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Research Paper

Antibacterial activity of silver doped hydroxyapatite toward multidrug-resistant clinical isolates of *Acinetobacter baumannii*

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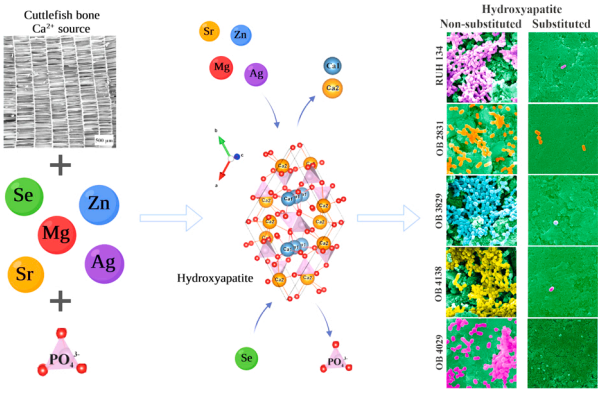
HIGHLIGHTS

- Hydroxyapatite (HAp) material doped with Ag, Sr, Zn, Mg and Se was synthesized.
- Metals enhance osteogenic properties of HAp and Ag provided antibacterial efficacy.
- Ag_HAp's were bactericidal toward drug resistant clinical isolates of *A. baumannii*.
- An option to prevent implant bone infections by multi-drug resistant bacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacteria *Acinetobacter baumannii* is a persistent issue in hospital-acquired infections due to its fast and potent development of multi-drug resistance. To address this urgent challenge, a novel biomaterial using silver (Ag^{+}) ions within the hydroxyapatite (HAp) lattice has been developed to prevent infections in orthopedic surgery and bone regeneration applications without relying on antibiotics. The aim of the study was to examine the antibacterial activity of mono-substituted HAp with Ag^{+} ions and a mixture of mono-substituted HAps with Sr^{2+}, Zn^{2+}, Mg^{2+}, SeO_3^{2-} and Ag^{+} ions against the *A. baumannii*.

The samples were prepared in the form of powder and disc and analyzed by disc diffusion, broth microdilution method, and scanning electron microscopy. The results from the disc-diffusion method have shown a strong antibacterial efficacy of the Ag-substituted and mixture of mono-substituted HAps (Sr, Zn, Se, Mg, Ag) toward several clinical isolates. The Minimal Inhibitory Concentrations for the powdered HAp samples ranged from 32 to 42 mg/L (Ag^{+} substituted) and 83–167 mg/L (mixture of mono-substituted), while the Minimal Bactericidal

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Concentrations after 24 h of contact ranged from 62.5 (Ag^+) to 187.5–292 mg/L (ion mixture). The lower substitution level of Ag^+ ions in a mixture of mono-substituted HAps was the cause of lower antibacterial effects measured in suspension. However, the inhibition zones and bacterial adhesion on the biomaterial surface were comparable. Overall, the clinical isolates of *A. baumannii* were effectively inhibited by substituted HAp samples, probably in the same amount as by other commercially available silver-doped materials, and such materials may provide a promising alternative or supplementation to antibiotic treatment in the prevention of infections associated with bone regeneration. The antibacterial activity of prepared samples toward *A. baumannii* was time-dependent and should be considered in potential applications.

1. Introduction

With the global rise of antibiotic resistance, alternative bactericidal means are becoming more appealing, and one of the historically well-known antibacterial agent is the silver ion, Ag^+. The Ag^+ exhibits antimicrobial activity through multiple mechanisms such as protein or nucleic acids denaturation, impairing respiratory electron chain and components of DNA replication, or increasing membrane permeability, all of which lead to cell death [1]. In more detail (Fig. 1), the Ag^+ can bind to sulfhydryl (thiol) groups in amino acids and inactivate enzymes or proteins. One of the main targets seems to be the enzymatic and protein system used for cell respiration, which leads to the uncoupling of the respiratory chain from oxidative phosphorylation at the cytoplasmatic membrane, leading to cell death [2]. Silver also inactivates enzymes responsible for peptidoglycan synthesis, which, together with binding to nitrogen, oxygen, or sulfur atoms in the DNA, prevents cell duplication [2–4]. Another interesting mode of action is facilitating the release of iron from organic molecules damaged by Ag^+, which causes the subsequent formation of reactive oxygen species, leading to protein, enzyme, or DNA disruption and cell death [2,3]. Furthermore, the Ag^+ tend to accumulate in periplasmatic space, leading to protein and enzyme inactivation, disrupting the various efflux and uptake molecular pumps, and leading to the disintegration of the plasma membrane [4,5]. From the latter aspect, the Gram-negative bacteria are probably more susceptible to the action of silver due to a thin peptidoglycan wall, allowing increased accumulation of ions in the periplasm [5,6]. Due to this variety of mechanisms, the formation of microbial resistance to Ag^+ is reduced, while at the same time, silver shows a low potential for cytotoxicity [7].

Commercially, silver is widely used in wound dressings or prostheses coatings and is considered safe and effective in the reduction of infections or re-infections [7,8]. However, the biomaterials that contain silver as an antimicrobial agent need to be carefully designed to avoid negative side effects. Recently, the toxic effect of nanomaterials on humans has gained increasing attention in the health industry. Several review papers have addressed the toxicological properties of silver nanoparticles during their use as antimicrobial agents. *In vivo* toxicity and biodistribution studies have established silver nanoparticles translocation, accumulation, and toxicity to various organs [9,10]. New approaches that include Ag^+ ions instead of nanoparticles need to be implemented for usage in human organism, while studies on silver nanoparticles remain valuable sources for a deeper understanding of the antibacterial mechanism of silver.

Hydroxyapatite (HAp) is the most widely used calcium phosphate for bone regeneration as a single ceramic phase in the form of granules, blocks and porous scaffolds, or as a composite with polymers or other ceramics materials [11]. In addition, HAp is used as a coating on metal orthopedic or dental implants because of its osteogenic property and ability to form strong bonds with the host bone tissues [12]. Biological HAp in its structure contains various trace elements which have a crucial role in bone growth [13]. Due to the flexible hexagonal structure of HAp, various cations (Sr^{2+}, Na^+, K^+, Mg^{2+} Zn^{2+}, Ag^+, etc.) and anions (CO_3^{2-}, SiO_4^{4-}, SeO_3^{2-}, SeO_4^{2-}, F^-, Cl^-, Br^-, etc.) can be substituted in its structure to obtain biomaterials with specific desirable properties dependent on the substituted ion [14]. In our previous study, scaffolds based on chitosan and calcium phosphates substituted with Sr^{2+}, Mg^{2+}, Zn^{2+} and SeO_3^{2-} ions have shown increased expression of osteogenesis-related markers and increased phosphate deposits, compared to the scaffolds with non-substituted calcium phosphates [15]. As Ag-substituted HAp has received great attention due to the possibility to control bacteria adhesion and prevent bacterial biofilm formation, it is of great interest to substitute Ag^+ ions along the Sr^{2+}, Mg^{2+}, Zn^{2+} and SeO_3^{2-} ions within calcium phosphates structure [16,17].

