A functional apatite with antibacterial efficacy for bone tissue infections

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Keywords: antibacterial, apatite, bone tissue infections, silicon, silver

Abstract
Silver, silicon co-substituted hydroxyapatite (Ag, Si-HA) was developed to provide bone repair coupled with antibacterial effect, with the aim to address the problems arise in the treatment of bone tissue infections. In this study, Ag, Si-HA demonstrated substantially reduced attachment of Staphylococcus aureus, Propionibacterium acnes, Escherichia coli, and Pseudomonas aeruginosa as compared to HA at 12 h. Being a prolific opportunistic pathogen in bone tissue infections, we investigated if P. aeruginosa could develop resistance against Ag, Si-HA. Our study showed that despite repeated exposure to fresh population of P. aeruginosa every 48 h, Ag, Si-HA exhibited effective antibacterial properties against the growth of P. aeruginosa over 168 h, indicating low risk of inducing bacterial resistance against Ag, Si-HA. As P. aeruginosa produces exotoxins and harbours endotoxins on its cell wall, together these toxins could delay healing process. We therefore examined if the effect of these toxins released by P. aeruginosa during the antibacterial assessment on the attachment of mesenchymal stem cells on HA and Ag, Si-HA. Unlike HA, cell attachment on Ag, Si-HA was not affected by the addition of supernatant obtained from the antibacterial assessment of HA and Ag, Si-HA. This demonstrated that Ag, Si-HA could inhibit the growth of bacteria as well as minimise or prevent the detrimental effect from bacteria toxins.

1. Introduction
Bone tissue infections including osteomyelitis, periodontitis and spondylodiscitis are some of the most frequently occurring complications in bone surgeries [1, 2], which compromise the self-healing capacity of bone tissues, leading to severe bone loss, implant failure or even amputation [3]. The microbial pathogens that are most commonly associated with such infections include gram-positive bacteria, such as Staphylococcus aureus, Propionibacterium acnes, Mycobacterium tuberculosis and Streptococcus spp.; gram-negative bacteria: Salmonella spp., Pseudomonas aeruginosa, Escherichia coli; and fungi: Candida spp. The treatment for bone tissue infections generally involves three weeks of intravenous therapy followed by three weeks of oral antibiotic [2]. However, as bone tissue is poorly vascularised, high dosage of antibiotic is generally required to achieve prolonged antibacterial concentration at the infected site. This in turn may cause systemic toxic effects like nephrotoxicity, ototoxicity, hepatotoxicity, allergy or gastrointestinal syndromes [3]. Furthermore, many bacterial species displayed resistance against antibiotic treatment. Owing to all these serious clinical problems arise in the treatment of bone infections, most studies have directed attention toward the development of novel biomaterials with both antibacterial and osteoinductive properties.

Hydroxyapatite (HA) is a material that is widely used in regenerative medicine, bone and dental surgery as it resembles the main inorganic component of mineralised tissues (biological apatite), thus, making it an asset for biomaterial engineering [4, 5]. HA is currently considered to be the gold standard in bone tissue regeneration. It has been used in the form of powders or granules as filler, porous structure as scaffold for bone replacement, and repair of post-resection defects in clinical practice [6, 7]. Due to its bioactivity, HA was also used as a coating...
material for metallic implants to enhance osseointegration process [8]. Despite the resemblance, it should be stressed that biological apatite is a carbonated hydroxyapatite, containing a number of trace amount of various ions, primarily magnesium, sodium, potassium, zinc, manganese, silicate and hydrogen phosphate [9]. Hence, over the years, research on HA was conducted to achieve two main goals: (1) to improve the biocompatibility of synthetic HA, and (2) to provide synthetic HA with supplementary functional properties that increase the success of implantation. Both goals could be achieved using partial ionic substitution in synthetic HA.

