substituted with Zn^{2+} / Cu^{2+} - as antibacterial systems

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Abstract

Recent research has focused on inorganic factors that were combined with ceramic and polymeric biomaterials and their possible applications. The aim of this study was to determine the antibacterial properties of calcium phosphate ceramics (hydroxyapatite) coupled with zinc and copper (CP-Zn, CP-Cu), obtained by co-precipitation. For this purpose, we synthesized calcium phosphates compounds substituted with zinc and calcium phosphates substituted with copper in percent of 1, 2 and 3%. Antimicrobial assays of these complexes were performed using three bacterial strains, two Gram negative (Pseudomonas aeruginosa ATCC 15692, Escherichia coli ATCC 8739) and one Gram positive (Staphylococcus aureus ATCC 6538).Ou studies showed significant antimicrobial properties of the calcium phosphates substituted with copper against S. aureus simultaneously with seeding of the strain and of the calcium phosphates substituted with copper against P. aeruginosa at one day after seeding and for the calcium phosphates substituted with zinc against P. aeruginosa at one day after seeding. These results have been confirmed by growth curves and the microscopic analysis.

Keywords: *calcium phosphates, hydroxyapatite nanoparticle, antimicrobial activity, P. aeruginosa, E. coli, S. aureus*

1. Introduction

Several studies suggest that the chemical and morphological nature of the biomaterials surface determine, to a large extent, how the biomaterial interacts with the host tissue and physiological fluids (1). Hydroxyapatite (HAp), the main component of bone tissue, is a crystalline mineral of calcium phosphate and due to its functional groups that consist of positively charged pairs of calcium ions and negatively charged oxygen atoms associated with triplets of crystal phosphates, distributed in a fixed topogeographic pattern on the crystal surface, it has been available for purification of proteins and nucleotides since 1956. This combination of active groups supports protein and nucleic acid retention by at least three distinct mechanisms: cation exchange with P-sites, calcium coordination with C-sites, and anion exchange with C-sites (2). Which mechanism or combination of mechanisms dominates, depends on the operating pH, buffer composition and the surface properties of the protein (or other solute) (3). Compared to conventional ceramic formulations, nanophase of HAp has specific properties such as surface grain size, pore size, wettability, and so on, that could control protein interactions (for example, adsorption, configuration and bioactivity); therefore, modulating subsequent enhances in osteoblast adhesion and long-term functionality. Surface adsorbed proteins can expose regions that are specific for receptors on biological cells and can greatly influence cell attachment, proliferation and the tissue

response to biomaterials (4). Recent trends in the field of biomaterials are focused on composites where calcium phosphate ceramics is the matrix for different trace elements. The following study is focused on fabrication of HAp substituted with zinc and copper ions. The calcium ions from HAp can be substituted with many ions such as Mg^{2+}, Mn^{2+}, Sr^{2+}, Na^{+}, K^{+} etc., whereas the OH- sites can be substituted with Cl^{-} and F^{-} ions and PO_4^{3-} with BO_3^{3-} etc. (5) and due to their biocompatibility in osteointegration and osteoconductivity, calcium phosphates compounds have been known to be efficient as local drug and bioactive delivery system. Many metals (e.g., Zn, Cu, Mn) are essential for the functioning of living organisms: as micronutrients, serving as structural proteins and pigment, used in the redox processes, regulation of the osmotic pressure, maintaining the ionic balance and enzyme component of the cells (6). Moreover, zinc and copper ions are trace elements that play a role of antioxidants that bind free radicals in the body thus preventing them to combine with proteins. Besides the role of antioxidant, zinc is also an activator for bone formation (7) and helps pathological calcification, is a cofactor for a lot of enzymes, plays a role in maintaining the structure and function of membranes, protein synthesis, in mitosis and cell proliferation. Copper is involved in iron absorption, in elastin and collagen function, and it is used in the treatment of rheumatoid arthritis. Both trace elements have anti-inflammatory and anti-infective properties, etc. (8,9). *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are an important cause of infection. *P. aeruginosa* an opportunistic bacterium is responsible for infections of the eyes, ears, urinary tract, hospital acquired intraabdominal complications, pneumonia, bloodstream infections, skin and soft tissue infections (10). On the other hand, *S. aureus* is considered a pathogen with high potential to cause multiple infections in humans and animals, it is the most relevant member of the group, considered the most virulent, responsible for a wide spectrum of diseases, ranging from infections of the skin and soft tissues to severe life threatening infections (11). *E. coli* is a bacterium that can cause infections in the human body, bloody diarrhea, urinary infection, hemolytic uremic syndrome, and kidney failure (12). Studies on the antimicrobial activity of calcium phosphate substituted zinc and copper have been performed; but the results were different: some authors have reported clear positive results for substituted phosphates with copper ions (13), substituted phosphates with zinc ions (14) and others for calcium phosphate (15).

2. Materials and Methods

Calcium phosphate nanoparticles were prepared as a support for copper and zinc ions, by co-precipitation. Copper and zinc ions were added in the form of sulphates: ($CuSO_4 \cdot 5H_2O$, 3M – Consors and $ZnSO_4 \cdot 7H_2O$, 3M - SC Silal Trading), in a percentage of 1, 2 and 3%, and the solution was added over a calcium nitrate solution ($Ca(NO_3)_2 \cdot 4H_2O$, 3M - Sigma - Aldrich). This mixture was added dropwise, with stirring, to the solution of ammonium phosphate ($(NH_4)_2HPO_4$, 0.3M - Sigma - Aldrich). The pH of the mixture was maintained at 9.5 with a solution of ammonium hydroxide (NH_4OH 25% - Sigma - Aldrich). After 24 hours the reaction mixture was filtered and washed until the pH value reached 8. This mixture was then dried at 100°C and heat treated at 500°C. The samples obtained were analyzed by X-ray diffraction using a SHIMATZU XRD 6000 diffractometer, with CuKα (λ=1.5405 Å) radiation, scanning speed 2°/min., in 2θ = 10 - 55° grd range and the micrographs were obtained using a Quanta Inspect F scanning microscope and the transmission electron micrographs were obtained using a Tecnai TM G2 F30 S-TWIN high resolution transmission electron microscope (HRTEM), equipped with STEM – HAADF detector, EDX and EELS, with the following characteristics: acceleration voltage of 300 KV obtained from a Shottky Field emitter with a high maximum beam current > 100 nA, high probe current 0.6 nA in a 1 nm spot, 15 nA in a 10 nm spot, small energy spread 0.8 eV and with a spot drift of 1 nm /