The bacterium *Acinetobacter baumannii* is well-known to everyone encountering hospital-acquired infections, including the ones of orthopedic implants [18] such as prosthetic joints [19,20]. This Gram-negative bacterium is considered to be of the highest concern, as it belongs to the so-called ESKAPE pathogens group [21], and requires immediate attention and the development of new and efficient antimicrobial treatments. Of special concern is the rapid and potent development of multidrug resistance by *A. baumannii*, and nowadays the carbapenem-resistance is almost ubiquitous [22–24]. As an example, in

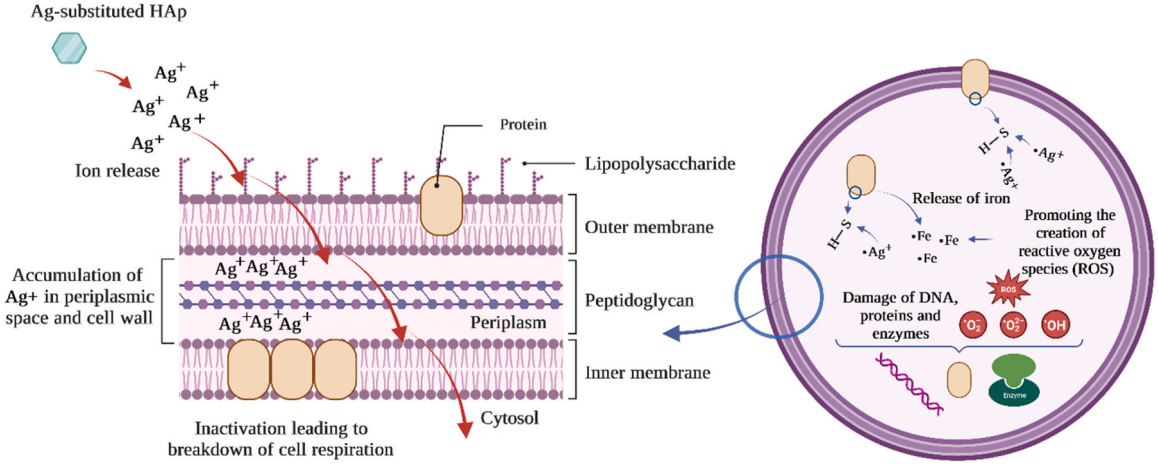

Fig. 1. Schematic of antibacterial mechanism of Ag^+ ions on Gram-negative bacteria. Created with BioRender.com.

2021 in Croatia, as much as 99.5 % of all *A. baumannii* clinical isolates were carbapenem-resistant (imipenem and/or meropenem) [25]. Therefore, any mean of antibacterial activity other than antibiotics towards these bacteria is very much desired. The study of Hetta et al. [26] on *A. baumannii* showed that bacterial multiplication and biofilm formation is highly interrupted by silver nanoparticles treatment; mainly by downregulation of the transcription level of important virulence and biofilm-related genes.

The silver-substituted HAp has a proven antibacterial activity but was always tested towards commonly used model organisms usually obtained from banks of microorganisms, such as *Escherichia coli* and *Staphylococcus aureus,* and determined by disc-diffusion assay and viability assay in a suspension [16,27,28]. Therefore, the goal of this study was to test HAp mono-substituted with Ag^{+}, Sr^{2+}, Mg^{2+}, Zn^{2+} and SeO_3^{2-} ions and a mixture of these mono-substituted HAps towards actual clinical isolates of the contemporary human pathogen, the *A. baumannii*. In addition, the minimal inhibitory and bactericidal concentrations of the powdered HAp were determined. This way, the possible usability of substituted HAp in real life conditions could be realistically assessed. The advantage of using Ag-substituted HAp powders compared to silver nanoparticles is that silver accumulation within the human organism can be avoided. According to the author's best knowledge, this is the first time that the antibacterial properties of substituted HAps were determined towards clinical isolates of *A. baumannii*.

2. Materials and methods

2.1. Synthesis of substituted hydroxyapatite

Non-substituted and Sr^{2+}, Mg^{2+}, Zn^{2+}, Ag^{+} and SeO_3^{2-} mono-substituted HAp powders were synthesized by the wet precipitation method as previously described in our studies with minor changes [28–31]. In brief, the HAp was prepared by dissolving calcium oxide (CaO) and strontium nitrate ($Sr(NO_3)_2$), sodium selenite pentahydrate ($Na_2SeO_3{\cdot}5H_2O$), zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$), magnesium chloride hexahydrate ($MgCl_2{\cdot}6H_2O$) or silver nitrate ($AgNO_3$) in demineralized water. Ammonium dihydrogen phosphate ($NH_4H_2PO_4$) was added to the solution to gain (Ca + metal)/P and Ca/(P + Se) molar ratio 1.67, required for stoichiometric HAp. Stirring was continued for 3 days at 60 ºC followed by overnight aging at room temperature. The selected Sr/(Ca +Sr), Mg/(Ca + Mg), Zn/(Ca + Zn), Ag/(Ca + Ag) and Se/(P + Se) was 2.5 mol% and the samples were designated as HAp_Sr, HAp_Mg, HAp_Zn, HAp_Ag and HAp_Se, while non-substituted powder was labeled as HAp. To determine the synergic effect of substituted elements prepared mono-substituted HAp powders were mixed and designed as HAp_MIX. The mass fraction of each substituted powder in the powder mixture was the same (20 wt%). To ensure that obtained mono-substituted HAp powders were homogeneously mixed, the appropriate amount of each powder was added to the mortar with ethanol (96 wt%) to form a paste. Powders were mixed with a pestle at room temperature till the ethanol was fully evaporated and the dried mixture of mono-substituted HAp remained.

2.2. Morphology and element composition of substituted-HAp

After synthesis and before powder filtering, a few drops of HAp_Sr, HAp_Mg, HAp_Zn, HAp_Ag and HAp_Se powders in the supernatant were diluted in ethanol (96 wt%). A drop of each mixture was set on the stub with carbon tape and let to dry at room temperature. The same procedure was used for HAp_MIX powder where powder after homogenization was diluted in ethanol and set at stubs. Further, filtered and dried powders were cold pressed at 250 MPa and cut into ~5 mm diameter discs or squares. The powder and disc morphology for all as-prepared powders was analyzed by scanning electron microscopy (SEM, TESCAN VEGA3 Easyprobe) at an electron beam energy of 13 keV. EDS analysis was performed at an electron beam energy of 20 kV, to investigate the elemental composition of the surface of the HAp_Sr, HAp_Mg, HAp_Zn, HAp_Ag, HAp_Se and HAp_MIX samples. Prior to analysis, dried discs were coated with gold and palladium plasma for 60 s.

Table 1
Characterization of *A. baumannii* isolates, adapted from Seruga Music et al. [32].

Isolate	Origin	Date of isolation (dd/mm/yy)	Sequence type	IC type
OB 3829	Tracheal aspirate	18/09/15	195	2
OB 3831	Sputum	11/09/15	1421	2
OB 4027	Sputum	24/09/15	1421	2
OB 4138	Bronchial aspirate	02/10/15	195	2
RUH 134	Urine	1982	2	2

2.3. Bacterial isolates

The *A. baumannii* clinical isolates (Table 1) were previously characterized [32] and used in this study. Briefly, isolates were recovered during 2015 from sputum and tracheal or bronchial aspirates of patients from the Special Hospital for Pulmonary diseases in Zagreb, Croatia. The patients were diagnosed with hospital-acquired pneumonia. The isolates were identified by standard biochemical tests (catalase, oxidase and growth on Kligler-iron agar) and Vitek2 system. Subsequent confirmation was performed via MALDI-TOF MS instrument and additional molecular identification was done by amplicon sequencing and multi-locus sequence typing, as described in detail in [32]. All isolates were classified as extensively-drug resistant, meaning non-susceptible to at least one agent in all but two or fewer antimicrobial categories. In more detail, all isolates were resistant to carbapenems (meropenem, imipenem), fluoroquinolones (ciprofloxacin, levofloxacin) and aminoglycosides (tobramycin, gentamicin, amikacin) but were susceptible to colistin [32]. The *A. baumannii* RUH 134 was used as a standard reference strain for international clonal lineage (IC) 2 [33,34].

2.4. Antibacterial activity

The antibacterial efficacy assay was performed according to EUCAST standard disc diffusion assay (https://www.eucast.org/) except the discs of HAp samples were used instead of antibiotic discs. The bacterial suspensions (~10^8 CFU mL^{-1} / 0.5 McFarland units) were smeared all over the Mueller-Hinton Agar plates (Biolife, Italy) with a sterile swab stick. The HAp samples were placed aseptically onto the agar surface and plates were incubated for 24 h at 37 °C. After the incubation, the inhibition zones, if present, were measured and reported. Aquacel®, a commercial antibacterial wound dressing, was used as a positive control, a material with known antibacterial efficacy.

