Supplementary Material

The combination of silver-containing hydroxyapatite coating and vancomycin has a synergistic antibacterial effect on methicillin-resistant *Staphylococcus aureus* biofilm formation.

Methods

Silver-hydroxyapatite (Ag-HA) coating - in vitro experiments. Pure titanium discs (14 mm diameter, 1 mm thickness; Kobe Steel, Kobe, Japan) were used as substrates for the coating deposition. One side was subjected to a K5 sandblasting machine (TKX Corp., Osaka, Japan) with a 180-grit aluminium oxide medium (Showa Denko K.K., Tokyo, Japan). Then, the discs were washed through ultrasonication in ethanol for three minutes. Powdered silver oxide (Kanto Chemical, Tokyo, Japan) was added to the powdered hydroxyapatite (HA; KYOCERA Corporation, Shiga, Japan) to prepare 3% Ag-HA, and the mixtures were stirred in plastic bags for five minutes. HA powders with and without silver oxide were thermally sprayed onto the sandblasted surface to coat the disc using a flame-spraying system (Oerlikon Metco Japan Ltd., Tokyo, Japan) with a flame temperature of approximately 2,700°C. The spraying powder was carried into the flame by a dry air carrier gas during spraying, melted by flaming, and then sprayed onto the disc. The coating process was conducted under normal atmospheric pressure. The physical and chemical properties of Ag-HA have been reported previously.[1,2] The discs were individually packaged and sterilized using a JS-8500 gamma sterilizer (MDS Nordion, Ontario, Canada).

Bacteria and culture conditions - in vitro experiments. The methicillin-resistant *Staphylococcus aureus* (MRSA) strain used was UOEH6 (University of Occupational and Environmental Health Hospital, Fukuoka, Japan). It was isolated from a blood sample of a septic patient and is a biofilm-producing strain. Bacteria were cultured overnight in tryptic soy broth (Eiken Chemical, Tokyo, Japan) at 37°C and then centrifuged. The pelleted cells were rinsed and resuspended in heat-inactivated 100% fetal bovine serum (Thermo Fisher Scientific, Wilmington, Delaware, USA) to obtain a cell concentration of a mean 43.0 (SD 8.4) x 10^7 colony-forming units (CFU) per millilitre. Immediately after inoculation, serial dilutions of the residual suspension were prepared, plated on agar plates, and incubated for 48 hours at 37°C. The colonies were counted, and CFUs per millilitre were determined.

3D confocal laser scanning microscopy (3D CLSM) protocol - in vitro experiments. MRSA cells were adhered onto the sample discs using the protocol used for the microbiological evaluation. All discs were rinsed twice with 500 µl of sterile phosphate-buffered saline (PBS) to remove nonadherent cells, stained with biofilm stain (FilmTracer calcein red-orange biofilm stain; Thermo Fisher Scientific) for one hour and then washed twice with 500 µl of sterile PBS. The stained biofilms were observed under a CLSM LSM880 microscope with a 20× air objective lens (Carl Zeiss AG, Jena, Germany) after excitation at 543 nm by a helium–neon (He–Ne) laser. An area of 0.208 µm (x-axis) × 0.208 µm (y-axis) was screened at 1 µm intervals along the z-axis (z-stack) in the red emission spectrum (548 nm to 640 nm) at a resolution of 1,024 × 1,024 pixels with a zoom factor of 2.0 and at a scan time of 8.8 seconds. The pinhole was adjusted to 32 µm. The total biofilm volume per area was determined using IMARIS (Carl Zeiss AG).

The morphological features of the biofilms in the Ti PBS, HA PBS, and Ag-HA PBS groups were observed under a CLSM LSM880 Airyscan unit with a 63× oil objective lens after excitation at 543 nm by a He–Ne laser. An area of 0.013 µm (x-axis) × 0.013 µm (y-axis) was screened at 0.3 µm intervals along the z-axis (z-stack) in the red region (emission long pass filter at 555 nm) at a resolution of 2,048 × 2,048 pixels with a zoom factor of 5.0 and at a scan time of 34.4 seconds. The pinhole was adjusted to 144 µm.

Airyscan is a novel 32-channel gallium arsenide phosphide-photomultiplier tube area detector positioned at the pinhole-plane of CLSM.[3] With Airyscan, additional light and spatial information are collected on top of typical CLSM imaging, resulting in substantial and simultaneous improvements in spatial resolution and signal-to-noise ratio.[3] It generates an optimal optical section and allows a structural resolution of 120 nm (in x- and y-dimensions) and 350 nm (in z-dimension) even in thick samples, as well as providing an instant 4× to 8× improvement in signal-to-noise ratio.[3]

Scanning electron microscopy protocol - in vitro experiments. MRSA cells were adhered onto the sample discs using the protocol used for the microbiological evaluation. All discs were rinsed twice with 500 µl of sterile PBS to remove the nonadherent cells and fixed in 2% glutaraldehyde for two hours at room temperature (~27°C). The fixed samples were washed with PBS and dehydrated by gentle washing in an ethanol series (50%, 75%, 90%, 99.5%, and 99.5%) for ten minutes each. The samples were frozen in butanol for ten minutes at 4°C, and the critical point drying was performed. The discs were then mounted on aluminium stubs and coated with gold before imaging. The morphological features of the biofilms of the Ti PBS, HA PBS, and Ag-HA PBS groups, as well as the surface features of each disc, were analyzed using SEM (JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 15 kV and 26 kV.

Microbiological evaluation by bacterial count determination - in vivo experiments. The explanted discs from each rat were agitated with a vortex mixer in 10 ml of sterile PBS, ultrasonically irrigated for five minutes, and then agitated with the vortex mixer again. Serial dilutions (ten-fold series) of the irrigated PBS solutions were plated on agar plates and incubated for 48 hours at 37°C; the colonies were counted, and the CFUs per disc were determined.

References

1. **Noda I, Miyaji F, Ando Y, et al.** Development of novel thermal sprayed antibacterial coating and evaluation of release properties of silver ions. *J Biomed Mater Res B Appl Biomater*. 2009;89(2):456-465.
2. **Shimazaki T, Miyamoto H, Ando Y, et al.** In vivo antibacterial and silver-releasing properties of novel thermal sprayed silver-containing hydroxyapatite coating. *J Biomed Mater Res B Appl Biomater*. 2010;92(2):386-389.
3. **Annette B, Joseph H.** ZEISS LSM 880 with Airyscan – introducing the fast acquisition mode. 2016. https://applications.zeiss.com/C125792900358A3F/0/8316EE885DEBC160C12580760046BE98/$FILE/EN_41_013_125_LSM880_Airyscan_Fast_Acquisition_Mode.pdf (date last accessed 10 March 2020).