minute; TEM point resolution of 2 Å and line resolution of 1 Å. In order to test the antibacterial activity of the calcium phosphate powder coupled with copper and zinc ions inhibition assays and growth curves were performed using three bacterial strains: two Gram negative (*P. aeruginosa* ATCC 15692, *E. coli* ATCC 8739) and one Gram positive strain (*S. aureus* ATCC 6538). Thus, the strains were grown on Luria Bertani medium containing NaCl 1%, peptone 1%, yeast extract, 0.5% and agar 2%. Before the experiments were carried out, the strains were activated by passaging the cells on solid medium and incubated for 18-24 hours at 30º C. Growth curves were obtained using a liquid medium containing the same ingredients as the solid medium, except agar. Briefly, each strain was seeded on plates in the exponential phase and in order to test the antimicrobial effect of HAp nanoparticles coupled with zinc and copper the treatment was achieved in two ways: simultaneously with seeding and one day after the seeding. The samples were marked as follows: I – CF unsubstituted, II.1 – CF – Zn 1%, II.2 - CF – Zn 2%, II.3 - CF – Zn 3%, III.1 - CF – Cu 1%, III.2 - CF – Cu 2%, III.3- CF – Cu 3%. Measurements of the kinetics of bacterial growth were assessed using a microplate spectrophotometer with temperature-controlled (TECAN) at 30°C; the microtiter plates were placed and mixed in a vortex for 5 seconds before each reading. Bacterial growth was estimated as a result of the changes in absorbance (OD 600 nm) in each well every 60 minutes for 8 hours. The amplitude effect of HAp nanoparticles un/substituted with zinc and copper has been observed through microscopic images of the bacterial cultures which were obtained using an optical microscope (Zeiss microscope, Germany) provided with immersion objective (100 X magnification).

3. Results and Discussion

XRD analysis (Fig. 1) showed that at 500°C, for unsubstituted sample, the diffraction peaks correspond to the HAp phase ($Ca_5(PO_4)_3(OH)$, Ref. Code 01-080-7086 and the monetite phase ($CaHPO_4$, Ref. Code 04-015 -9193).

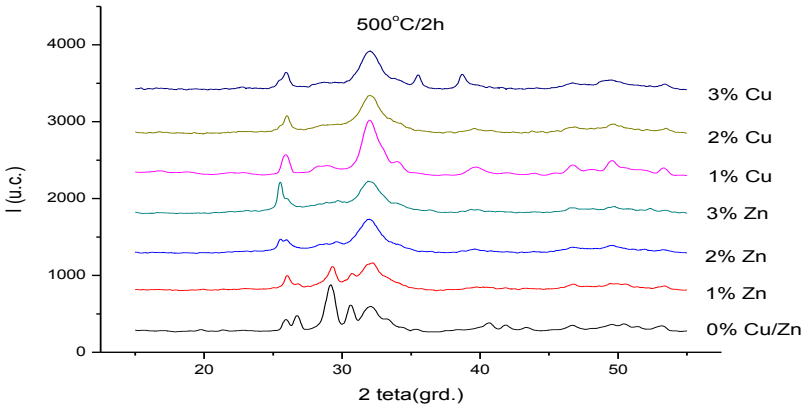


Fig. 1. XRD pattern of the unsubstituted sample and for samples substituted with zinc ions and with copper ions, annealed at 500°C/2h

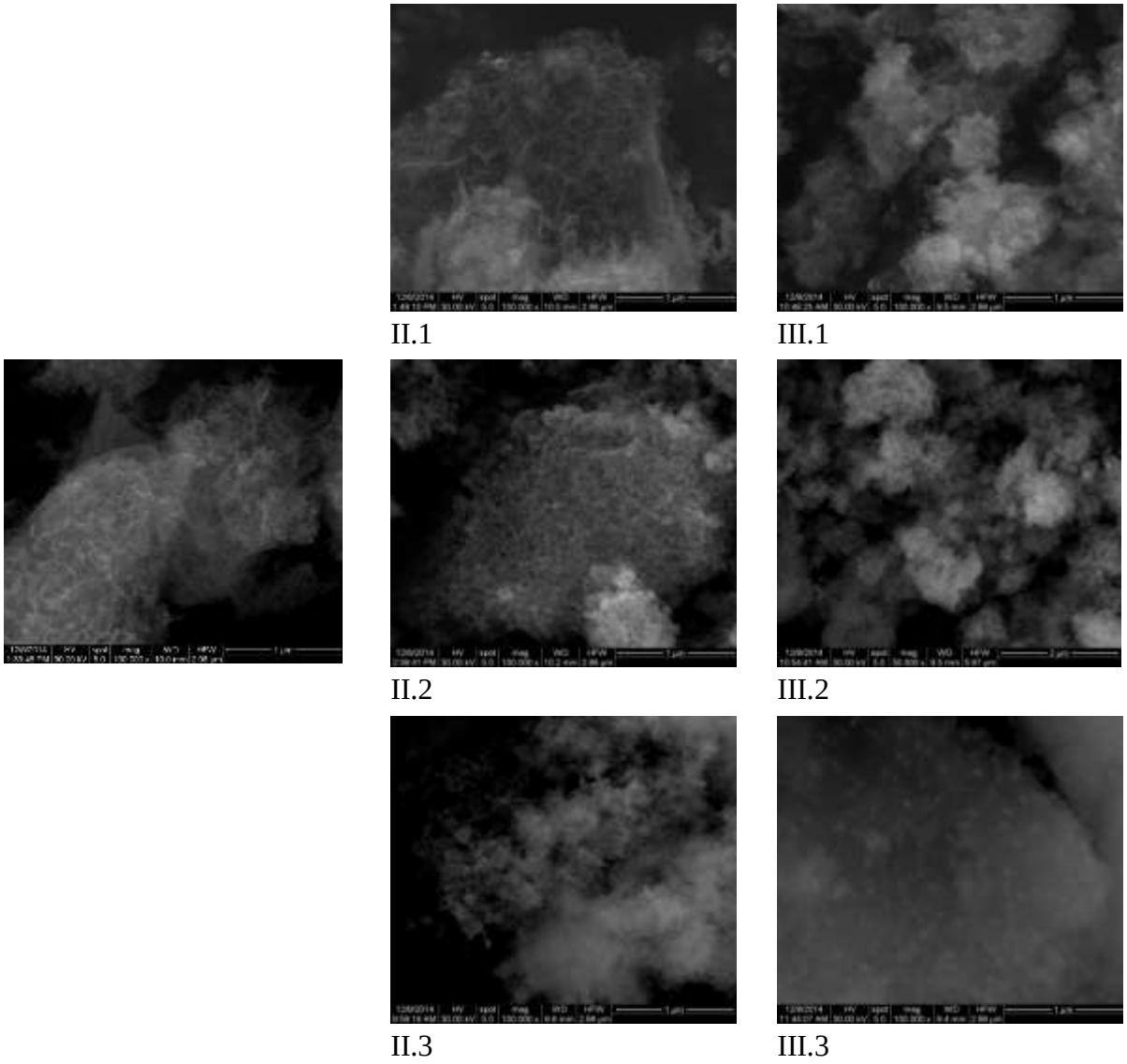


Fig. 2. SEM images for the unsubstituted sample and for samples substituted with zinc ions and copper ions, annealed at 500^{o}C