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by broth microdilution method, using powdered samples, as follows. A ~5×10^5 CFU/mL bacterial suspension in Mueller-Hinton Broth (Biolife, Italy) was resuspended in a series of 1.5 mL sterile plastic vials (eppendorf type). In the first vial, that contained 1 mL of bacterial suspension, 4 mg of powdered HAp sample was added, yielding concentration of 4000 mgHAp/L. Next, double dilutions up to 1.95 mgHAp/L were done and vials were incubated for 24 h at 37 °C. After 24 h, the vials were inspected and MIC value was reported as the lowest concentration without visible bacterial growth in liquid medium (remains transparent).

During the incubation, after 1, 3, 6, 24, 48 h and 7 days, an aliquot of 10 μL was taken from each vial, inoculated onto agar plate and incubated. This way, the MBC was determined, as a lowest concentration

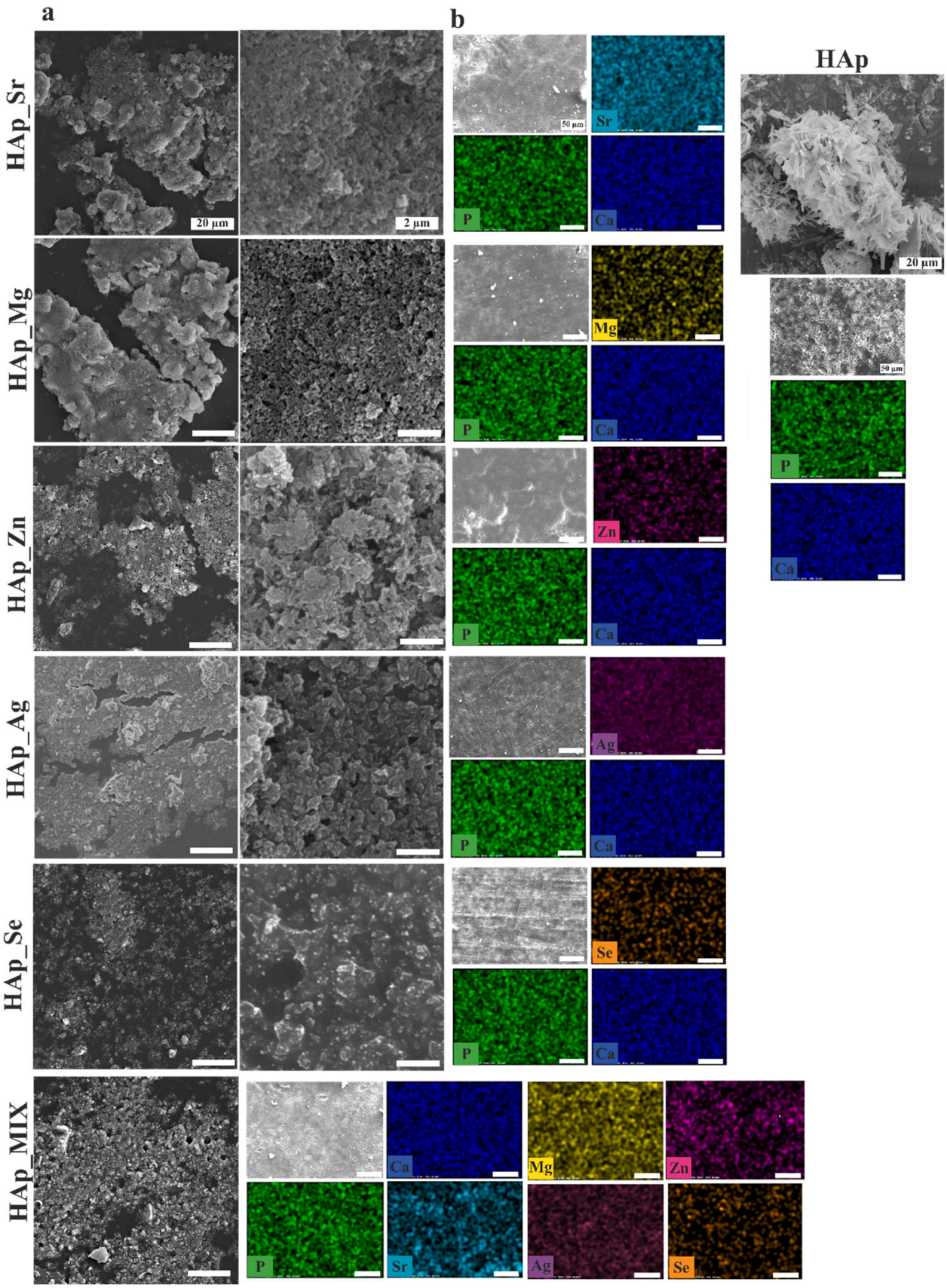

Fig. 2. (a) SEM micrographs of as-prepared powders and (b) EDS elemental mapping results of prepared discs. Scale bar: (a) 20 and 2 µm and (b) 50 µm.

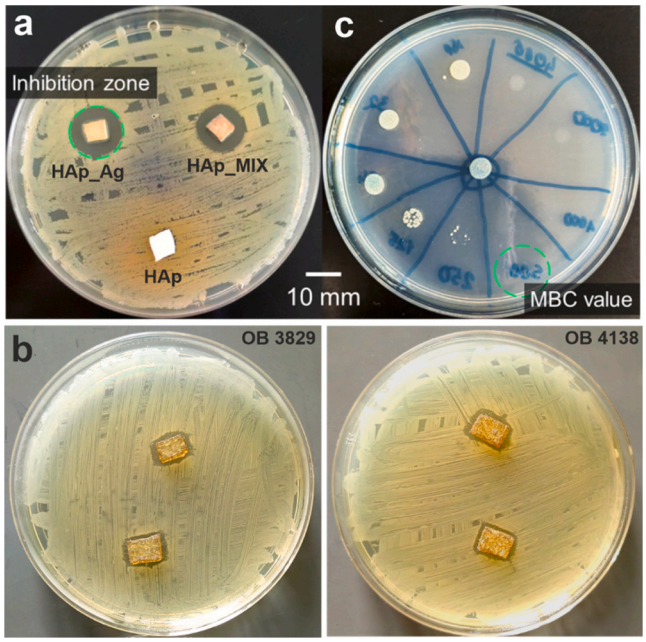

Fig. 3. Representative agar plates of disc-diffusion test: (a) *A. baumannii* isolate marked 4138 with HAp_Ag and HAp_MIX exhibiting inhibition zones and HAp without the inhibition zone and (b) Aquacel® (positive control) activity towards isolates marked 3829, and 4138. (c) The example on how *Minimal Bactericidal Concentration* (MBC) was determined, the lowest concentration without any visible colony growth.

without any visible colony growth. All the experiments were done in triplicate.

2.5. Surface morphology after diffusion assay

After the diffusion test, an analysis of the surface morphology was performed as previously described in our study [28]. In brief, after the disc diffusion assay, the samples were lifted off the agar surface and aseptically placed into a sterile 30 mm Petri dish so that the side in contact with the inoculated agar was facing up. Then, a 2 % solution of paraformaldehyde in phosphate buffered saline (PBS) was poured into the Petri dish to cover the samples and fixate the bacterial cells for 24 h at 4 °C. Then, the samples were washed with sterile PBS and dehydrated by immersion in a series of ethanol solutions as follows; 30 %/2 min, 50 %/2 min, 70 %/5 min, 96 %/5 min, 99.9 %/5 min, 99.9 %/5 min. The dehydrated samples were dried at 50 °C for 30 min. After drying, the disc surface morphology was analysed by SEM at electron beam energy of 7 keV. Bacteria and sample substrate were false-coloured in Adobe Photoshop CC 2018.

3. Results

3.1. SEM and element mapping analysis

Several substituted HAp samples were prepared, namely mono-substituted (HAp_Sr, HAp_Mg, HAp_Zn, HAp_Ag, HAp_Se) and a mixture of mono-substituted HAps with all of the mentioned ions (the HAp_MIX). The SEM micrographs of as-prepared powders and EDS elemental mapping results of samples in disc shape are given in Fig. 2. SEM analysis of as-prepared HAp powder shows plate-shaped crystals in aglomerated flower form. The powder substituted with Sr^{2+}, Mg^{2+}, Zn^{2+}, Ag^{+} and SeO_3^{2-} did not show characteristic flower-shaped agglomerates due to the effect of the different ions on crystal growth as explained in our previous studies [28–31]. Size of substituted HAp particles obtained in this work is smaller compared to our previous studies [28–31], which might be due to slight modifications in the synthesis protocol (CaO was used instead of calcium carbonate as a Ca^{2+} precursor). According to the SEM images, particle size is lower than 1 μm where spherical (Sr^{2+}, Mg^{2+}, Ag^{+}, SeO_3^{2-} and mixture) or elongated (Zn^{2+}) crystals are evident.

