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Development of New Generation Bone Graft Material: Silver, Silicon Co-Substituted Apatite with Bi-Functional Properties

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ABSTRACT

With the rise of ageing population, the need to restore the function of degenerative bone greatly drives the market for bone grafts. Hydroxyapatite (HA) is chemically similar to natural bone mineral and has been widely used in bone graft applications. However, its slow osseointegration process and lack of antibacterial property could lead to implant-related infection, resulting in implant failure. Studies on ionic substitution of apatite have gained attention in recent years with greater understanding of the composition of bone mineral being a multi-substituted apatite. An integrated approach is proposed by co-substituting silver (Ag) and silicon (Si) into HA (Ag,Si-HA) to modify its surface for bi-functional properties. Incorporation of Si can enhance the biomineralization of HA and introduction of Ag can create antibacterial property. Ag,Si-HA containing 0.5 wt.% of Ag and 0.8 wt.% of Si was prepared by a wet precipitation method. A phase-pure apatite with a nanorod morphology of dimensions 60 nm in length and 10 nm in width was synthesized. Surface Ag⁺ ions of Ag,Si-HA were demonstrated to prevent the replication of adherent *Staphylococcus aureus* bacteria for up to 120 h. Biocompatibility tests revealed that human adipose-derived mesenchymal stem cells (hMSCs) proliferated well on Ag,Si-HA with culturing time. Enhanced cell attachment in turn permitted greater bone differentiation as evidenced in the increase of collagen type I and osteocalcin expressions of hMSCs cultured on Ag,Si-HA as compared to HA from day 14 onwards. Overall, co-substitution of Ag and Si could complement the benefits of each substituent by endowing HA with antibacterial property, and concurrently promoting its biological performance. Their synergistic effects can serve unmet medical needs and solve the problem of implant-related infection. This work also enhances the understanding of substituted apatite with multiple ions for bi-functional properties.

INTRODUCTION

Bioactivity of the material plays an integral role for bone regeneration, and optimal healing of the defect region is heavily reliant upon the prevention of bacterial infection after implant placement. Therefore, apart from accelerating the osseointegration process, incorporating the apatite with antibacterial property is crucial for a successful bone repair. The unique crystal structure of HA makes it susceptible to a variety of cations and anions, permitting ionic substitutions to take place simultaneously. Substitution of Si into HA facilitates early precipitation of carbonated-containing apatite layer, thereby creating and conditioning a surface associated with increased adsorption of proteins that promotes osteoblasts to mineralize [1]. On the other hand, Ag^+ ions interact with thiol groups of the bacterial proteins so as to inactivate its replication [2]. Hence, an effective solution against implant-related infection is proposed in this study by co-substituting Ag and Si into HA (Ag,Si-HA) for bi-functional properties. Ag,Si-HA will be investigated for its synergistic effects in term of antibacterial action and bioactive behavior.

EXPERIMENTAL DETAILS

$\text{Ag}_{0.05}\text{Ca}_{9.95}(\text{PO}_4)_{5.68}(\text{SiO}_4)_{0.32}\text{OH}_{1.63}$ was synthesized using a wet precipitation method according to the method described elsewhere [3]. Ag,Si-HA nanopowder was autoclaved or heat-treated at 1200 °C for 2 h in air with a heating rate of 2.5 °C/min. Elemental and phase composition of Ag,Si-HA were determined by X-ray fluorescence (XRF) spectroscopy and X-ray diffraction (XRD), respectively. Microstructural feature of Ag,Si-HA was studied using a field emission scanning electron microscope (FESEM).

Ag,Si-HA powder was compacted uni-axially into Ø12 mm discs before dry heating at 600 °C for 2 h in air. Ag,Si-HA discs were sterilized by rinsing three times with phosphate buffer saline (PBS) solution, followed by ultraviolet exposure for 30 min. Sterilized Ag,Si-HA discs were added into 1×10^7 CFU/ml of *staphylococcus aureus* (*S. aureus*) and incubated at 37 °C for 24 h. A period of up to 120 h was further examined for antibacterial effect. At the end of each incubation time, Ag,Si-HA discs were removed from the test solution and gently rinsed with PBS solution, before putting into a new tube containing 5 ml peptone water, to vortex vigorously for 60 s. The vortex solution was then diluted in steps. For each time point, 25 µl of each diluted vortex solution was added onto a tryptone soya agar in triplicate, and incubated at 37 °C for another 24 h to allow enumeration of viable bacterial count.