For samples substituted with 1, 2, 3% Zn / Cu is noted that the diffraction peaks are corresponding to HAp phase ($Ca_5(PO_4)_3(OH)$, Ref. Code 01-080-7086). In addition to HAp phase, it was also formed a byproduct of monetite, which has no siginificance to our study, because the experimental data shows that *in vivo* monetite turns into HAp (16). The unit cell parameters of HAp are $a = b = 9.45$ Å and $c = 6.90$ Å have lower values in the samples with zinc and copper substitution. Thus, unit cell parameters of HAp substituted with zinc were $a = b = 9.42$ Å and $c = 6.88$ Å and of HAp substituted with copper was $a = b = 9.42$ Å and $c = 6.88$ Å. Such an effect would be expected when an ion is substituted with one smaller in volume, which is also the case of Ca^{2+} and Cu^{2+}/Zn^{2+}, so this is a proof that the substitution took place.

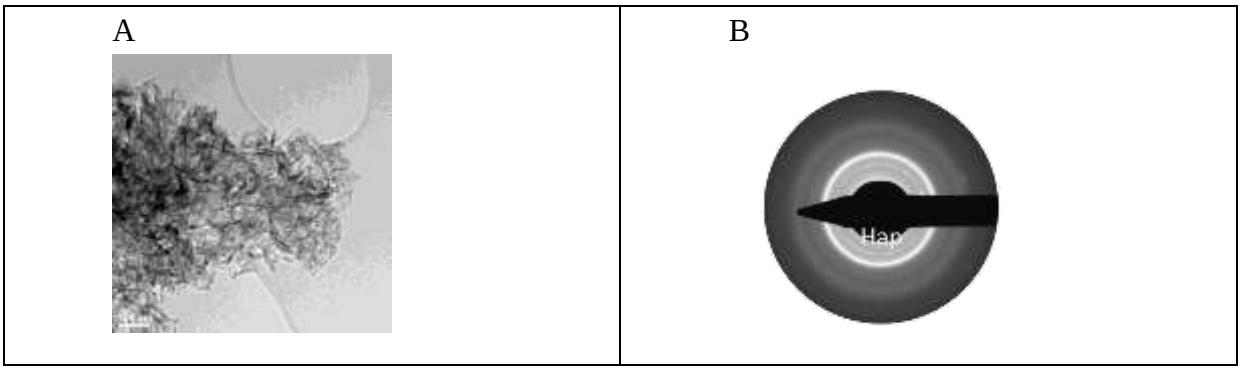


Fig. 3. TEM (a) image and SAED (b) pattern for Hap substituted with zinc ions powder, annealed at 500^{o}C/2h

The shape and the size of the hydroxyapatite particles observed on the powder with copper substitution (III.1) are smaller, needle–like shaped and similar in regard to compactness (Fig. 4a).

The SAED patterns presented in figures 3 b (II.1) and 4 b (III.1) indicate that the powders have fine crystalline structures. In the SAED patterns were identified (211), (112) and (300) crystallographic planes corresponding to HAp with hexagonal structure.

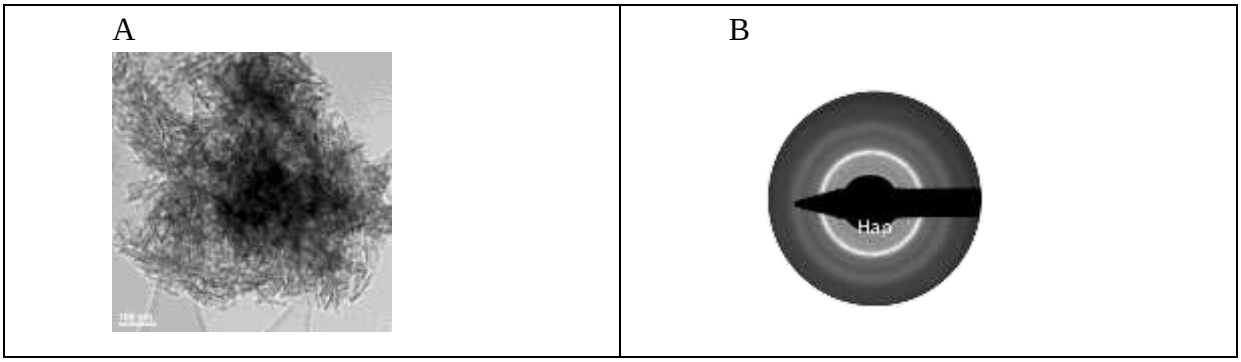


Fig. 4. TEM (a) image and SAED (b) pattern for Hap substituted with coper ions powder, annealed at 500^{o}C/2h

The morphological characterization of the obtained powders was performed by scanning electron microscopy. Thus, the geometry of the calcium phosphate particles observed in the powders with zinc substitution (II.1, II.2, II.3) differs from that of powders with copper substitution (III.1, III.2, III.3) as follows: the nanoparticles that contain Zn are more elongated and the others appear as aggregates of round-shaped nanoparticles. These differences can be seen in Fig. 2; where it can also be observed that their size decreases when the substitution rate increases. The morphology of the samples was also characterized using TEM analysis. TEM analysis of hydroxyapatite powders with zinc substitution (II.1) accurately confirm nanometric size of these particles, with a particle size distribution in the range of 10–50 nm (Fig. 3a). In Fig. 5 are highlighted the EDAX spectra of the powders of hydroxyapatite substituted with zinc (a) and copper ions (b). The presented spectra show the ratio of the constituents of the materials and the presence of zinc and copper in the structure of hydroxyapatite. The results of SAED and HRTEM analyzes obtained in the present investigation are in good agreement with the results of XRD analysis and with other authors (17, 18).

Bacterial adhesion and cell adsorption onto HA is known to depend on the surface properties of both the biomaterial and the bacterial strain, and may be due to both nonspecific bonding and specific interactions, including nonspecific van der Waals, electrostatic forces, and lectin-like adhesion-receptor interactions (19, 20). Some studies have confirmed the antibacterial efficiency of hydroxyapatites substituted with copper ions (copper ions were introduced into the structure using copper acetate $(CH_3COO)_2 \cdot H_2O$) against *E. coli* bacteria. Hydroxyapatites substituted with copper ions to the concentration of 3.3% inhibited the growth of *E. coli* bacteria, while a content of 0.66% of Cu^{2+} was deemed to be insufficient to inhibit bacteria (21).

