Materials Science & Engineering C 102 (2019) 299–304

Contents lists available at ScienceDirect

Materials Science & Engineering C

journal homepage: www.elsevier.com/locate/msec

Directly bonding antimicrobial peptide mimics to steel and the real world applications of these materials

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A B S T R A C T

Despite increased sterilisation and education campaigns, hospital acquired infections have not been eradicated. Bacterial colonisation of frequent touch surfaces is key in the transmission of infection. Most current technologies cannot provide a material which can rapidly kill bacteria. Here we report a novel surface technology, which uses synthetic mimetics of human defensin proteins on a surface. The surface shows excellent antibacterial efficacy against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Both microbiology laboratory tests and trials in hospital settings of this new antimicrobial material (AMS) showed > 99% efficacy over a year *in situ*. It maintains its efficacy through accelerated ageing tests and has shown to kill bacteria far more rapidly (45 min) than the commercially available technologies (24 h).

1. Introduction

Nosocomial infections often occur on environmental surfaces in hospitals and the bacteria can survive for long periods [1]. These bacteria are often resistant to cleaning regimes and are easily transmitted around a hospital setting [2,3]. Studies have shown a link between hospital-acquired infection in patients and bacterial load on surfaces in the ward [4]. Surface coatings using titanium dioxide have also been increasingly reported in the literature, yet these rely on high levels of UV flux to initiate antibacterial activity which is not often available in hospital settings [5]. The most established surface technologies, silver and copper, while highly efficacious with limited human health concerns, have significant financial implications affecting their uptake into the healthcare setting.

Antimicrobial peptides have been applied as antibacterial coatings *in vivo* [6]. These peptides are increasingly a favoured approach as it is difficult for bacteria to develop resistance to their mode of action [7]. Several recent studies demonstrated that antimicrobial peptides can be successfully immobilised onto titanium while maintaining their antimicrobial efficacy [8,9]. However, none of these are translatable to steel surfaces due to the absence of attachment groups and the lack of reactivity of steel surfaces. Antimicrobial peptides can be deposited onto multilayer systems using layer by layer techniques and attaching the peptide to polymers layered onto the surface [10]. As steel is the most ubiquitous metal used in healthcare, setting the creation of antibacterial surfaces for steel is an imperative unmet need in the fight against hospital infection.

In this work, we report a novel system for attaching antibacterial peptides (based on human antimicrobial defensin proteins) to steel surfaces creating durable materials with excellent antimicrobial properties.

2. Results and discussion

Plasma nitriding is a well-established process to increase mechanical properties such as wear resistance; however, its effect on the chemical activity is not well known [11]. Nitriding provides a nitrogen-rich metal surface where the nitrogen is linked to iron atoms in the compound layer. In this work, we have shown that under the correct catalytic conditions the nitrogen of the nitride is also able to interact with the peptide at the surface. X-ray photoelectron spectroscopy (XPS) analysis of the AMS was used to detect elements and chemical bonding

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https://doi.org/10.1016/j.msec.2019.03.064
Received 19 December 2018; Received in revised form 25 February 2019; Accepted 18 March 2019
Available online 19 March 2019
0928-4931/