In-vitro cell viability behavior was evaluated for 5 days of incubation using human adipose-derived mesenchymal stem cells (hMSCs) at 10^5 cells/sample, and viable cells were counted using alamarBlue™ assay. At each time point, samples were cultured in 10 % alamarBlue™ medium for 4 h. The absorbance was then monitored at a wavelength of 570 nm, with a reference wavelength of 600 nm. Cell number was obtained by cross-referencing to a standard calibration curve of hMSCs count against absorbance done at the beginning of the study to obtain the live cell count at each time point. Collagen type I (COL I) and osteocalcin (OCN) expressions of hMSCs cultured on the samples for 7, 14 and 21 days were determined according

to the manufacturer's protocol shown on the Metra™ CICP EIA kit (Quidel) and Metra™ Osteocalcin ELISA kit (Quidel), respectively.

A *t*-test was used to determine whether any significant differences existed between the mean values of the experimental groups. A difference between groups was considered to be significant at $p < 0.05$. Five replicates were measured for alamarblue™, and the mean value was calculated. Four replicates were measured for COL I and OCN, and the mean value was calculated.

RESULTS & DISCUSSION

Characterization of Ag,Si-HA nanopowder

Ag,Si-HA displayed a nanorod morphology with an average length of ~60 nm and width of ~10 nm, mimicking the bone mineral (Figure 1). 0.5 wt.% of Ag and 0.8 wt.% of Si (Table 1) were incorporated in Ag,Si-HA, giving rise to a (Ag+Ca)/(P+Si) atomic ratio of 1.66, which was close to the theoretical value of 1.67 for apatite. A phase-pure apatite was identified for Ag,Si-HA and remained stable at 1200 °C, as observed from the X-ray diffraction patterns (Figure 2). Hence, it was demonstrated that Ag and Si were structurally incorporated into HA to form a single-phase, rather than existing as a second phase with HA.

Figure 1 FESEM morphology of Ag,Si-HA nanopowder

Table 1 Measured weight percentage of silver and silicon using XRF

	wt.% Ag	wt.% Si
Amount	0.5 ± 0.1	0.7 ± 0.1
Line	AgK α	SiK α

Figure 2 X-ray diffraction patterns of Ag,Si-HA (a) autoclaved and (b) heat treated at 1200 °C

Antibacterial action of Ag,Si-HA

The log reduction assay is represented in Figure 3. Adherent *S. aureus* on HA grew steadily from a starting population of approximately 1×10^7 CFU/ml to a population of 7×10^7 CFU/ml over a period of 120 h. On the other hand, the number of adherent *S. aureus* on Ag,Si-HA was reduced by 2-order to approximately 4×10^5 CFU/ml at 6 h, and further reduced to 8×10^3 CFU/ml at 12 h. At 18 h, the number of adherent *S. aureus* on Ag,Si-HA was reduced to zero, and this remained till 120 h. Overall, a 7-log reduction of adherent *S. aureus* was observed for Ag,Si-HA as compared to HA over a culture period of 120 h. This suggested that Ag^+ ions on the crystal surface were producing an antibacterial effect against the adherent bacteria.

Figure 3 Log reduction assay of HA and Ag,Si-HA discs cultured with *S. aureus*

Bone growth and differentiation of Ag,Si-HA

Preliminary *in-vitro* study compared the responses of hMSCs cultured on HA and Ag,Si-HA. Results demonstrated increasing growth activity of hMSCs cultured on HA and Ag,Si-HA (Figure 4). Despite the presence of Ag, the growth of hMSCs of HA and Ag,Si-HA were comparable. In addition, it was interesting to note that the expression of COL I and OCN (Figure 5) was statistically higher ($p < 0.05$) for hMSCs cultured on Ag,Si-HA than HA, indicating the efficacy of Si in driving osteoblast cells towards the maturation stage. Therefore, results showed that the substitution of Si was desirable for improved osseointegration.

Figure 4 Cell proliferation of hMSCs on HA and Ag,Si-HA at different time points, * $p < 0.05$

Figure 5 Quantitative measurement of (a) COL I and (b) OCN expressions of hMSCs cultured on HA and Ag,Si-HA, * $p < 0.05$

CONCLUSIONS

This study demonstrated the feasibility of co-substituting Ag and Si into HA simultaneously to achieve bi-functional properties of antibacterial and favourable bioactive properties. A phase-pure apatite with a nanorod morphology of dimensions 60 nm in length and 10 nm in width was synthesized for Ag,Si-HA. On the whole, effective inhibition of the adherent *S. aureus* was observed for Ag,Si-HA as a result of the interaction between the Ag⁺ ions on the surface crystal of Ag,Si-HA and bacteria. Despite the presence of Ag, comparable hMSCs proliferation was demonstrated in Ag,Si-HA and HA. Furthermore, enhanced bone differentiation were observed in Ag,Si-HA than HA, as reflected from the elevated levels of COL I and OCN expressions. Overall, Ag,Si-HA has demonstrated to be a potential bone substitute material in minimizing post-infection complication.

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