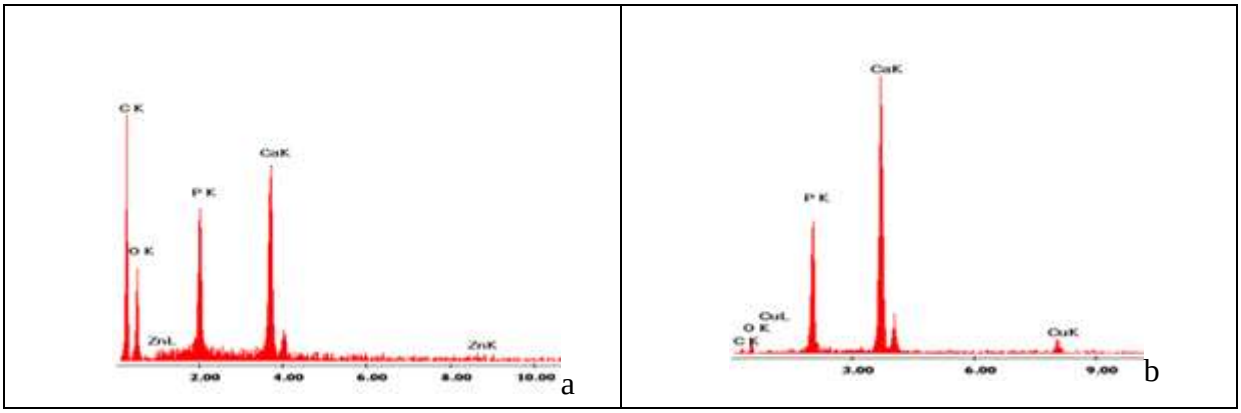
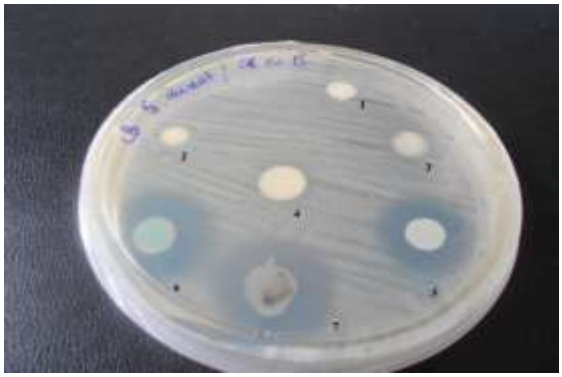


Fig. 5 EDAX spectrum of Hap substituted with zinc (a) and copper ions (b)

Berry and Siragusa (1997) have demonstrate that HAp non-specifically bind microorganisms such *Carnobacterium divergens*, *Brochothrix thermosphacta*, *E. coli*, *Enterococcus faecalis*, *Liseria monocytogenes*, *Pseudomonas fescens*, *Salmonela thyphimurium*, *S. aureus*, *Yersinia enterocolitica*, and potentially other bacteria. The adsorption of bacteria, which possess a net negative charge to HAp appears to be similar to the adsorption of negatively charged proteins, which is due to the electrostatic attraction between the negatively charged molecule and the positively charged calcium atoms of the HAp (22). Moreover, studies concerning adhesion process of different *Streptococcus* and *Lactobacillus* strains on powdered HAp was shown to change with Ca^{2+} concentration, and this effect depends on the surface properties of each strain (23). Many metals ions (e.g., Zn^{2+}, $Cu^{2+,}$ etc.) are essential for the functioning of living organisms and at in present, a fairly common tendency is to use the antibacterial properties of certain ions, including silver, copper and zinc ions (6).

A

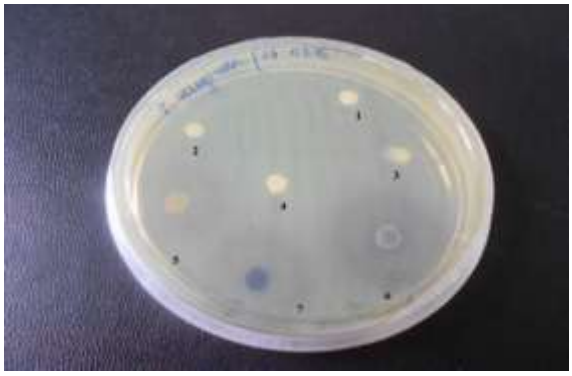
a’



B

b’

C



c’

***Fig. 6. E. coli* (ATCC 8739) (a, a’), *S. aureus* (ATCC 6538) (b, b’), *P. aeruginosa* (ATCC 15692) (c, c’) treatment performed simultaneously with seeding (a, b, c) and treatment performed at one day after the seeding (a’, b’, c’): 1-I, 2-II.1, 3-II.2, 4-II.3, 5-III.1, 6-III.2, 7-III.3**

The disk diffusion test (Fig. 6) show how the nanoparticles CP-Zn and CP-Cu affect bacterial strains growth (*P. aeruginosa ATCC 15692*, *S. aureus ATCC 6538* and *E. coli ATCC 8739)*. In the presence of calcium phosphate substituted with 1, 2, 3% copper and zinc there were not present areas of inhibition against *E. coli ATCC 8739*. Treatments performed simultaneously with seeding showed good results against *S. aureus ATCC 6538* in contact with copper substituted calcium phosphate (ratio D1/D2 approx. 2). Against *P. aeruginosa ATCC 15692*, at one day after treatment, calcium phosphates substituted with copper (ratio D1/D2 in the range 1.2 – 1.5) and those substituted with zinc (ratio D1/D2 approx. 1,2) showed good antibacterial activity. Also, unsubstituted calcium phosphates gave good results against *P. aeruginosa ATCC 15692* in a day of sowing.

In this regard, our results show a similar inhibitory effect on Gram negative and Gram positive bacteria and Zn^{2+}/Cu^{2+} HAp nanoparticles treatment. Thus, the diameter of the inhibition aria was observed and measured and the results are listed in Table 1.