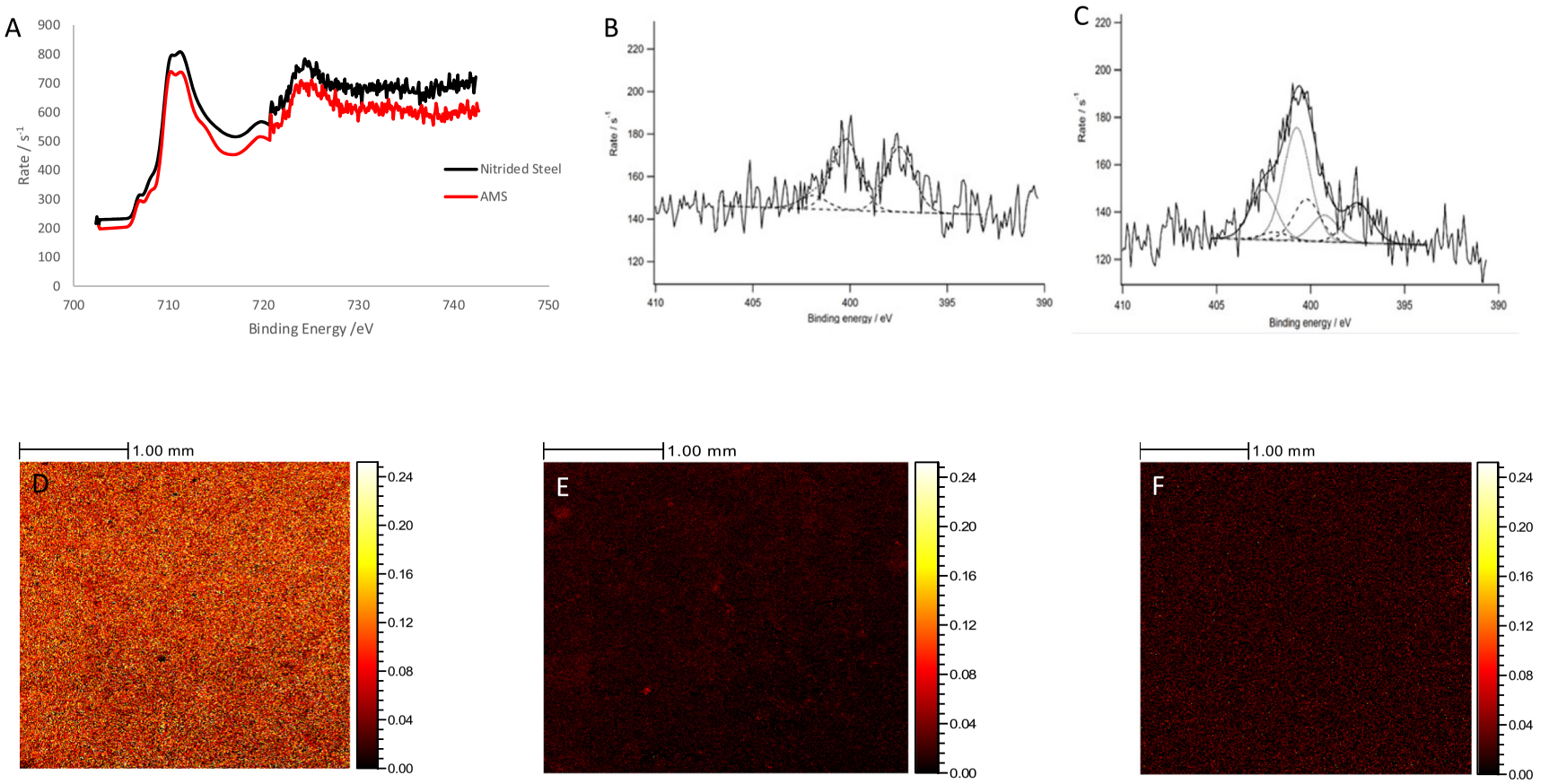

Fig. 1. Analysis of surface: demonstrating bonding of peptide to the surface.
A) XPS scan showing Fe 2p XP spectra for nitride steel (black) and AMS (red) demonstrating the similar shape and intensity noting no chemical difference in the Fe environment. B) XPS scan showing Nitrogen region of the spectra of nitrided steel. C) Nitrogen binding energies and the different chemical environments of the nitrogen after AMS formation. D) ToF SIMS 4 × 4 mm scan of AMS. E) ToF SIMS 4 × 4 mm scan of nitride surface F) ToF SIMS 4 × 4 mm scan of untreated steel surface. The intensity of colour is directly related to the presence of peptide ions CH_4N^+ (glycine), $CH_3N_2^+$ (arginine), $C_2H_6N^+$ (alanine) and $C_5H_{11}N^+$ (leucine) on the surface. All images are composite of multiple amino acid peaks and are representative of all surfaces (n = 4) in the treatment group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