Silver (Ag) and Ag nanoparticles exhibit a wide spectrum of oligodynamic actions against bacteria, viruses and fungi with a relatively low risk of developing resistance against the metal, and can be easily synthesised [10–13]. Hence, Ag has been studied widely for its application, which include forming biomaterial nanocomposite with HA and polymers [14–16] or incorporated into HA to form a single phase material for excellent antibacterial property [17, 18]. Recently, silver, silicon co-substituted hydroxyapatite (Ag, Si-HA) that comprises an adequate amount of Ag and silicon (Si) was incorporated in HA exhibited a balance between antibacterial activity and biocompatibility [19]. Despite the substitution of Ag, Ag, Si-HA was shown to be cytocompatible in supporting the growth of human adipose derived mesenchymal stem cells over 28 d [19]. In addition, it exhibited optimal osteogenic properties with increased bone differentiation markers including alkaline phosphatase, collagen type I and osteocalcin expression as compared to HA and AgHA, as well as inhibiting the growth of S. aureus for up to 7 d [19]. This demonstrated that the incorporation of Si in Ag, Si-HA helped to mitigate the reduced cell differentiation caused by Ag.

It is the interest of this study to evaluate the antibacterial efficacy of Ag, Si-HA against four different bacterial pathogens that are commonly found in bone tissue infections. We have chosen two species of gram-positive bacteria: S. aureus and Pp. acnes, and two species of gram-negative bacteria: E. coli and P. aeruginosa [20–23]. Furthermore, as P. aeruginosa has an established bacterial pathogen in bone tissue infections, the risk of P. aeruginosa developing resistance against Ag, Si-HA was also investigated by repeat exposing Ag, Si-HA to fresh population of P. aeruginosa at every 48 h over a period of 168 h. It was known that P. aeruginosa produced exotoxins and harbour endotoxins on its cell wall [24, 25], which could delay healing process, the effect of these toxins released by P. aeruginosa during the antibacterial assessment on the attachment of cells on HA and Ag, Si-HA was also examined.

2. Materials and methods

2.1. Preparation of apatite discs

The synthesis of apatite and characterisation of the apatite powders were reported previously in detail [19]. Briefly, Ag, Si-HA comprising 0.5 wt% Ag and 0.8 wt % Si was synthesised based on a wet precipitation reaction between calcium hydroxide (Merck) containing silver nitrate (Merck) and orthophosphoric acid (Merck) containing tetraethyl orthosilicate (Sigma Aldrich). The precipitation reaction was carried out under continuous stirring at 25 °C, and aqueous ammonia (Sigma Aldrich) was added to maintain the pH value above 10.5. Precipitates were aged, autoclaved and washed. Similarly, HA was synthesised based on the same technique via an aqueous precipitation reaction between calcium hydroxide and orthophosphoric acid. Phase-pure synthesised powders made up of nanorods with the dimensions of ~60 by 10 nm [26] were compacted to form into a 12 mm disc, and dry heated at 600 °C in air for the antibacterial assessments. Sample discs were sterilised by soaking in 70% ethanol for 4 h, and dried overnight.

2.2. Bacteria culture

The four different bacteria strains used in the antibacterial effect study were S. aureus (ATCC 25923), Pp. acnes, (ATCC 51277), E. coli (ATCC 25922) and P. aeruginosa (ATCC 27853). Each Ag, Si-HA disc was immersed in 1 ml of respective medium (peptone water (Oxoid) for S. aureus and E. coli, fastidious anaerobe broth (Lab M) for Pp. acnes and Mueller Hinton broth (Oxoid) for P. aeruginosa) containing 1–2 × 10⁶ CFU ml⁻¹ of each bacterial strain in a 24-well plate and incubated at 37 °C for 12 h. Similar procedures were repeated on HA discs incubated with the 4 different bacterial species, respectively.