The EDS mapping of prepared discs was used to confirm the atomic composition of the prepared substituted HAp samples. EDS mapping showed a uniform distribution of the elements Ca, P, Sr, Mg, Zn, Ag and Se. The uniform distribution of elements is also evident in the sample HAp_MIX indicating the appropriate homogenization of HAp_Sr, HAp_Mg, HAp_Zn, HAp_Ag and HAp_Se powders. The homogeneous distribution of elements is crucial in biomaterials development as it allows uniform element release and interaction with the surrounding environment.

Table 2
The inhibition zones and MIC of powdered HAp samples.

		Acinetobacter baumannii isolates				
	Sample	RUH 134	OB 3829	OB 3831	OB 4027	OB 4138
Inhibition zone (mm ±1 mm)	HAp_Ag	16	14	15	15	15
	HAp_MIX	14	12	14	14	15
	Aquacell®	11	10	10	12	10
MIC value (mg/L, mean ± SD)	HAp_Ag	42 ± 18	42 ± 18	32 ± 0	42 ± 18	42 ± 18
	HAp_MIX	167 ± 72	104 ± 36	83 ± 36	125 ± 0	167 ± 72

3.2. Antibacterial activity

Of all the tested samples, only the HAp_Ag, HAp_MIX and Aquacel® commercial wound dressing (gauze), used as positive control, have shown antibacterial activity by the qualitative disc-diffusion method (Fig. 3a,b). These two HAp samples were thus chosen to test the MIC and MBC values (Fig. 3c).

From the inhibition zone measurements (Table 2) it can be concluded that the HAp_Ag samples, when compared to HAp_MIX samples, exhibited slightly stronger antibacterial activity (by slightly wider inhibition zone). However, as disc diffusion is a qualitative method, and the zone sizes were only slightly different, this could be considered just as an indication. The SEM analysis of the tested samples also show a similarly reduced number of bacterial cells on the surface of HAp_Ag and HAp_MIX when compared to abundant bacteria on the control HAp (Fig. 4). It is worth to notice the various degree of a reduced amount of cells compared to control and dependent on the bacterial isolate, on the surface of other ion-substituted HAps as well, even though no apparent antibacterial activity was noticed by the disc diffusion test.

The MIC values (Table 2) are results of a more precise method allowing quantification, and were lower for the HAP_Ag sample when compared to HAp_MIX sample, confirming that HAp substituted solely with Ag^{+} ions had stronger bactericidal activity when compared to the mixed sample. Such conclusion was further supported by the determined MBC values which were lower (meaning better bactericidal activity) for HAp_Ag when compared to the HAp_MIX sample (Fig. 5). The MBC values were measured over time, and show apparently reduced antibacterial activity of powdered samples in a suspension after 1 day mark, up to the final measurement at 7 days mark (Fig. 5).

4. Discussion

The nanocrystalline silver dressings are reported to be useful in infected wounds or burns, although there is little evidence for added benefit in clean wounds or closed surgical incisions [1,7]. Specifically for *A. baumannii*, Selcuk et al. [35] reported that silver dressing "Acticoat" was more effective than octenidine, mupirocin and sulfadiazine in rat burn model. We compared the HAps mono-substituted with Ag^{+} and

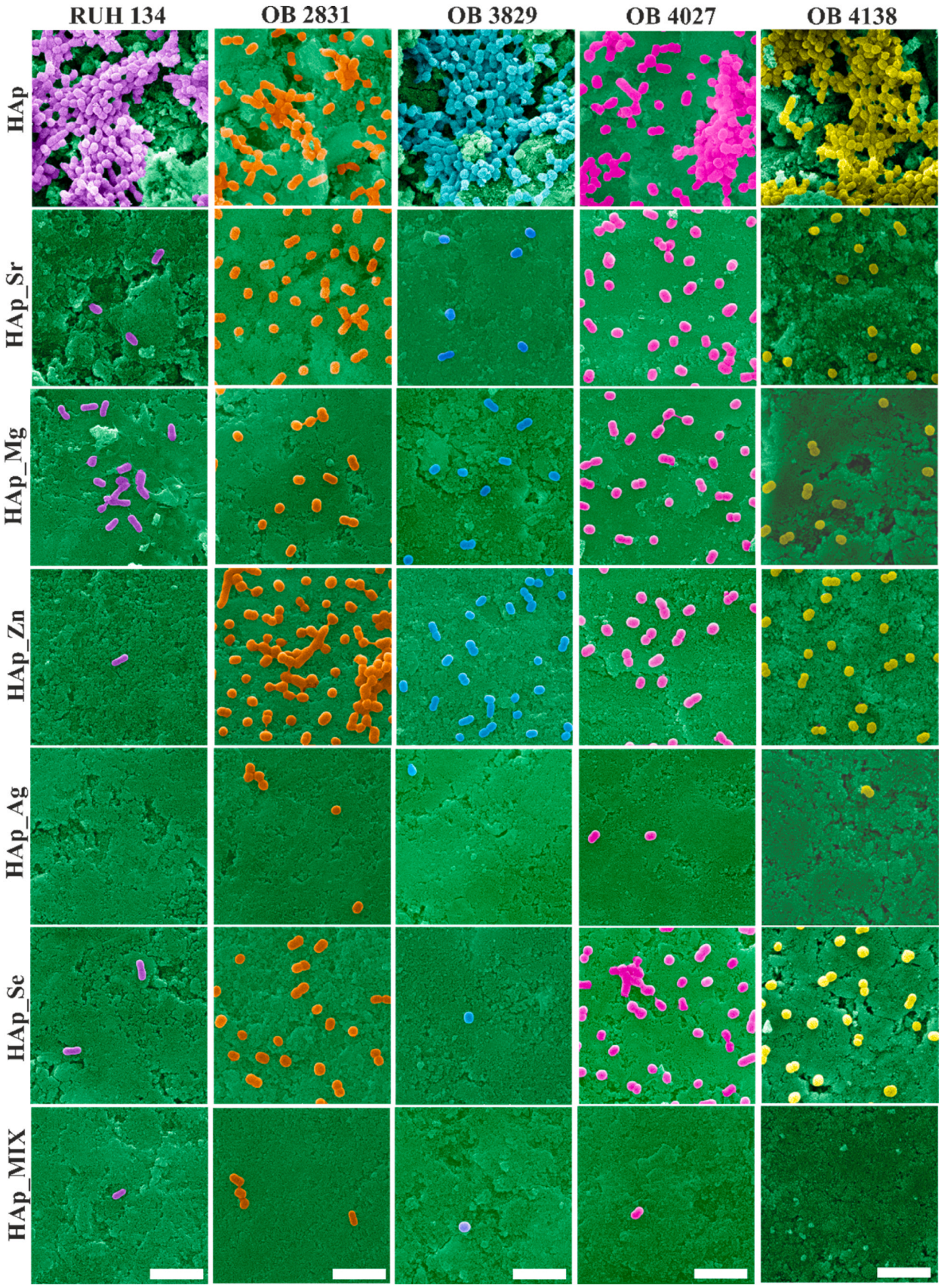

Fig. 4. SEM images of *A. baumannii* isolates marked RUH 134, OB 2831, OB 3829, OB 4027 and OB 4138 on the discs of the prepared samples after inhibition zone analysis. The RUH 134 cells were colored in purple, OB 2831 in orange, OB 3829 in blue, OB 4027 in pink, OB 4138 in yellow, while the HAp surface was stained in green. Scale bar: 4 μm.