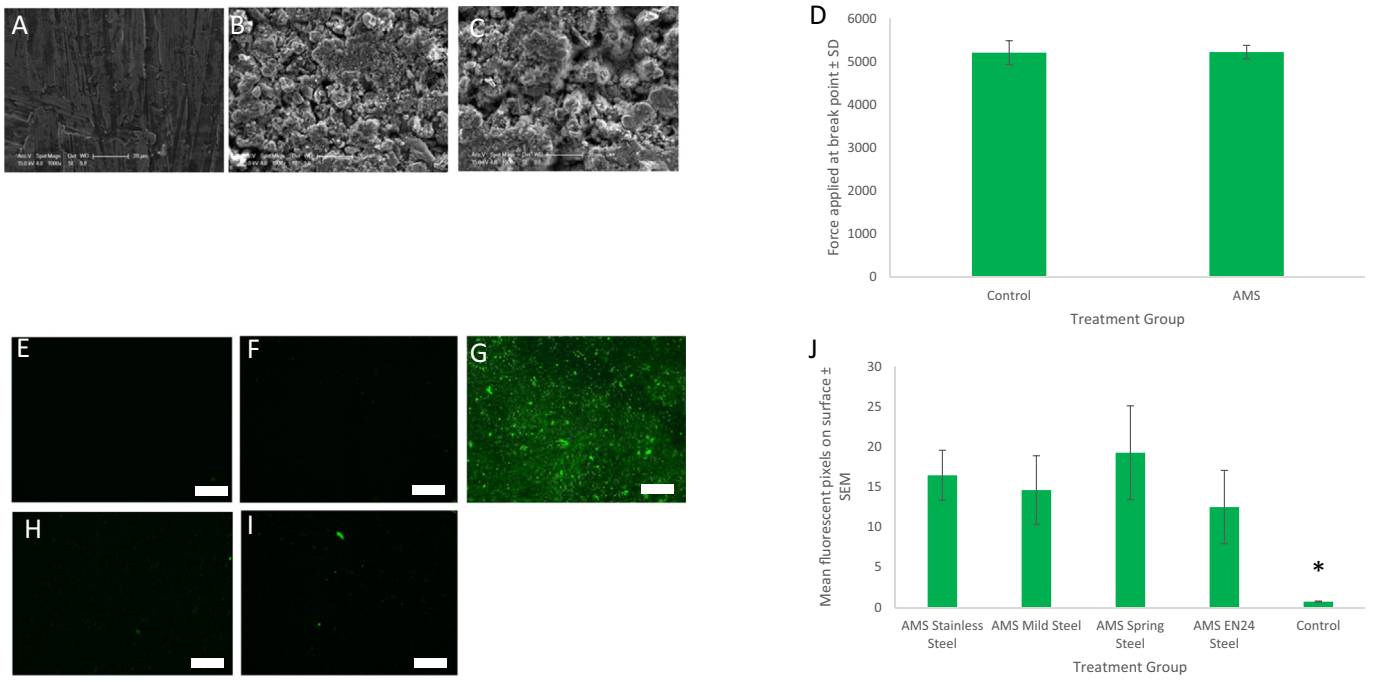

Fig. 2. Surface imaging of peptide-coated structures and their material properties.
A) SEM image of steel, B) SEM image of nitride steel and C) SEM image of AMS. Scale bars are 20 μm all images are representative of all samples in the treatment group. D) Ultimate tensile strength of nitride steel surfaces compared to AMS surfaces. Error bars show standard deviation, $n = 3$, $p > 0.05$. E) Fluorescence microscopy of steel. F) Fluorescence microscopy of nitrided steel, G) Fluorescence microscopy of AMS. H) Fluorescence microscopy of AMS without HBTU/DIEA in the reaction mixture. I) Fluorescence microscopy of AMS (no nitriding). Images are representative of all samples in the treatment group. Scale bar is 100 μm. J) Quantification of fluorescence observed on AMS on different steel substrates. Error bars show standard deviation, n = 4, * denotes statistical significance < 0.05.

in the uppermost 10 nm of the surface over a large analysis area (~1 mm^2). While the binding energy of iron in nitrided steel with and without an amide bond formation with an amino acid does not change (Fig. 1A), the nitrogen spectra of the material clearly does (Fig. 1B–C). While the data envelope for the nitrogen binding energy is complex, the clear shift in binding energy does confirm an interaction between the nitrided surface and the amino acid. Time of Flight Secondary Ion Mass Spectrometry (ToF SIMS) scans of the peptide across the samples show a uniform coating of peptide across the surface with well-defined peaks for glycine, leucine, alanine and multiple peaks for arginine (supplementary information) all of which correspond to the attached peptide sequence (Fig. 1D) compared to controls (Fig. 1E–F).