2.3. Attachment of bacteria

Following 12 h incubation, the Ag, Si-HA discs were removed from the plate and washed with phosphate saline buffer solution (PBS). The adherent bacteria on the surface of the disc were fixed with 10% formalin, dehydrated sequentially through a series of ethanol concentrations (25%, 50%, 75%, 95%, 100%), and vacuum-dried before viewing under scanning electron microscope (Hitachi S-4300).
2.4. Antibacterial effect of Ag, Si-HA
In a separate experiment, antibacterial action of the Ag, Si-HA discs against \( \sim 10^4 \text{ CFU ml}^{-1} \) \( P. \ aeruginosa \) was examined over a period of 168 h, using log reduction assay. In this study, following 24 h incubation, the original bacterial population was removed and replaced with fresh population of \( \sim 10^6 \text{ CFU ml}^{-1} \) \( P. \ aeruginosa \). This process was repeated every 48 h till 168 h. After each incubation period, discs were removed from the test solution, and placed into a new tube containing 5 ml peptone water, and vortexed for 60 s to remove the adhering bacteria. An aliquot of 100 \( \mu l \) was retrieved and serially diluted for the enumeration of surviving bacteria on the surface of the disc. An aliquot of 20 \( \mu l \) of various dilutions was then added onto a Mueller Hinton agar in triplicate and incubated at 37 \( ^{\circ} \text{C} \) for 24 h. The number of colonies formed was enumerated and recorded. Similarly, 100 \( \mu l \) was retrieved from the test solution, and then subjected to the same procedures to determine the surviving colonies in the medium.

2.5. Antagonistic effect of supernatant on cell culture
The test solution of Ag, Si-HA and HA of respective bacterial cultures at 24 h was centrifuged, and each supernatant was retrieved and added in the cell culture with the discs. Aliquot containing \( \sim 1.3 \times 10^3 \) human bone marrow-derived mesenchymal stem cells (purchased commercially from Life Technologies Holdings Pte Ltd—Invitrogen, Stempro® human bone marrow-derived stem cell kit, passage 5) were seeded onto each disc in a 24-well plate containing 200 \( \mu l \) supernatant from Ag, Si-HA and 800 \( \mu l \) Dulbecco’s modified Eagle medium supplemented—GlutaMAX with 10% fetal bovine serum medium. The cell-supernatant mixtures were maintained in a humidified atmosphere of 95% air and 5% carbon dioxide at 37 \( ^{\circ} \text{C} \) for 24 h. The process was repeated using the supernatant retrieved from HA. Cells seeded on tissue culture plate without discs were used as the control.

After 24 h, the discs were washed three times in PBS solution, and cells were fixed with 4% formaldehyde in PBS solution for 10 min, followed by washing with PBS solution and permeabilised using 0.1% Triton X-100 for 10 min. The supernatant treated discs were then incubated with 1% bovine serum albumin-PBS solution at 25 \( ^{\circ} \text{C} \) for 30 min to block the non-specific binding. DAPI (1:1000 in PBS solution) was added to the discs at 25 \( ^{\circ} \text{C} \) and treated for 5 min. The nuclei of the cells were stained by DAPI [27]. The samples were then given a final wash with PBS (5 min \( \times \) 3) before mounting under vactashiel antifade mounting medium, and viewed under a confocal laser scanning microscope (CLSM, Nikon).

2.6. Statistical analysis
A two-way analysis of variance, followed by Turkey’s post-hoc testing was used to determine any significant differences existed between the mean values of the experimental groups for log reduction assay. A difference between groups was considered to be significant at \( p < 0.05 \). Three replicates were measured for log reduction assay with 2 independent repeats, and the mean value was calculated.

3. Results and discussion
Bone tissue infections were caused by bacterial adhesion and biofilm formation [28]. In this study, bacterial cells of \( S. \ aureus \), \( Pp. \ acnes \), \( E. \ coli \) and \( P. \ aeruginosa \) were clearly seen to attach on the surface of HA after 12 h (figure 1). On the other hand, a substantially reduced number of the bacterial cells of \( S. \ aureus \), \( E. \ coli \), \( Pp. \ acnes \) were seen on the surface of Ag, Si-HA discs (figures 2(a)–(c)). A closer observation on these surfaces showed that most of these bacterial cells were shown to be disintegrated. In particular, it was worthwhile to note that there was no \( P. \ aeruginosa \) cells observed on Ag, Si-HA (figures 2(d)). Furthermore, the amount of attached bacteria on HA and Ag, Si-HA were quantified in figure 3. Approximately 4-log reduction of the bacterial population for \( S. \ aureus \), \( E. \ coli \), \( Pp. \ acnes \) and approximately 7-log reduction of the bacterial population for \( P. \ aeruginosa \) could be observed on Ag, Si-HA as compared to HA. The attachment of bacteria suggested that HA had no antibacterial effect against all the four bacterial strains tested whilst the reduced attachment of bacteria or disintegrated cells on Ag, Si-HA demonstrated strong antibacterial effect on the growth of all the four bacterial stains tested. Therefore, the substitution of Ag into apatite demonstrated improved antibacterial activity against \( S. \ aureus \), \( Pp. \ acnes \), \( E. \ coli \), and \( P. \ aeruginosa \) for bone tissue infections.