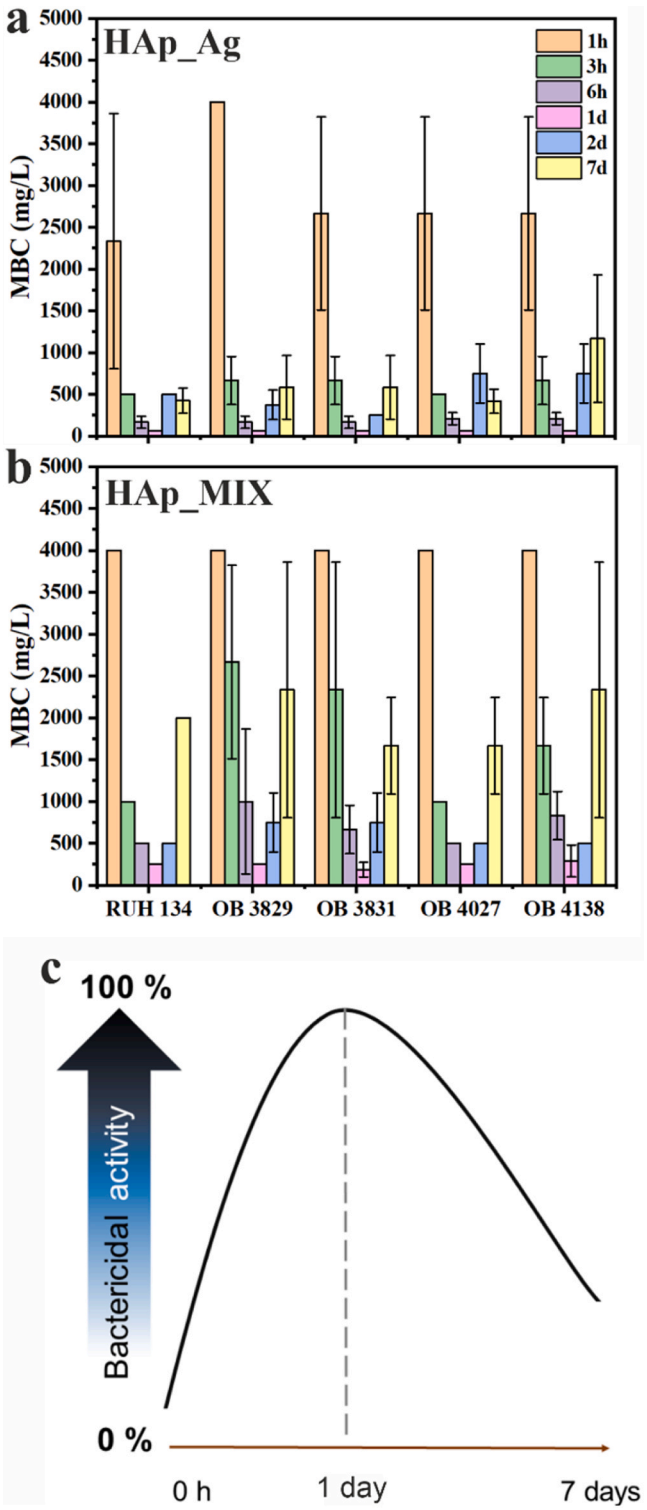

Fig. 5. The MBC values (mg/L) of HAp_Ag (a) and HAp_MIX samples (b) after set time period. (c) Proposed schematic representation of bactericidal activity of HAp_Ag and HAp_MIX depending on the contact time.

a mixture of HAps mono-substituted with Ag^{+}, Sr^{2+}, Mg^{2+}, Zn^{2+} and SeO_3^{2-} to Aquacel® gauze, a widely used commercial antibacterial product. The HAp_Ag and HAp_MIX seemingly outperformed the silver wound dressing at antibacterial efficacy towards clinical *A. baumannii* isolates. However, the system more related to the experiments presented in this study are the silver coated prostheses. According to the latest systematic review on the subject, there are three commercially available silver coatings for orthopedic prostheses and, generally, a statistically significant efficacy of silver coatings in the prevention of prosthetic joint infections was reported [36]. The review lists several local and systemic complications, such as argyria or increased silver concentrations in the blood, respectively. However, the possible toxicity of silver prostheses was found to be minimal, as reported blood concentrations (0.02–30 ppb) were under the toxic levels for human cells (levels <200 ppb are considered normal) [36,37]. The reported local silver concentrations were higher, which could cause a condition in which the skin becomes blue or bluish gray (more related to wound dressings), generally benign, but cosmetically an irreversible condition [36,38].

In our previous studies, HAps substituted with up to 5 mol% of Sr^{2+}, Zn^{2+} and Mg^{2+} were prepared by wet precipitation method. *In vitro* cytocompatibility evaluation showed a non-cytotoxic effect with emphasis on human embryonic kidney cell proliferation [29,31]. Further, 5 mol% substitution with SeO_3^{2-} ions caused human embryonic kidney cells apoptosis, while 1 mol% substitution was non-cytotoxic towards human cells. However, 1 mol% substitution with SeO_3^{2-} led to selective anticancer properties of calcium phosphate powders, causing apoptosis of osteosarcoma human cells and a positive effect towards healthy cells [30]. In another of our previous studies [28], the viability of human embryonic kidney cells cultured for 3 days in extracts of HAp substituted with 2.5 and 5 mol% of Ag^{+} ions significantly decreased, whereas no significant difference in cell viability was observed for the 1 mol% substitution compared with non-substituted HAp. To further evaluate cytocompatibility, human mesenchymal stem cells were cultured in extracts of HAp samples substituted with 1 and 2.5 mol% Ag^{+} ions. Non-cytotoxicity was confirmed after 3 days of cell culture, in which cell viability increased for examined samples [28]. According to the obtained studies, SeO_3^{2-} and Ag^{+} substitution level in HAp needs to be carefully selected to avoid cytotoxic effects towards human healthy cells. Substitution levels of SeO_3^{2-} below 1 mol% and Ag^{+} ions below 2.5 mol% should have a positive effect towards human healthy cells [28,30]. The HAp_MIX sample tested in the presented study contains 0.5 mol % of each substituted element, leading to a multi-functional biomaterial that is non-cytotoxic towards human cells and has anti-bacterial and possible anti-cancer properties. Additionally, co-substitution with different ions (e.g. Sr^{2+}, Mg^{2+}, Zn^{2+}) that promote cell proliferation and differentiation can counterbalance potential Ag^{+} cytotoxicity in HAp structure [39]. A schematic illustration of the antibacterial and biological properties of HAp substituted with Sr^{2+}, Zn^{2+}, Mg^{2+}, SeO_3^{2-} and Ag^{+} according to our previous and the present study is shown in Fig. 6.

In our previous study [28], the antibacterial effect of Ag-substituted HAp was confirmed by disc diffusion and viability assay. However, for comparison with literature reports, the quantitative measurements of MICs and MBCs are preferable. In the present study using clinical isolates of *A. baumannii*, the MICs and MBCs of HAp_Ag were 32–42 and 63 mg/L, respectively, and for HAp_MIX were 83–167 (MIC) and 188–292 (MBC) mg/L. Although the MBC at different time points was measured, here for comparison with literature data, MBCs after 24 h is presented. Wiglusz et al. [6] tested non-substituted HAp and HAp substituted with silver and europium as carriers of silver nanoparticles toward several model bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*). When HAp was substituted with Ag^{+} ions, reported MIC and MBC values were ranging from 200 to 500 mg/L. When HAp was co-substituted with silver and europium, the MIC and MBC values were significantly higher (500–1900 mg/L). Similarly, when silver was incorporated in HAp as nanoparticles rather than ionic silver, the MIC and MBC values were again higher, meaning reduced antibacterial activity (500 – 3700 mg/L). However, in that study [6], the amount of added/substituted silver was not defined. The results reported by Phatai et al. [40] have shown the MBC value of HAp co-substituted with Zn^{2+} and Ag^{+} (0.0, 0.25 and 1.0 mol%) toward Gram-positive *Bacillus subtilis* to be > 40,000 mg/L. However, it is worth noting that *B. subtilis* is a sporogenic bacteria, and endospores are highly resistant to antibacterial agents. High efficiency of