Examination of the AMS using scanning electron and fluorescent microscopy demonstrates that the nitriding process does alter the material properties of the surface (Fig. 2A, B). Images clearly show the introduction of the compound layer and the associated physical changes of the nitriding process. However, no further structural changes were observed on the addition of the peptide to the nitride in the AMS formation (Fig. 2C), and the ultimate tensile strength of the material before and after the peptide treatment was not significantly different (Fig. 2D).

Fluorescence imaging using the peptide with an inbuilt fluorescent tag showed that the peptide is only present in the AMS formation when the steel has been nitrided, and when the catalyst mixture and the peptide are all present (Fig. 2E–I). If any of the components (i) nitriding, ii) catalyst (HBTU/DIEA) or iii) peptide) is missing, no fluorescence is seen. This shows that the nitride is essential for attachment, which matches the data in the XPS showing that the nitrogen peak shifts on bonding. The fluorescence images also show a uniform covering of the AMS. This corroborates the ToF SIMS images which also demonstrated a uniform coating of the AMS and matches the literature on surface coatings for implantable materials [12]. Quantification of the fluorescent peptide on a range of steel varieties demonstrates that the type of steel substrate used is not important as long as the plasma nitriding process can be successfully applied (Fig. 2J). This also supports the fluorescence and XPS data demonstrating that the nitride is essential for the peptide to be applied to the surface.

To simulate the bacterial contamination with which environmental surfaces are challenged in a clinical setting, high concentrations of bacteria in small volumes were added to the surfaces. The bacteria levels on the materials were monitored over short periods of time; this demonstrated that the AMS could achieve a 3 fold log reduction in bacteria number in < 45 min and that this was observed for both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*) (Fig. 3A). This is comparable to the activity of human defensin proteins which show efficacy against both Gram positive and Gram negative bacteria [12]. Further studies demonstrated that the AMS could achieve a greater than threefold log reduction in bacteria number in a range of bacteria isolated from patient infections including methicillin-resistant *S. aureus*, *S. epidermidis*, *Enterococcus* spp., *E. coli* and *P. aeruginosa* in under 45 min (Fig. 3B). To determine the resilience of the AMS the materials were subjected to repeat inoculations using methicillin resistant-*S. aureus*. The materials were inoculated and incubated for 45 min before the bacteria were harvested from the surfaces (Fig. 3C). The materials were then reinoculated twice more to determine the ability of the AMS to keep preventing bacterial colonisation of the steel. At each inoculation, the AMS significantly lowered the bacteria number compared to the steel. This is in sharp contrast to the current cleaning regimes used in hospitals where repeat cleaning is required or bacteria colonise surfaces and a direct increase in patient infections is observed [4]. The bacterial reduction was also observed for the AMS with other antibacterial agents attached (Supplementary information Figs. 1–3). This demonstrates the potential for this material to be a platform technology, which can have a range of antimicrobial actives bonded to the material for use in different environments.

SEM imaging of the stainless steel surfaces following *S. aureus* inoculation demonstrate the affect bacteria have on the surface. Surfaces which did not receive the AMS showed high levels of corrosion at the