Table 1. Results of antibacterial treatment of samples of HAp substituted with Zn^{2+} and Cu^{2+}

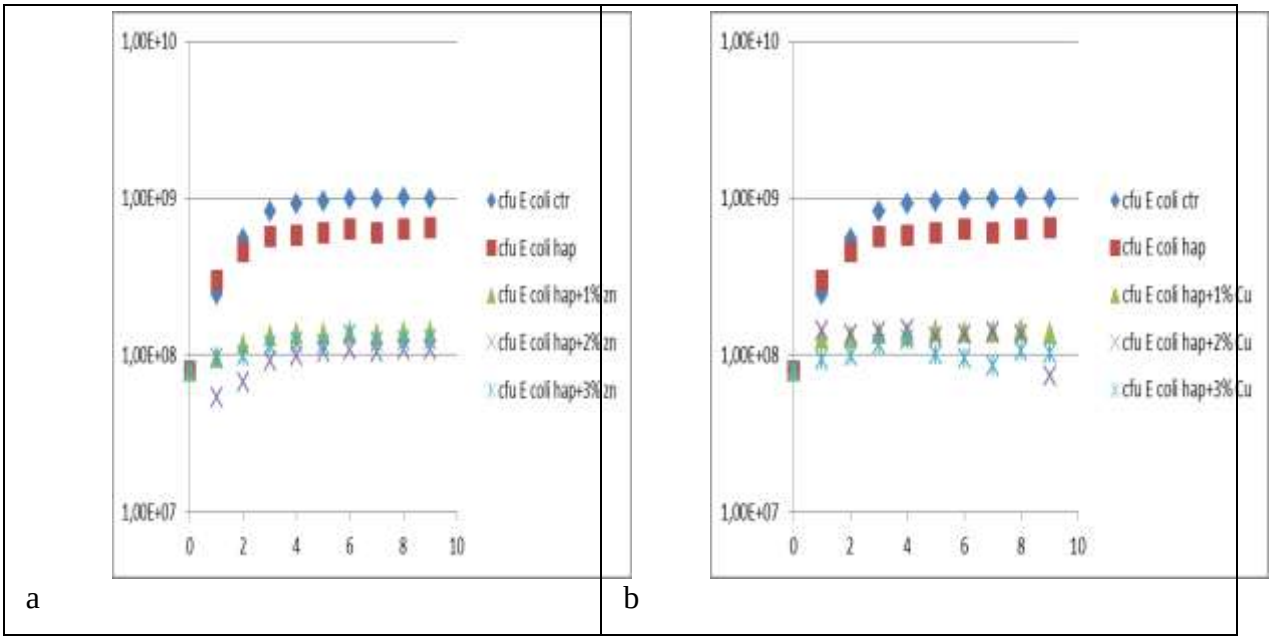
	Treatment performed simultaneously with seeding			
Strains+treatment	Lysis stretch	Diameter lysis stretch (D1)	Diameter spot (D2)	Ratio D1/D2

E. coli				
I	-	-	-	-
II.1	-	-	-	-
II.2	-	-	-	-
II.3	-	-	-	-
III.1	-	-	-	-
III.2	-	-	-	-
III.3	-	-	-	-
S. aureus				
I	-	-	-	-
II.1	-	-	-	-
II.2	-	-	-	-
II.3	-	-	-	-
III.1	+	1,4±0,5cm	0,7cm	2,00
III.2	+	1.9±0,5cm	0,9cm	2,11
III.3	+	2±0,5cm	0,9cm	2,22
P. aeruginosa				
I	-	-	-	-
II.1	-	-	-	-
II.2	-	-	-	-
II.3	-	-	-	-
III.1	-	-	-	-
III.2	-	-	-	-
III.3	-	-	-	-
Treatment performed at one day after the seeding (after growing)				
E. coli				
I	-	-	-	-
II.1	-	-	-	-
II.2	-	-	-	-
II.3	-	-	-	-
III.1	-	-	-	-
III.2	-	-	-	-
III.3	-	-	-	-
S. aureus				
I	-	-	-	-
II.1	-	-	-	-
II.2	-	-	-	-
II.3	-	-	-	-
III.1	-	-	-	-
III.2	-	-	-	-
III.3	-	-	-	-

P. aeruginosa				
I	+	0,6±0,5cm	0,5cm	1,2
II.1	+	0,5±0,5cm	0,4 cm	1,25
II.2	+	0,7±0,5cm	0,6 cm	1,16
II.3	+	0,6±0,5cm	0,5cm	1,2
III.1	+	0,6±0,5cm	0,5cm	1,2
III.2	+	0,6±0,5cm	0,5cm	1,2
III.3	+	0,6±0,5cm	0,4cm	1,5

There is evidence that infecting bacteria are often surface-associated, and their cell surface can therefore be expected to be more similar to that of bacteria grown on solid medium than the ones found in liquid media (24). Bacterial cells grown on solid medium differ to those grown in liquid medium in expression of cell-associated molecules (25). Kiers, and coworkers have explained that this pattern is due by the presence of liquid layers of polyelectrolyte surrounding bacterial wall and membrane who decreases the energy barrier due to electrostatic repulsion in the interactions of the organisms with negatively charged substrates (26).

In consequence we tested the growth ability of the three bacterial strains at two different HAp Cu/Zn substituted concentrations. Fig. 7 show the effect of HAp and Cu^{2+} and Zn^{2+} substituted HAp on growth curves of *P. aeruginosa* ATCC 15692, *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 in comparison with HAp alone and bacterium growth control.