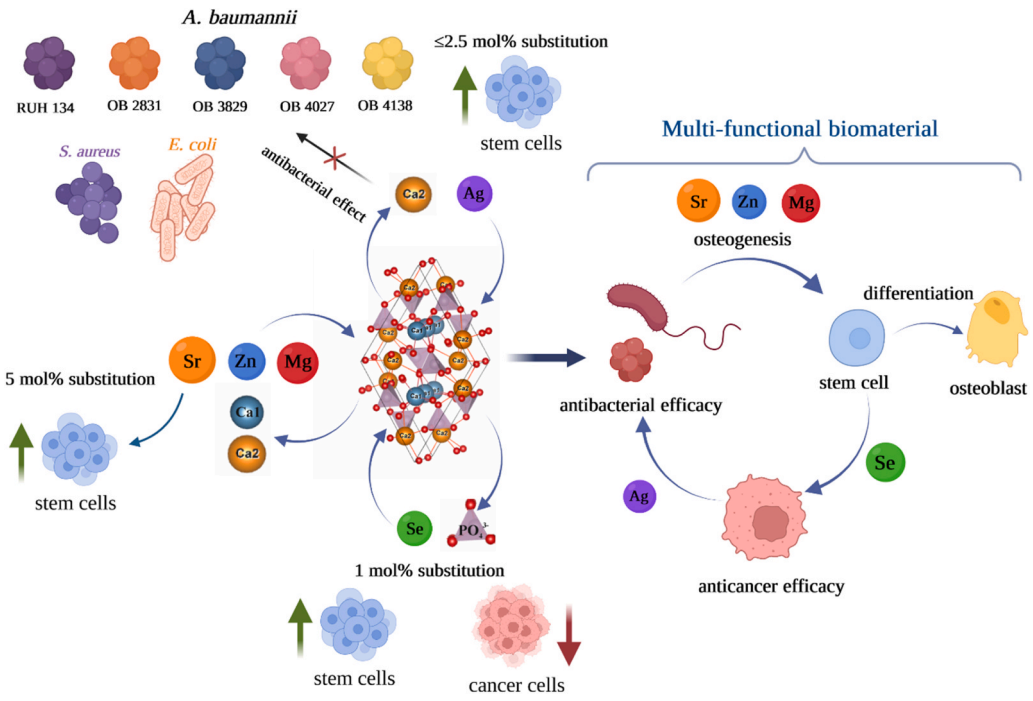

Fig. 6. Schematic illustration of antibacterial and biological properties of HAp substituted with Sr^{2+}, Zn^{2+}, Mg^{2+}, SeO_3^{2-} and Ag^{+} according to our previous [28–31] and the present study. Created with Biorender.com.

silver-nanoparticles/HAp nanocomposites toward *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* was reported by Ni et al. [41] with MIC and MBC values ranging from 3.9 to 15.6 and 7.8 – 62.5 mg/L, respectively. González-Torres et al. [42] reported a MIC value of 75 mg/L for Ag-substituted HAp and 200 mg/L for fluorine-substituted HAp toward Gram-positive bacteria *Enterococcus faecalis*. A very comprehensive report was provided by Cavassin et al. [43], which included many bacteria, including 21 different isolates of *A. baumannii* whose susceptibility to different types of silver nanoparticles yielded MICs and MBC values ranging from 3.4 to 54 mg/L. It must be noted that these were sole nanoparticles, not in any way connected to HAp. In general, the silver nanoparticles are highly effective toward bacteria and can be effective in combination with HAp, while effectiveness is dependent on the synthesis process. Results reported in this study on the antibacterial efficacy of silver incorporated in mono-substituted HAp and a mixture of mono-substituted HAps are comparable to literature reports of MIC and MBC values, both for substituted HAp and for the pure silver nanoparticles.

Along with the antibacterial activity of Ag^{+}, the present study highlights the importance of the electrostatic interaction between biomaterials and bacteria cells in order to prevent bacteria cell adhesion. The surface charge of HAp is one factor affecting bacterial attachment. Bacterial cells possess a net negative charge due to the high amount of carboxyl, amine, and phosphate groups [44]. In our previous studies [28,31], Ag^{+} and Sr^{2+} co-substituted HAp powders have shown a negative surface charge that led to lower bacteria adhesion compared to the non-substituted HAp, while substitutions with Mg^{2+} and Zn^{2+} ions in multi-phase calcium phosphate systems led to a positive surface charge causing good bacteria cell adhesion on the surface of the samples. Therefore, the charge of the surface could have an important role in bacteria adhesion.

An interesting observation was a seemingly acquired resistance of all the tested *A. baumannii* isolates to HAp_Ag and HAp_MIX samples, conferred from the measurement of MBC which showed the highest bactericidal activity after 1 day of contact, and reduction of activity after 2 and 7 days of contact. Several explanations are possible; the released Ag^{+} ions irreversibly attach to the dead bacterial cells or cell debris, preventing further activity. Or, the cells enter the so-called *Viable but Non-Culturable* (VBNC) state in the suspension, and when transferred to an agar plate regain their composure and become viable [45]. Another possibility is that the *A. baumannii* isolates carry either intrinsically or plasmid resistance to silver. Although considered to be rare, the resistance to silver has been well documented [46,47]. In many cases, the same plasmid carries genes responsible for silver, but also antibiotic resistance, even to multiple antibiotics [46]. Various isolates of *A. baumannii*, whether environmental or clinical, were shown to carry resistance to silver, and it correlated with the production of *β*-lactamase [48,49]. From the available literature, the plasmid-mediated silver resistance was detected in the clinical isolates from wounds and burns (19 silver-resistant out of 150 isolates of various species), including in two out of 14 *A. baumannii* isolates [50]. It was also demonstrated how an *A. baumannii* strain that was not inherently resistant to silver, developed a "slower-to-kill" phenotype to silver nanoparticles after prolonged exposure [24]. One could consider that possible solution to increase the antibacterial properties of obtained biomaterials could be the increase of the Ag^{+} substitution level in HAp structure. However, increasing the amount of the Ag^{+} substitution higher than 2.5 mol% causes a toxic effect on human cells as previously determined in our study [28]. Therefore, Ag^{+} substitution level of 2.5 mol% is the maximum to obtain the non-cytotoxic biomaterial.

Doubtless, *A. baumannii* is one of the bacterial species that can be resistant to silver, whether ionic or in the form of nanoparticles. Whether isolates tested in here presented study carry silver resistance, either plasmid acquired or intrinsic, could be determined by additional analysis, but perhaps is not necessary. The aim of this work was to test the Ag-substituted HAp as proposed material with antibacterial activity, not just toward the usual model bacteria such as *E. coli* or *S. aureus* but to actual organisms of concern, clinical isolates of the extensively-drug resistant *A. baumannii*, and the antibacterial activity was proven.

5. Conclusions

The HAp substituted with Ag^{+}, and mixture of mono-substituted HAps with Ag^{+}, Sr^{2+}, Mg^{2+}, Zn^{2+} and SeO_3^{2-} ions exhibited

antibacterial efficacy toward drug-resistant clinical isolates of *A. baumannii*. Lower substitution level of Ag^{+} ions in HAp_MIX (0.5 mol %) compared to the HAp_Ag (2.5 mol%) sample led to the lower antibacterial effect measured in bacterial suspension but showed similar antibacterial effect during 24 h of disc-diffusion assay, resulting in similar inhibition zones and bacterial adhesion on the biomaterial surface. The substitution level in HAp_MIX could be increased to the 2.5 mol% substitution level to increase the antibacterial effect while ensuring non-cytotoxicity.

The clinical isolates of *A. baumannii* were affected by silver in mono-substituted HAp with Ag^{+} ion and mixture of mono-substituted HAps probably in the same amount as by other commercially available silver-doped materials. As an added result, the apparent time dependent diminishment of bactericidal activity of silver toward *A. baumannii* should be kept in mind.