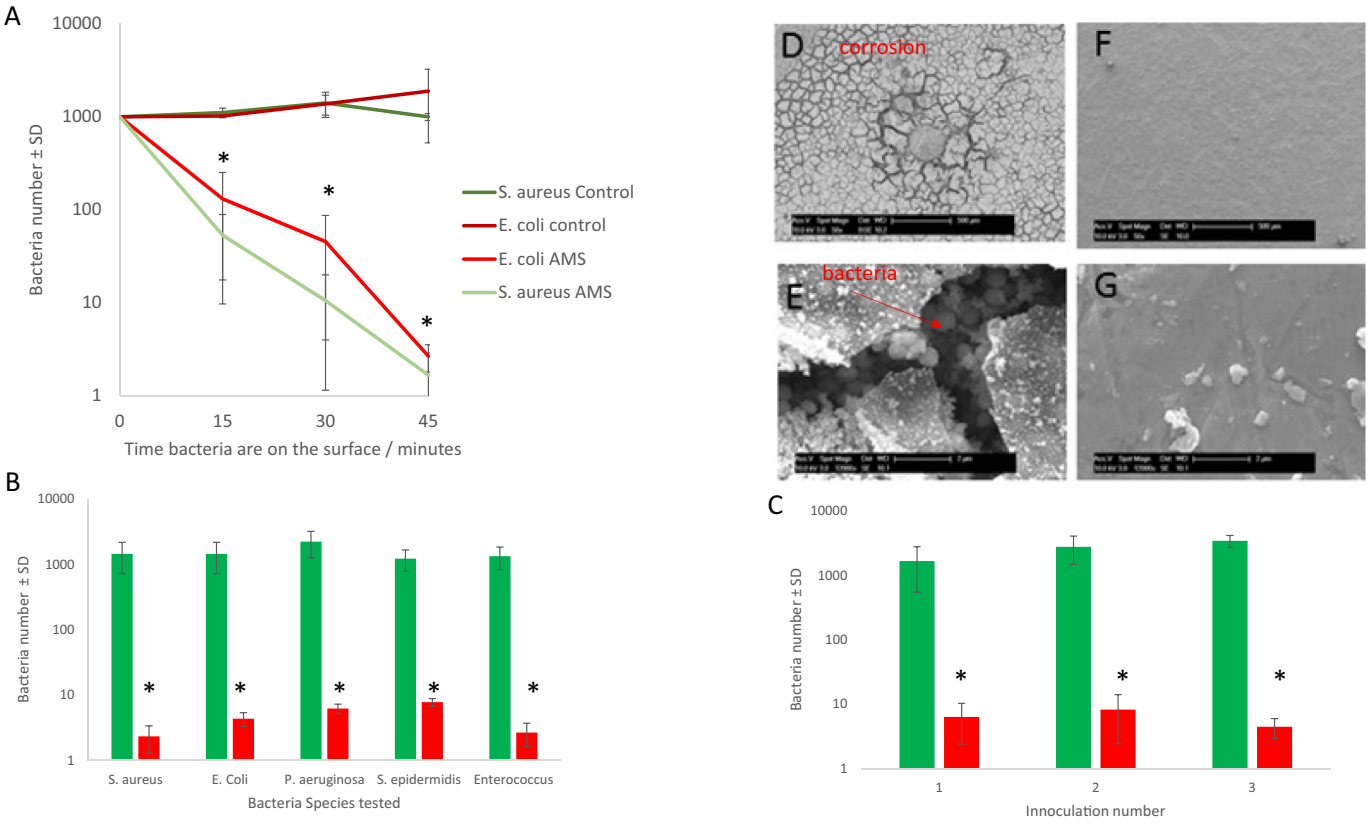

Fig. 3. Antibacterial efficacy of surfaces.
A) Antibacterial efficacy of AMS, compared to steel against *S. aureus* and *E. coli* over 45 min. B) Antibacterial efficacy of AMS (red), compared to steel (green) against *S. aureus*, *E. coli*, *P. aeruginosa*, *S. epidermidis*, *Enterococcus* spp. following 45 min incubation on the surface. C) Antibacterial efficacy of AMS (red), compared to steel surfaces (green) against *S. aureus* with repeat inoculations of bacteria onto the surface. In all graphs * denotes statistical significance $p < 0.05$, $n = 3$. D) Low magnification SEM image of steel inoculated with *S. aureus* cells after 16 h incubation, showing the corrosion caused by the presence of the bacteria suspension on the surface, scale bar is 500 µm. E) High magnification SEM image of steel inoculated with *S. aureus* cells after 16 h incubation, showing the bacteria present in the corroded surface, scale bar is 2 µm. F) Low magnification SEM image of AMS inoculated with *S. aureus* cells after 16 h incubation showing no corrosion on the coated samples, scale bar is 500 µm. G) High magnification SEM image of AMS inoculated with *S. aureus* cells after 16 h incubation showing no bacteria present on the surface, scale bar is 2 µm. All images are representative of all samples in the group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