a b

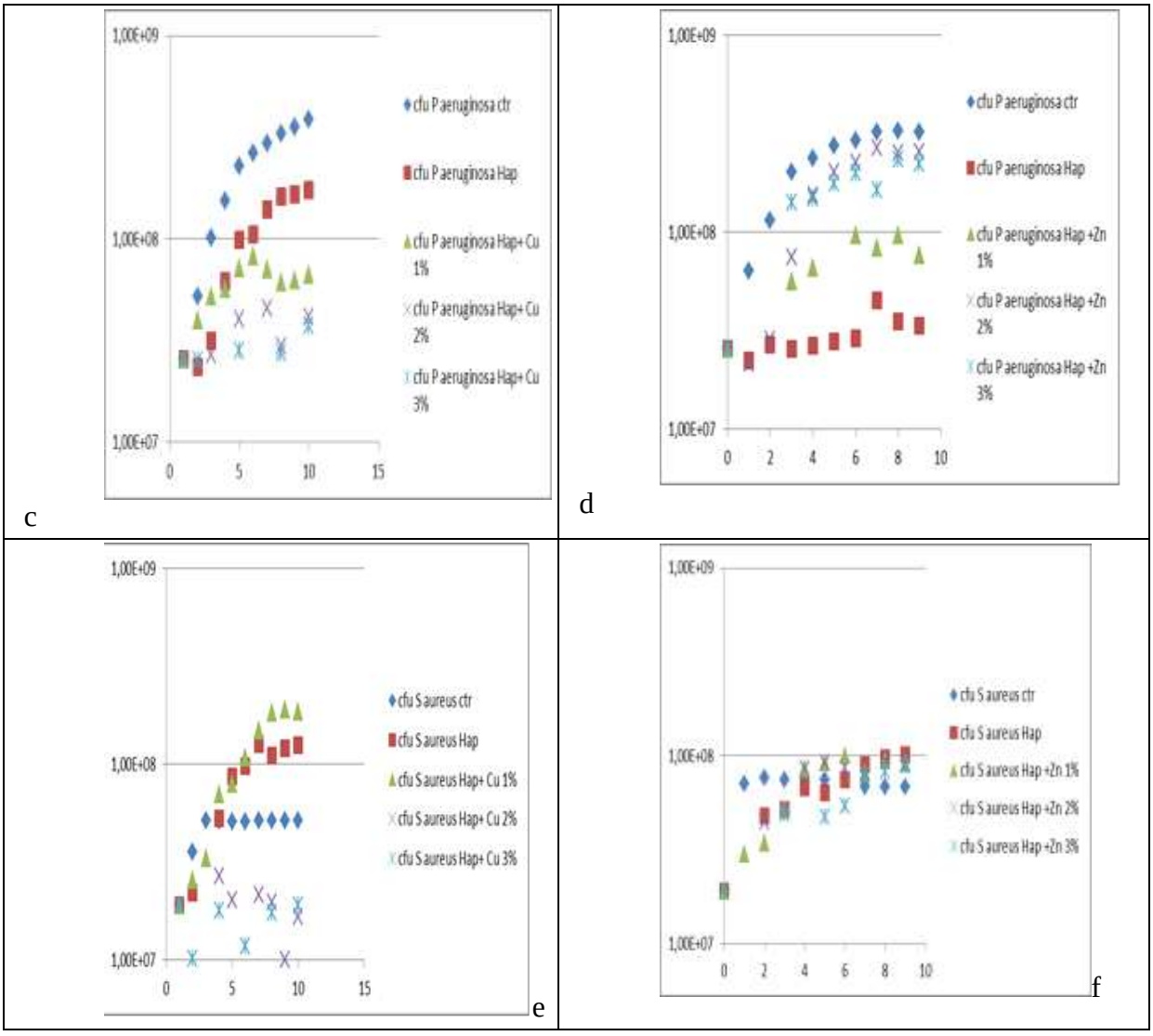
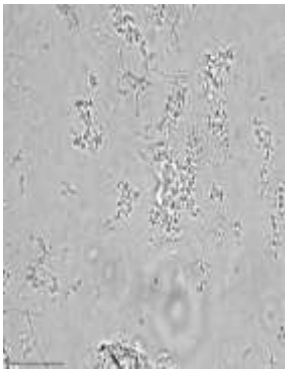


***Fig. 7* Effect of HAp and HAp substituted with Cu2+ and Zn 2+ on growth curves of *P. aeruginosa* ATCC 15692, *E. coli* ATCC 8739 and *S. aureus* ATCC 6538**

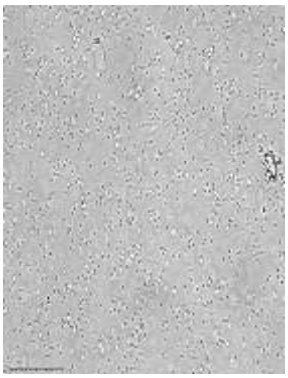
The growing curves show that the bacterial sensitivity of the tested strains to the added Zn/Cu HAp substituted nanoparticles was different: in some bacteria, low concentrations resulted in complete inhibition, whereas in others, complete inhibition was obtained only at higher concentrations. From the limited number of tested bacteria, it appears that the *S. aureus ATCC 6538* (Gram positive) growth was affected to a greater extent by the added Cu/Zn HAP substituted nanoparticles, compared with that of the Gram negative strains tested, similar with the results obtained by Beyth and coworkers (27), who have studied surface antimicrobial activity of incorporated polyethylenimine nanoparticles. Therefore, this effect can be attributed to the considerable differences in cell wall structure. In the Gram negative bacteria *P. aeruginosa ATCC 15692b* and *E. coli ATCC 8739* the presumed mechanism of action of the antimicrobial polycations (i.e. quaternary ammonium polyethyleneimine as well as hydroxyapatite, a chemical complex structure with P and C sites on its surface, alone or Zn/Cu substituted) involves displacement of the divalent cations

that hold together the negatively charged surface of the lipopolysaccharide network, thereby disrupting the outer membrane (28).

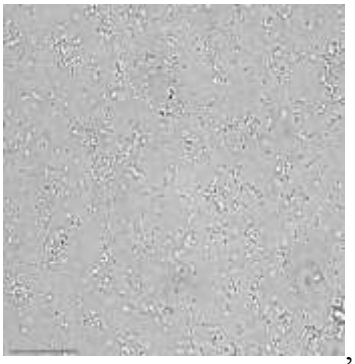
Optical microscopy images show that the bacterial sensitivity of the tested strains to Zn/Cu HAp substituted nanoparticles follows the same pattern with the diffusion tests and growing curves. It appears that *S. aureus ATCC 6538* has been affected to a greater extent by the added Cu/Zn HAp substituted nanoparticles, compared with that of the Gram negative strains tested. Cu^{2+} appears to be more effective then Zn^{2+} as the bacterial cell numbers has decreased dramatically. Also, biologic effect of HAp Cu/Zn substituted nanoparticles has a dose dependent inhibition effect.



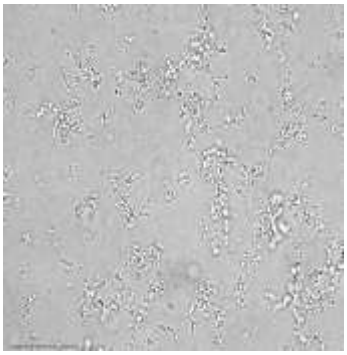
a



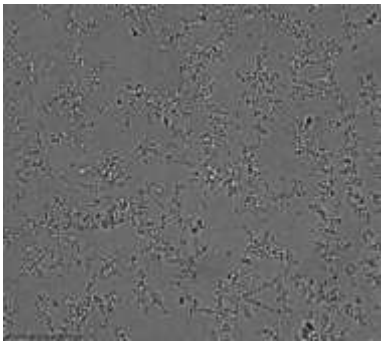
b



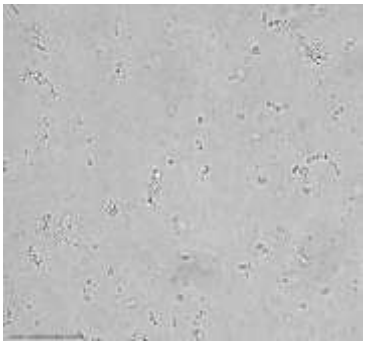
c



c’