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CRediT authorship contribution statement

Tomislav Ivankovic: Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Methodology, Validation, Visualization, Project administration, Writing – original draft. **Helena Turk:** Formal analysis, Investigation. **Jasna Hrenovic:** Funding acquisition, Resources, Writing – review & editing. **Zdravko Schauperl:** Investigation, Methodology, Resources, Writing – review & editing. **Marica Ivankovic:** Funding acquisition, Resources, Writing – review & editing. **Antonia Ressler:** Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Project administration, Methodology, Validation, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Environmental Implication

The bacteria *Acinetobacter baumannii* is nowadays notorious in hospital-acquired infections. Increasing concern is the rapid and potent development of multi-drug resistance by *A. baumannii* so, at this point in time, the carbapenem-resistance is almost ubiquitous and pan-drug resistance is not rare. To face this challenge, in this study, silver ions (Ag^{+}) were substituted within hydroxyapatite lattice along with magnesium (Mg^{2+}), zinc (Zn^{2+}), selenite (SeO_3^{2-}) and strontium (Sr^{2+}) to prevent infections without the need for antibiotic in bone regeneration applications.

References

[1] Khansa, I., Schoenbrunner, A.R., Kraft, C.T., Janis, J.E., 2019. Silver in wound care—friend or Foe?: A comprehensive review. Plast Reconstr Surg Glob Open 7, e2390. https://doi.org/10.1097/GOX.0000000000002390.

[2] Gordon, O., Slenters, T.V., Brunetto, P.S., Villaruz, A.E., Sturdevant, D.E., Otto, M., et al., 2010. Silver coordination polymers for prevention of implant infection: Thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. Antimicrob Agents Chemother 54, 10. https://doi.org/10.1128/AAC.01830-09.

[3] Ishida, T., 2018. Antibacterial mechanism of Ag^{+} ions for bacteriolyses of bacterial cell walls via peptidoglycan autolysins, and DNA damages. MOJ Toxicol 4, 345–350. https://doi.org/10.15406/mojt.2018.04.00125.

[4] Jung, W.K., Koo, H.C., Kim, K.W., Shin, S., Kim, S.H., Park, Y.H., 2008. Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli. Appl Environ Microbiol 74, 7. https://doi.org/10.1128/AEM.02001-07.

[5] Randall, C.P., Gupta, A., Jackson, N., Busse, D., O'Neill, A.J., 2015. Silver resistance in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms Antimicrob. Chemother 70, 1037–1046. https://doi.org/10.1093/jac/dku523.

[6] Wiglusz, R.J., Kedziora, A., Lukowaik, A., Doroszkiewicz, W., Strek, W., 2012. Hydroxyapatites and europium(III) doped hydroxyapatites as a carrier of silver nanoparticles and their antimicrobial activity. J Biomed Nanotechnol 8, 605–612. https://doi.org/10.1166/jbn.2012.1424.

[7] May, A., Kopecki, Z., Carney, B., Cowin, A., 2021. Antimicrobial silver dressings: a review of emerging issues for modern wound care. ANZ J Surg 92, 379–384. https://doi.org/10.1111/ans.17382.

[8] Fiore, M., Sambri, A., Zucchini, R., Giannini, C., Donati, D.M., De Paolis, M., 2021. Silver-coated megaprosthesis in prevention and treatment of peri-prosthetic infections: a systematic review and meta-analysis about efficacy and toxicity in primary and revision surgery. Eur J Orthop Surg Traumatol 31, 201–220. https://doi.org/10.1007/s00590-020-02779-z.

[9] Ferdous, Z., Nemmar, A., 2020. Health impact of silver nanoparticles: A review of the biodistribution and toxicity following various routes of exposure. Int J Mol Sci 21, 2375. https://doi.org/10.3390/ijms21072375.

[10] Ahmed, K.B.R., Nagy, A.M., Brown, R.P., Zhang, Q., Malghan, S.G., Goering, P.L., 2017. Silver nanoparticles: Significance of physicochemical properties and assay interference on the interpretation of in vitro cytotoxicity studies. Toxicol Vitr 38, 179–192. https://doi.org/10.1016/j.tiv.2016.10.012.

[11] Legeros, R.Z., Legeros, J.P., 2008. Hydroxyapatite. In: Kokubo, T. (Ed.), Bioceramics and their clinical applications, hydroxyapatite. Woodhead Publishing, Cambridge, pp. 367–394.

[12] Bose, S., Tarafder, S., Bandyopadhyay, A., 2015. Hydroxyapatite coatings for metallic implants. In: Mucalo, M. (Ed.), Hydroxyapatite (HAp) for biomedical applications. Woodhead Publishing, Amsterdam, pp. 147–157.

[13] Dorozhkin, S.V., Epple, M., 2002. Biological and medical significance of calcium phosphates. Angew Chem Int Ed Engl 41 (17), 3130–3146. https://doi.org/10.1002/1521-3773(20020902)41:17<3130::AID-ANIE3130>3.0.CO;2-1.

[14] Wopenka, B., Pasteris, J.D., 2005. A mineralogical perspective on the apatite in bone. Mater Sci Eng C 25 (2), 131–143. https://doi.org/10.1016/j.msec.2005.01.008.

[15] Ressler, A., Antunovic, M., Teruel-Biosca, L., Gallego Ferrer, G., Babic, S., Urlic, I., et al., 2022. Osteogenic differentiation of human mesenchymal stem cells on substituted calcium phosphate/chitosan composite scaffold. Carbohydr Polym 277, 118883. https://doi.org/10.1016/j.carbpol.2021.118883.

[16] Yuan, Q.H., Xu, A.P., Zhang, Z.Q., Chen, Z.H., Wan, L., Shi, X., et al., 2018. Bioactive silver doped hydroxyapatite composite coatings on metal substrates: synthesis and characterization. Mater Chem Phys 218, 130–139. https://doi.org/10.1016/j.matchemphys.2018.07.038.

[17] Kolmas, J., Piotrowska, U., Kuras, M., Kurek, E., 2017. Effect of carbonate substitution on physicochemical and biological properties of silver containing hydroxyapatites. Mater Sci Eng C 74, 124–130. https://doi.org/10.1016/j.msec.2017.01.003.

[18] Choe, H., Tatro, J.M., Hausman, B.S., Hujer, K.M., Marshall, S.H., Akkus, O., et al., 2022. Greenfield EM. *Staphylococcus aureus* and *Acinetobacter baumannii* inhibit osseointegration of orthopedic implants. Infect Immun 90. https://doi.org/10.1128/iai.00669-21 e00669-21.

[19] Vasiliadis, A.V., Poutoglidou, F., Chatziravdeli, V., Metaxiotis, D., Beletsiotis, A., 2021. Acute periprosthetic hip joint infection caused by multidrug-resistant *Acinetobacter baumannii*: Is debridement, antibiotics, irrigation, and implant retention a viable treatment option? Cureus 13, e13090. https://doi.org/10.7759/cureus.13090.

[20] Vila, A., Pagella, H., Amadio, C., Leiva, A., 2016. Acinetobacter prosthetic joint infection treated with debridement and high-dose tigecycline. Infect Chemother 48, 324–329. https://doi.org/10.3947/ic.2016.48.4.324.

[21] Mulani, M.S., Kamble, E.E., Kumkar, S.N., Tawre, M.S., Pardesi, K.R., 2019. Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. Front Microbiol 10, 539. https://doi.org/10.3389/fmicb.2019.00539.

[22] Moubareck, C.A., Halat, D.H., 2020. Insights into *Acinetobacter baumannii*: a review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen. Antibiotics 9, 119. https://doi.org/10.3390/antibiotics9030119.

[23] Weinberg, S.E., Villedieu, A., Bagdasarian, N., Karah, N., Teare, L., Elamin, W.F., 2020. Control and management of multidrug resistant Acinetobacter baumannii: a review of the evidence and proposal of novel approaches. Infect Prev Pract 2, 100077. https://doi.org/10.1016/j.infpip.2020.100077.

[24] McNeilly, O., Mann, R., Cummins, M.L., Djordjevic, S.P., Hamidian, M., Gunawan, C., 2023. Development of nanoparticle adaptation phenomena in acinetobacter baumannii: physiological change and defense response. Bacteriol 11. https://doi.org/10.1128/spectrum.02857-22 e02857-22.