point of application (Fig. 3D). High magnification images of the steel show large numbers of bacteria in the corroded surface (Fig. 3E). The AMS samples had no corrosion or damage (Fig. 3F) and bacteria were not visible on the surface (Fig. 3G), demonstrating that the AMS not only has inhibited bacterial growth but has also protected the surface from the corrosive properties bacteria colonisation can have on surfaces [13]. To determine the stability of the interaction between the peptide and the surface established methods were used to attempt to remove any physically adsorbed peptide [14]. The presence of the peptide was confirmed using ToF SIMS (Supplementary information) and antibacterial efficacy (Fig. 4A). The washing protocols did not have any effect on the efficacy of the surfaces demonstrating the peptide was still present following the test. This suggests a very strong interaction between the peptide and the steel surfaces. Following these positive tests, the AMS system was installed in a clinical environment for an extended period of time to determine their efficacy *in situ*. The AMS were applied to a variety of surfaces on board a Royal Fleet Auxiliary (RFA) ship, within the medical department. The ship was deployed for 11 months. The frequent touch surfaces included toilets, keys, and handles. Bacteria numbers on the AMS and steel surfaces were monitored throughout the deployment. Both the AMS and steel controls were subject to standard cleaning regimes. The AMS inhibited > 95% of bacteria growth compared to control surfaces (Fig. 4B). Literature shows that repeated cleaning regimes, including enhanced cleaning regimes could not prevent recolonisation of surfaces with MRSA unlike the efficacy reported here [15]. It has also been demonstrated in intensive care units that the levels of *Acinetobacter baumannii* found on environmental surfaces and the cleanliness of these surfaces were linked to the increase of patient infections with *Acinetobacter baumannii* [16]. The trial on board the RFA ship demonstrates the suitability of the AMS to prevent infection in a clinical environment long term. To determine the stability of the AMS to frequent touch surfaces the surfaces were subjected to durability testing where the surfaces were continually abraded to mimic the repeated touch on surfaces over 10 years. After this accelerated process, the surfaces still maintained both their excellent efficacy and kill rate (Fig. 4C). The AMS was also compared to commercially available technologies which use the current market-leading technologies, silver and copper (Fig. 4D). The AMS demonstrated a much faster kill rate than both silver and copper as it has a much quicker efficacy time (45 min). This matches literature data on human defensin efficacy (1 h) [13]. The technology has a much faster efficacy than silver which is reported in the literature to be between 8 and 24 h [17,18]. The technology displays a much higher efficacy than trials of copper materials used by hospital staff in a hospital setting [19,20]. The technology reported also shows better efficacy than silver impregnated technologies [21].