d



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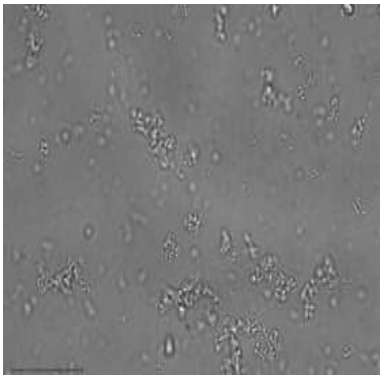
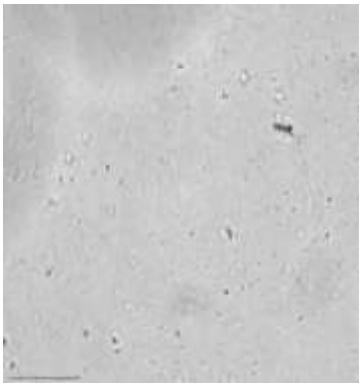


e



e’

Fig. 8 Optical microscopy images of E. coli ATCC 8739 grew on ***un/substituted*** HAP ***with zinc and copper ions. Legend: Hap-b, Hap +Zn (1%-c, 2%-d, 3%-e) and Hap + Cu (1%-c', 2%-d', 3%-e'), a - Control***

The mechanisms of the antibacterial activity of metal ions are not fully explained. However, the literature provides information on three hypothetical mechanisms (29): (a) the ions penetrate into the bacterial cell and, by affecting the production of intracellular ATP+ they disrupt the process of DNA replication; (b) the accumulation of ions in the cell membranes of bacteria will change their permeability (the gradual release of proteins and lipopolysaccharides). The transportation of protons through the cell membrane is prevented, and consequently it leads to the destruction of the cell membrane and the death of the bacterial cell; (c) ion induction of reactive oxygen species (ROSs), causing irreversible changes in their structure and thus the death of the bacterial cell.

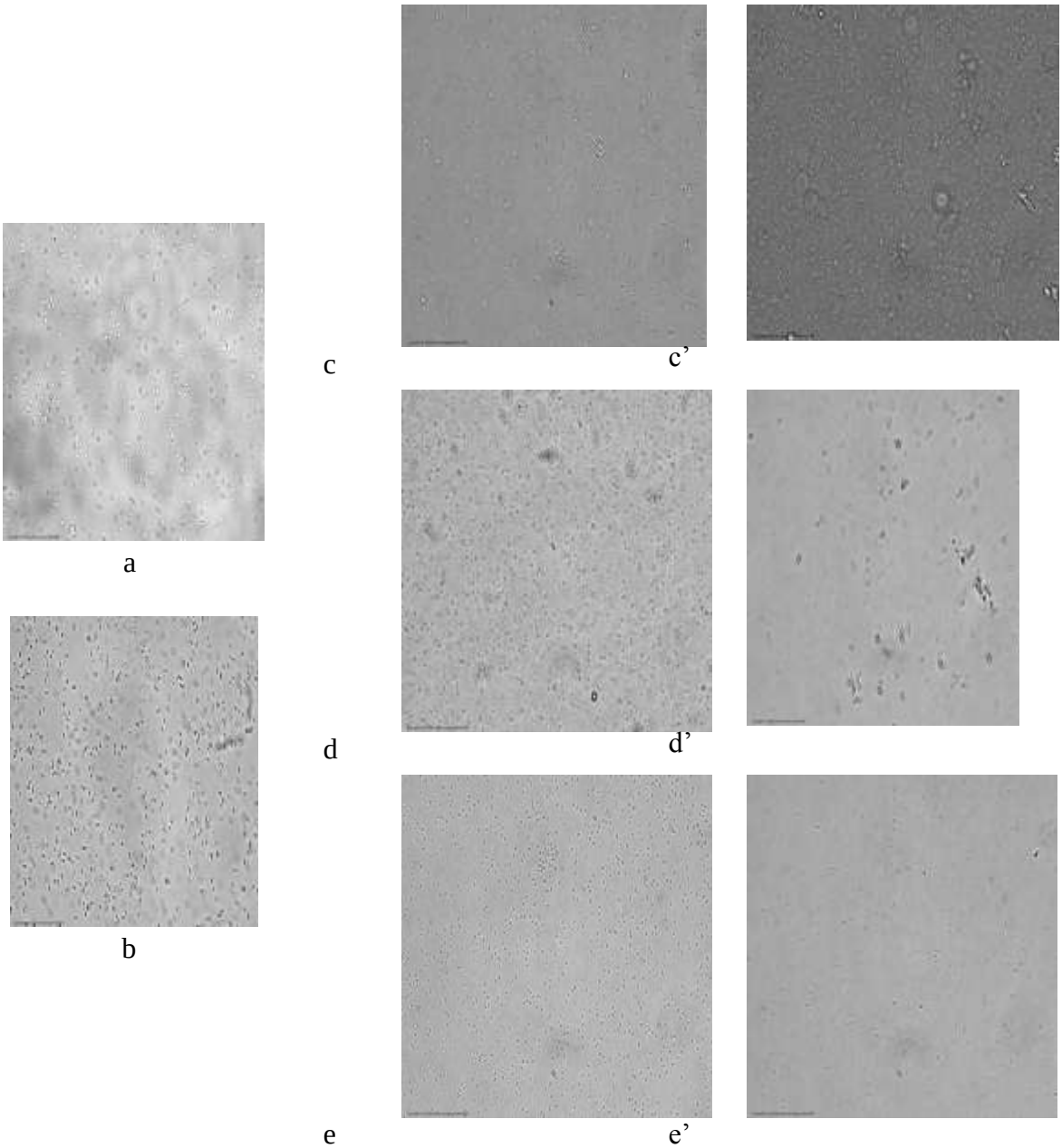


Fig. 9. Optical microscopy images of S. aureus ATCC 6538 grown on ***un/substituted*** HAP ***with zinc and copper ions. Legend: Hap-b, Hap +Zn (1%-c, 2%-d, 3%-e) and Hap + Cu (1%-c', 2%-d', 3%-e'), a- control***

Although some ions such as Zn^{2+} are essential trace elements for bacterial growth, at high concentrations of Zn^{2+}, most bacteria are inhibited. This is mainly due to the fact that heavy metals alter the conformational structures of nucleic acids and proteins, and consequently form complexes with protein molecules, which render them inactive, (e.g., inactivation of enzymes, slow growth and destruction cell membrane integrity) (30).