[25] CAMS 2021 -The Croatian Academy of Medical Sciences, Antibiotic resistance in Croatia; 2021. ⟨https://iskra.bfm.hr/otpornost-bakterija-na-antibiotike-u-hrvatskoj/⟩.
[26] Hetta, H.F., Al-Kadmy, I.M.S., Khazaal, S.S., Abbas, S., Suhail, A., El-Mokhtar, M. A., et al., 2021. Antibiofilm and antivirulence potential of silver nanoparticles against multidrug-resistant Acinetobacter baumannii. Sci Rep 11 (1), 10751. https://doi.org/10.1038/s41598-021-90208-4.
[27] Riaza, M., Ziaa, R., Ijaza, A., Hussainb, T., Mohsina, M., Malikc, A., 2018. Synthesis of monophasic Ag doped hydroxyapatite and evaluation of antibacterial activity. Mater Sci Eng C 90, 308–313. https://doi.org/10.1016/j.msec.2018.04.076.
[28] Ressler, A., Ivankovic, T., Polak, B., Ivanisevic, I., Kovacic, M., Urlic, I., et al., 2022. A multifunctional strontium/silver-co-substituted hydroxyapatite derived from biogenic source as antibacterial biomaterial. Ceram Int 48, 18361–18373. https://doi.org/10.1016/j.ceramint.2022.03.095.
[29] Ressler, A., Cvetnic, M., Antunovic, M., Marijanovic, I., Ivankovic, M., Ivankovic, H., 2020. Strontium substituted biomimetic calcium phosphate system derived from cuttlefish bone. J Biomed Mater Res 108 (4), 1697–1709. https://doi.org/10.1002/jbm.b.34515.
[30] Ressler, A., Antunovic, M., Cvetnic, M., Ivankovic, M., Ivankovic, H., 2021. Selenite substituted calcium phosphates: preparation, characterization, and cytotoxic activity. Materials 14 (12), 3436. https://doi.org/10.3390/ma14123436.
[31] Ressler, A., Ivankovic, T., Ivanisevic, I., Cvetnic, M., Antunovic, M., Urlic, I., et al., 2023. Multiphase zinc and magnesium mono-substituted calcium phosphates derived from cuttlefish bone: a multifunctional biomaterials. Ceram Int 49 (7), 11005–11017. https://doi.org/10.1016/j.ceramint.2022.11.295.
[32] Seruga Music, M., Hrenovic, J., Goic-Barisic, I., Hunjak, B., Skoric, D., Ivankovic, T., 2017. Emission of extensively-drug-resistant Acinetobacter baumannii from hospital settings to the natural environment. J Hosp Infect 96, 323–327. https://doi.org/10.1016/j.jhin.2017.04.005.
[33] Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M.E., Garaizar, J., et al., 1996. Comparison of outbreak and nonoutbreak Acinetobacter baumannii strains by genotypic and phenotypic methods. J Clin Microbiol 34, 1519–1525. https://doi.org/10.1128/jcm.34.6.1519-1525.1996.
[34] Dijkshoorn, L., Brouwer, C.P.J.M., Bogaards, S.J.P., Nemec, A., Van den Broek, P. J., Nibbering, P.H., 2004. The synthetic N-terminal peptide of human lactoferrin, hLF(1-11), is highly effective against experimental infection caused by multidrug-resistant Acinetobacter baumannii. Antimicrob Agents Chemother 48, 4919–4921. https://doi.org/10.1128/AAC.48.12.4919-4921.2004.
[35] Selçuk, C.T., Durgun, M., Ozalp, B., Tekin, A., Tekin, R., Akçay, C., et al., 2012. Comparison of the antibacterial effect of silver sulfadiazine 1 %, mupirocin 2 %, Acticoat and octenidine dihydrochloride in a full-thickness rat burn model contaminated with multi drug resistant *Acinetobacter baumannii*. Burns 38, 1204–1209. https://doi.org/10.1016/j.burns.2012.04.009.
[36] Fiore, M., Sambri, A., Zucchini, R., Giannini, C., Donati, D.M., De Paolis, M., 2021. Silver-coated megaprosthesis in prevention and treatment of peri-prosthetic infections: a systematic review and meta-analysis about efficacy and toxicity in primary and revision surgery. Eur J Orthop Surg Traumatol 31, 201–220. https://doi.org/10.1007/s00590-020-02779-z.
[37] Donati, F., Di Giacomo, G., D'Adamio, S., Ziranu, A., Careri, S., Rosa, M.A., et al., 2016. Silver-coated hip megaprosthesis in oncological limb savage surgery. Biomed Res Int 2016, 9079041. https://doi.org/10.1155/2016/9079041.
[38] Lansdown, A.B.G., 2010. A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. Adv Pharmacol Sci 2010, 910686. https://doi.org/10.1155/2010/910686.
[39] Li, P., Jia, Z., Wang, Q., Tang, P., Wang, M., Wang, K., et al., 2018. A resilient and flexible chitosan/silk cryogel incorporated Ag/Sr co-doped nanoscale hydroxyapatite for osteoinductivity and antibacterial properties. J Mater Chem B 6, 7427–7438. https://doi.org/10.1039/C8TB01672K.
[40] Phatai, P., Prachumrak, N., Kamonwannasit, S., Kamcharoen, A., Roschat, W., Phewphong, S., et al., 2022. Zinc-silver doped mesoporous hydroxyapatite synthesized via ultrasonic in combination with sol-gel method for increased antibacterial activity. Sustainability 14, 11756. https://doi.org/10.3390/su141811756.
[41] Ni, Z., Gu, X., Wang, Z., Zou, X., Zhao, Y., Sun, L., 2018. Synthesis of silver nanoparticle-decorated hydroxyapatite (HA@Ag) poriferous nanocomposites and the study of their antibacterial activities. RSC Adv 8, 41722–41730. https://doi.org/10.1039/C8RA08148D.
[42] González-Torres, V., Hernández-Guevara, E., Castillo-Martínez, N.A., Rosales-Aguilar, M., Díaz-Trujillo, G.C., 2021. Antibacterial activity analysis of hydroxyapatite based materials with fluorine and silver. Rev Mex De Ing Biomed 42, 49–57. https://doi.org/10.17488/RMIB.42.2.4.
[43] Cavassin, E.D., de Figueiredo, L.F.P., Otoch, J.P., Seckler, M.M., de Oliveira, R.A., Franco, F.F., et al., 2015. Comparison of methods to detect the in vitro activity of silver nanoparticles (AgNP) against multidrug resistant bacteria. J Nanobiotechnol 13, 64. https://doi.org/10.1186/s12951-015-0120-6.
[44] Mediaswanti, K., 2016. Influence of physicochemical aspects of substratum nanosurface on bacterial attachment for bone implant applications. J Nanotechnol 2016, 1–6. https://doi.org/10.1155/2016/5026184.
[45] Zhao, X., Zhong, J., Wei, C., Lin, C.W., Ding, T., 2017. Current perspectives on viable but non-culturable state in foodborne pathogens. Front Microbiol 8, 580. https://doi.org/10.3389/fmicb.2017.00580.
[46] Gupta, A., Phung, L.T., Taylor, D.E., Silver, S., 2001. Diversity of silver resistance genes in IncH incompatibility group plasmids. Microbiology 147, 3393–3402. https://doi.org/10.1099/00221287-147-12-3393.
[47] Silver, S., 2003. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev 27, 341–353. https://doi.org/10.1016/S0168-6445(03)00047-0.
[48] Deshpande, L.M., Chopade, B.A., 1994. Plasmid mediated silver resistance in *Acinetobacter baumannii*. Biometals 7, 49–56. https://doi.org/10.1007/BF00205194.
[49] Deshpande, L.M., Kapadnis, B.P., Chopade, B.A., 1993. Metal resistance in Acinetobacter and its relation to β-lactamase production. Biometals 6, 55–59. https://doi.org/10.1007/BF00154233.
[50] Hosny, A.E.D.M.S., Rasmy, S.A., Aboul-Magd, D.S., Kashef, M.T., El-Bazza, Z.E., 2019. The increasing threat of silver-resistance in clinical isolates from wounds and burns. Dovepress 2019, 1958–2001. https://doi.org/10.2147/IDR.S209881.