3. Conclusions

We have demonstrated a method for applying synthetic peptide

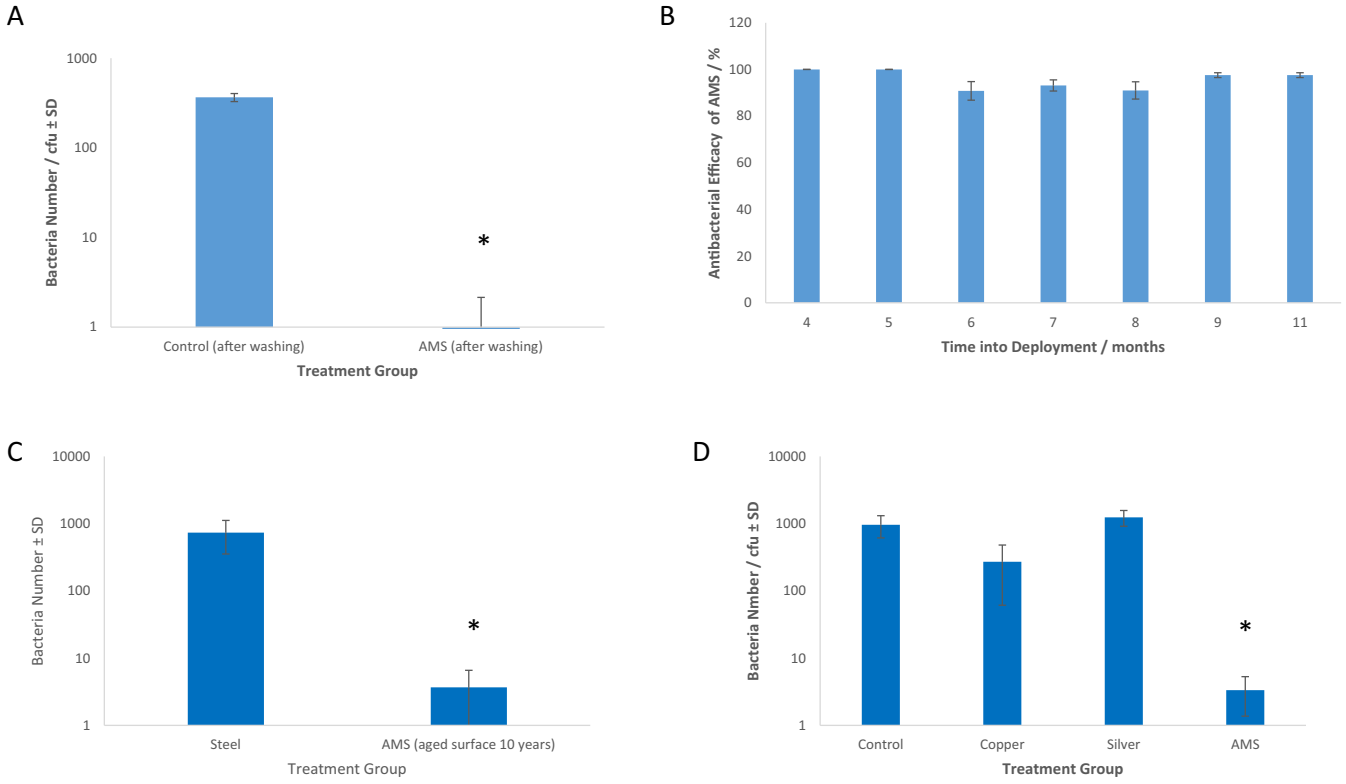

Fig. 4. Efficacy tests in a clinical environment, stability and comparison to commercially available technologies.
A) Antibacterial efficacy following literature washing protocols to remove adsorbed material from the surface, * denotes statistical significance $p < 0.05$, n = 3. B) Antibacterial efficacy of AMS calculated as a percentage of the bacteria growing on control surfaces matched by location, usage and environment over 11 months on board a Royal Fleet Auxiliary Ship. C) Antibacterial efficacy of AMS after ageing of the surface, * denotes statistical significance $p < 0.05$, $n = 6$. D) Comparison of AMS with commercially available silver and copper antibacterial technologies, * denotes statistical significance $p < 0.05$, n = 6.

mimics to steel using a simple 2-step process. We have demonstrated the uniformity and efficacy of the AMS material and shown that it is active against five different bacteria, which are responsible for hospital-acquired infections. The AMS technology has shown excellent efficacy in a clinical trial and showed much greater efficacy than the current commercially available technologies. We believe it is widely applicable to prevent the spread of infection in a healthcare environment.

4. Experimental

Unless otherwise stated, all materials were sourced from Sigma-Aldrich, UK. Bacteria were collected and identified by the Queen Elizabeth Hospital and were kindly gifted to this project by the Infection control team at QEHB.

4.1. AMS formation

Steel surfaces were obtained and nitrided commercially by Metaltech Ltd. (Consett, UK). The surfaces were polished and cleaned using acetone. The surfaces were incubated in *N*,*N*-dimethylformamide (DMF) (1 mL), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Scientific Laboratory Supplies, Hessle, UK) in DMF (0.052 M), N,N-diisopropylethylamine (DIEA) (0.1 mL) and antimicrobial peptide RRRRRRGALAGRRRRRRGALAG/5(6)-RRRRRR-GALAGRRRRRRGALAG (0.38 M) (GenScript, Piscataway, USA) per square centimetre of surface with continuous agitation for 16 h. The surfaces were washed three times with DMF and three times with acetone and air dried. Control samples were cleaned with acetone and DMF and air dried.

4.2. XPS analysis

To simplify the spectra, AMS were prepared with a single amino acid (glycine 0.38 M) instead of the full peptide sequence to examine the surface binding. Samples were analysed using the Kratos AXIS ULTRA with a monochromated Al kα X-ray source (1486.6 eV) operated at 10 mA emission current and 12 kV anode potential (120 W). Casaxps (version 2.3.18dev1.0x) software was used for quantification and spectral modelling.

4.3. ToF-SIMS

A ToF-SIMS IV instrument (IONTOF GmbH) was used with a 25 keV Bi3 + primary ion source and 10 keV acceleration voltage to analyse the AMS samples. Data were acquired over a 4 × 4 mm area at a resolution of 100 pixels per mm. Data acquisition and retrospective data analysis was performed using SurfaceLab6 (IONTOF GmbH).