\( P. \ aeruginosa \) is an opportunistic bacterial pathogen, which is commonly found in bone tissue infections [29]. Therefore, a more extensive investigation of the antibacterial assessment of Ag, Si-HA with \( P. \ aeruginosa \) was performed. As shown in figures 2(d) and 3, there was hardly any bacteria observed and detected on Ag, Si-HA at 12 h. By extending the culture period to 168 h and quantifying the bacteria, it was demonstrated that there was indeed no bacteria found on (Ag, Si-HA vortex) and surrounding Ag, Si-HA (Ag, Si-HA medium) as shown in figure 4(a) over the extended 168 h period. In contrast, bacteria were shown to propagate on (HA vortex) and surrounding HA (HA medium) reaching to a bacterial population ranging from \( 10^6 \) to \( 10^8 \text{ CFU ml}^{-1} \) over 168 h.
Figure 1. Bacteria attachment on HA. Bacterial cells are seen on HA disc cultured with (a) *S. aureus*, (b) *Pp. acnes*, (c) *E. coli* and (d) *P. aeruginosa* on HA, after 12 h.

Figure 2. Bacterial attachment on Ag, Si-HA. Reduced or absence of (a) *S. aureus*, (b) *Pp. acnes*, (c) *E. coli* and (d) *P. aeruginosa* on Ag, Si-HA, after 12 h.
These findings demonstrated that Ag, Si-HA exhibited effective antibacterial properties against *P. aeruginosa* as compared to HA (figure 4(b)). Furthermore, there was no sign of bacterial growth on Ag, Si-HA despite repeated exposure of fresh population of *P. aeruginosa* every 48 h. When a microbe resists the effects of a substance that once could successfully treat the microbe, it develops antimicrobial resistance against it [30]. Since the added *P.
**P. aeruginosa** was not propagating on Ag, Si-HA at 120 and 168 h when fresh *P. aeruginosa* was added on 72 and 120 h, respectively, it could be suggested that there was a very low risk of inducing bacterial resistance in *P. aeruginosa* against Ag, Si-HA. It would thus be fair to conclude that Ag, Si-HA was shown to have the potential of having an advantage over usage of antibiotic in treating bone tissue infections by providing direct antibacterial effect at the infected bone site, and with a low risk of inducing *P. aeruginosa* to develop resistance against Ag, Si-HA.

*P. aeruginosa* secretes exotoxins during growth phase, and this could cause damage to the host by destroying cells or disrupting normal cellular metabolism [24]. As a gram-negative bacteria, *P. aeruginosa* harbours endotoxins on its cell wall, and there could be a possibility of releasing these endotoxins when it is killed [25], which could delay healing process [31]. Therefore, infection caused by gram-negative bacteria could become more complicated, and it would be essential to determine if there was any effect of the toxins released by *P. aeruginosa* from the antibacterial assessment on HA and Ag, Si-HA. As *P. aeruginosa* was growing on HA, exotoxins could possibly be present in the test solution of HA. Since no growth of *P. aeruginosa* was observed in the antibacterial assessment of Ag, Si-HA, it might suggest that endotoxins could be released from the dead *P. aeruginosa* to the test solution of Ag, Si-HA. Hence, the supernatant obtained from the antibacterial assessment of Ag and Si-HA was added into the mesenchymal cell culture cultured in Ag, Si-HA for 24 h to assess the attachment of cells by staining the nuclei of the cells with DAPI (figure 5). It could be observed that the attachment of cells in the control (cells seeded on tissue culture plate) and HA was affected by the addition of supernatant obtained from the antibacterial assessment of HA as much lesser nuclei was observed (figures 5(g) and (h)). Thus, this suggested that the supernatant obtained from the antibacterial assessment of HA contained exotoxins, which was detrimental to the attachment of cells, and could in turn affect the cell growth [32].