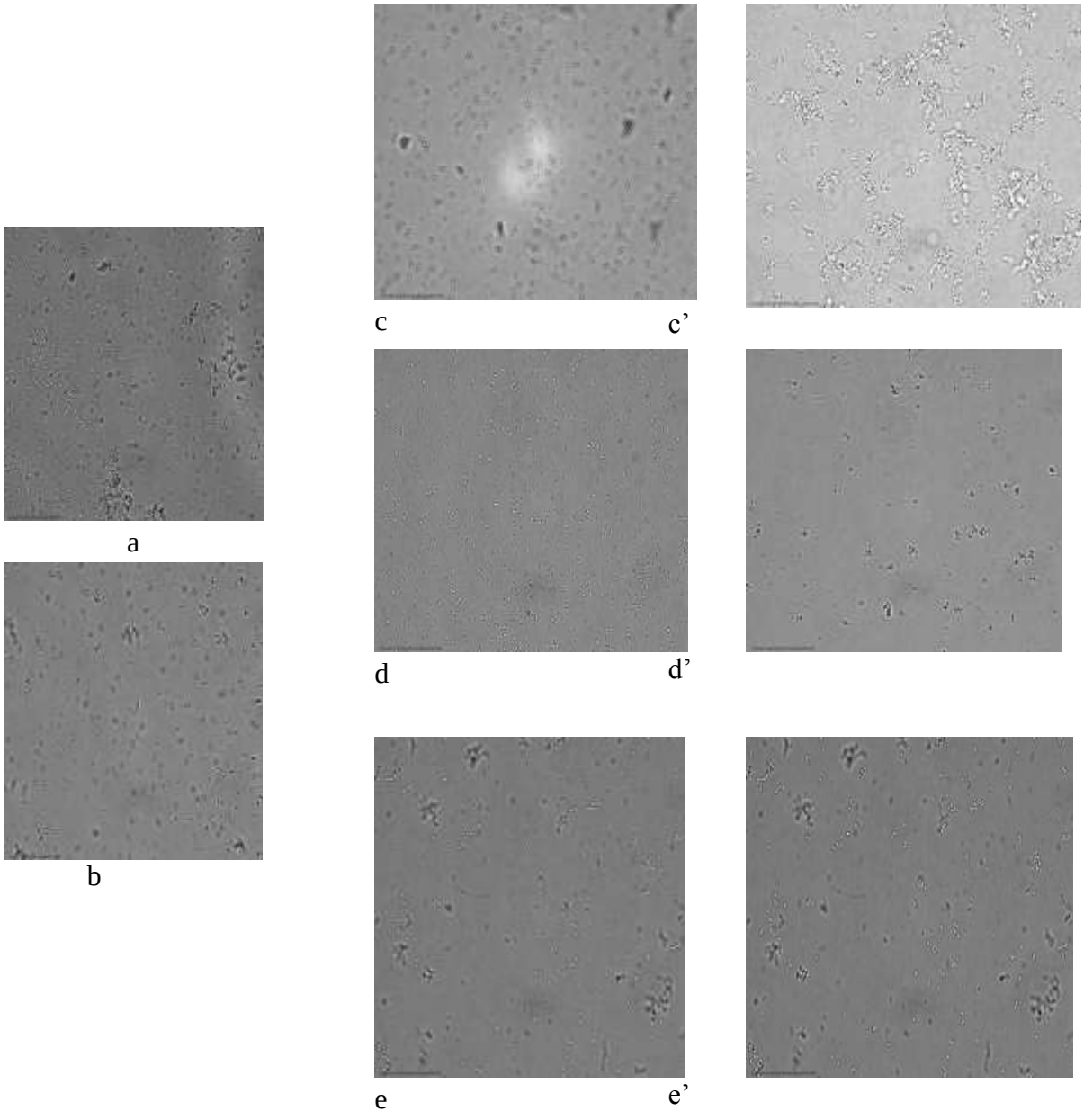


Fig. 10. Optical microscopy images of P. aeruginosa ATCC 15692 grown on ***un/substituted*** HAP ***with zinc and copper ions. Legend: Hap-b, Hap +Zn (1%-c, 2%-d, 3%-e) and Hap +Cu (1%-c', 2%-d', 3%-e'), a - control***

Comparative studies showed an increased antimicrobial activity with increasing the concentration of Zn^{2+} incorporated in the HAp powder exhibited the similar antimicrobial activity against all the tested species of microorganism, except against *S. aureus*, which was more susceptible (31) and results of Liu et al. (2009) have indicate that Zn-HAp may distort and damage bacterial cell membrane, resulting in a leakage of intracellular contents and eventually the death of bacterial cells (32). Thus, Zn-HAp exhibited antimicrobial capability since there was a significant decrease in the number of viable *S. aureus* bacteria (33).

Microorganisms including bacteria require copper at low concentrations as essential micronutrients, but at high concentrations, copper can cause inhibition of cell growth or even death of the cells (34). Copper ions have been used to control the growth of microorganisms; however, the mechanism of this antimicrobial activity on the survival and growth of bacterial cells is not well understood. Normally, copper at high concentration is toxic to microorganisms because it can mediate cell membrane damage, interact with nucleic acids and mediate protein damage (35, 36).

It is also possible that, having destroyed the outer membrane permeability barrier, the cationic groups further penetrate into the inner membrane, causing leakage (37). On the other hand, Gram positive bacteria like *S. aureus*, have a simple cell wall structure consisting only of a rigid peptidoglycan layer. This layer, though thick, has numerous pores, which allow different molecules too readily penetrate the thick cell wall and reach the cytoplasmatic membrane (38).

In conclusion, by precipitation we have obtained calcium phosphate powder substituted with zinc and copper. SEM images have highlighted the clusters of very fine particles in the nanometer range. Studies have shown that the calcium phosphate nanoparticles coupled with copper and zinc, exhibit antimicrobial capability against *P. aeruginosa* and *S. aureus* bacteria. In disc diffusion experiments, calcium phosphate nanoparticles coupled with zinc and copper showed no antibacterial properties towards *E. coli* ATCC 8739 bacteria strain. Antimicrobial studies using disc diffusion assay against *P. aeruginosa* ATCC 15692, *S. aureus* ATCC 6538 showed that 3% CP-Cu exhibited maximum inhibition and hence is an ideal material to be used for biological applications. However, the strains behavior in liquid medium is different, all strains are inhibited at HAp 1% substituted, growing curves and microscopic images are in a good correlation with literature data. CP-Cu and CP-Zn nanoparticles can be used to fight against *P. aeruginosa* and *S. aureus* bacteria, as antibacterial system. However, this broad spectrum antibacterial effect of the HAp nanoparticles substituted with Zn and Cu, merits future screening. Additional studies focusing on the incorporation of other ions in nanoparticles relevant against various harmful bacteria are also required.

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