4.4. Scanning electron microscopy

Samples were studied on a Philips XL30 FEG scanning electron microscope. Imaging was carried out in triplicate, and the images shown are representative of each surface. For bacterial SEM, surfaces were inoculated with 1000 cells of *S. aureus* in LB broth. The surfaces were incubated at 37 °C for 16 h. The surfaces were fixed using a 3% solution of glutaraldehyde in 0.1 M phosphate buffer for 4 h and then washed with deionised water. Images were taken on a Philips XL30 FEG scanning electron microscope. Imaging was carried out in triplicate, and the images shown are representative of each surface.

4.5. Fluorescence microscopy

AMS formation was carried out with using a range of methods to isolate the key factors for attachment. AMS were prepared: 1) With only the nitriding process and no further attachment, 2) nitriding and attachment without the HBTU/DIEA catalyst mixture, 3) Full AMS formation using steel without nitriding, 4) Full AMS formation and 5) control steel. Surfaces were viewed with an Axioplan-2 fluorescence microscope and images of their surfaces obtained with AXIOVISION software (both Carl Zeiss Ltd., Cambridge, UK) to detect residual fluorescent peptide. Peptide on the surface was quantified using established methods [22].

4.6. Ultimate tensile strength

AMS and steel surfaces were cut into a 10 cm long strip with 3 cm wide shoulder and 1 cm wide neck. The tensile strength was monitored using a twin screw driven with auto ranging ± 200 kN load Universal Test Machine (Model 1484, Zwick). The analysis was carried out on Zwick TestXpert2 software.

4.7. Antibacterial efficacy

AMS and steel substrates were placed in 12 well tissue culture plates (Corning, UK). Methicillin-resistant-*S. aureus* (MRSA) and *E. coli* were isolated from infected patient wounds from the Queen Elizabeth Hospital, Birmingham. The bacteria were diluted in LB broth, and 1000 cells in 1 μL drops were applied 9 times to each surface. At 15, 30 and 45 min surfaces were removed from the experiment and suspended in Dey Engels neutralising solution (10 mL) and vortexed with zirconium oxide beads (VWR International, Lutterworth, UK). They were plated onto LB agar plates and incubated for 16 h and the colonies counted. Following this *S. aureus*, *S. epidermidis*, *Enterococcus* spp., *P. aeruginosa* and *E. coli* all isolated from patient infections in the Queen Elizabeth Hospital, Birmingham were tested against the materials as described above.

4.8. Testing durability of surfaces

Following a protocol from Naderi et al. surfaces were washed to remove any adsorbed material [14]. Surfaces were made as previously described, they were then washed with PBS five times and water three times. The surfaces were placed in a pre-warmed 0.2 M SDS solution and held at 70C for 15 min. The surfaces were then washed a further five times with PBS and three times with water before undergoing ToF SIMS analysis and antibacterial efficacy testing as described above.

4.9. Clinical trial on board RFA ship

Target areas on board the RFA ship were selected. These included 1) the key in the operating theatre drugs cabinet, 2) the dispensary drawer in casualty emergency receiving area, 3) the door handle in the casualty emergency receiving area, 3) an equipment drawer in operating theatre 4) a toilet plate in the communal facilities 5) a drawer in the ward area. Surfaces treated with the peptide coating were installed before the ship's deployment. Control surfaces were identified which were also made of steel but did not have the peptide treatment. Both peptide treated and control surfaces were subjected to standard daily cleaning regimes during times of active deployment. Both treated and control surfaces were swabbed on a weekly basis, and the number of colonies on each plate was counted.

4.10. Comparison of AMS with commercially available antibacterial products

AMS samples were prepared as described above. Strips of antibacterial silver and copper were cut into 1 × 1 cm^2 and the efficacy tested as described above.

4.11. Surface stability

AMS samples were prepared as previously described. Both AMS and control steel samples were loaded into separate chambers with zirconium oxide beads. The chambers were subjected to agitation to ensure the beads abraded the surface twice a second continuously for 72 h. The surfaces were then tested for antibacterial efficacy as described above.

Acknowledgments

This research has been funded by Royal Academy of Engineering through an Enterprise Fellowship and the University of Birmingham through a Birmingham Fellowship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2019.03.064.

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