On the other hand, the attachment of cells on Ag, Si-HA did not seem to be affected by the addition of supernatant obtained from the antibacterial assessment of HA (figure 5(c)) as the number of nucleus was comparable to the samples with no addition of supernatant (figures 5(d)–(f)). Furthermore, it was interesting to note that despite the addition of supernatant obtained from the antibacterial assessment of Ag, Si-HA, the

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**Figure 5.** Characterising the effect of post-bacterial culture supernatants on mesenchymal cell cultures. DAPI stained cells of (a) control, (b) HA, (c) Ag, Si-HA cultured with the addition of supernatant obtained from the antibacterial assessment of HA; (d) control, (e) HA, (f) Ag, Si-HA cultured with no addition of supernatant; (g) control, (h) HA, and (i) Ag, Si-HA cultured with the addition of supernatant obtained from the antibacterial assessment of Ag, Si-HA, after 24 h.
attachment of cells on the control (cells seeded on tissue culture plate), HA and Ag, Si-HA (figures 5(g)–(i)) were also comparable to the samples with no addition of supernatant (figures 5(d)–(f)). This might suggest that there might be no endotoxin released by P. aeruginosa in the antibacterial assessment of Ag, Si-HA. Nevertheless, all these findings might also suggest that Ag, Si-HA was able to minimise or prevent the detrimental effect of the bacterial toxins in the supernatant obtained from the antibacterial assessment of HA and Ag, Si-HA on the attachment of cells. Bacterial toxins consisted of proteins [33] that could interact with Ag⁺ ions. Wigginton et al [34] observed that the binding of bacterial protein with Ag nanoparticles, which inhibited its enzymatic activity. Braunwarth et al [35] reported on effective antibacterial property of Ag⁺ ions releasing wound dressing against the endotoxins of P. aeruginosa. Therefore, it could be postulated that the minimised effect of the bacterial toxins on the cell attachment of Ag, Si-HA could be due to the binding of bacterial toxins with the surface-bound Ag⁺ ions of Ag, Si-HA or the releasing of Ag⁺ ions from Ag, Si-HA could have reacted with the bacterial toxins [19]. It could also be the possibility the result of both modes of actions. Further characterisation and investigation such as the co-culture study of cells and bacteria with Ag, Si-HA would be required to further ascertain and explain it. Ag, Si-HA was demonstrated to be biocompatible, supporting cell proliferation and bone differentiation of human adipose derived stem cells over 28 d in previous in vitro study [19]. There was also an increased expression of alkaline phosphatase, collagen type I and osteocalcin produced by cells cultured on Ag, Si-HA than HA and AgHA. Taken together, the preliminary results obtained in this study demonstrated that AgSi-HA could potentiate as an effective antibacterial material for bone tissue infections.

5. Conclusions

This study showed that Ag, Si-HA demonstrated substantially reduced attachment of S. aureus, Pp. acnes, E. coli, and P. aeruginosa as compared to HA at 12 h. By introducing fresh population of P. aeruginosa every 48 h, Ag, Si-HA exhibited effective antibacterial properties against P. aeruginosa over 168 h. Results suggested that Ag, Si-HA had low risk of inducing bacterial resistance against it. Furthermore, the attachment of cells on Ag, Si-HA was not affected by the addition of supernatant obtained from the antibacterial assessment of HA and Ag, Si-HA. This demonstrated that Ag, Si-HA not only had the potential to inhibit the growth of bacteria but was also able to minimise or prevent the detrimental effect from bacterial toxins, thereby representing a promising antibacterial material for bone tissue infections.

Acknowledgments

This research is supported by the Singapore Ministry of Health’s National Medical Research Council under its NMRC Open Fund Individual Research Grant Number OFIRG15nov026.

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