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New antimicrobial contact catalyst killing antibiotic resistant clinical and waterborne pathogens

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ABSTRACT

Microbial growth on medical and technical devices is a big health issue, particularly when microorganisms aggregate to form biofilms. Moreover, the occurrence of antibiotic-resistant bacteria in the clinical environment is dramatically growing, making treatment of bacterial infections very challenging. In search of an alternative, we studied a novel antimicrobial surface coating based on micro galvanic elements formed by silver and ruthenium with surface catalytic properties.

The antimicrobial coating efficiently inhibited the growth of the nosocomial pathogens *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Enterococcus faecium* as demonstrated by the growth inhibition on agar surface and in biofilms of antibiotic resistant clinical *E. faecalis*, *E. faecium*, and *S. aureus* isolates. It also strongly reduced the growth of *Legionella* in a drinking water pipeline and of *Escherichia coli* in urine. We postulate a mode of action of the antimicrobial material, which is independent of the release of silver ions. Thus, the novel antimicrobial coating could represent an alternative to combat microbial growth avoiding the toxic side effects of high levels of silver ions on eukaryotic cells.

1. Introduction

Antimicrobial resistance threatens the effective prevention and treatment of an ever-increasing range of infections caused by microorganisms. Very high rates of resistance have been observed in bacteria that cause common health-care associated and community-acquired infections (e.g., urinary tract infections, pneumonia) in all WHO regions [1]. The situation is aggravated by the shrinking of the antibiotic development pipeline [2]. Thus, there is an urgent need for alternative strategies to combat microbial infections; one of those is anti-microbial silver (Ag) which is increasingly used in the clinic and in general healthcare [3,4]. Antimicrobial silver technologies are based on the release of silver ions, which are the active components in disinfection with silver. The antimicrobial efficacy is dependent on the availability of a sufficient amount of silver ions, thus the higher the microbial load the higher the silver ion concentration has to be. To obtain sufficiently high silver ion concentrations modern silver technologies make use e.g., of nano-silver. According to the high surface area of nano-silver particles higher silver ion concentrations are generated by dissolving the thin silver oxide layer on top of the silver particle surface. However, in medical or technical applications sulfur containing components (e.g., proteins) cause strong reduction of free silver ion concentrations due to the formation of hardly soluble silver sulfide (Ag_2S) [5–8]. Besides silver this drawback also holds true for the other so called oligodynamic metals (e.g., copper).

In this study we compared the antimicrobial activity of AgXX® consisting in micro galvanic elements formed by silver and ruthenium with an electroplated silver coating applied onto a V2A stainless steel surface. Both materials were tested for bacterial growth inhibition on agar surfaces and in batch cultures. Strong growth inhibition of Gram-negative as well as Gram-positive pathogens was demonstrated for AgXX® coatings. Bacterial growth curves in the presence of AgXX® revealed a significant reduction amounting to a decrease of 10^3 CFU ml^{-1}. Moreover, AgXX® efficiently reduced the growth of the waterborne

Abbreviations: MH, Mueller–Hinton; TSB, Tryptic Soy Broth.

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pathogen *Legionella* in a simulated drinking water pipe. A mechanism of action independent of the release of silver ions is postulated for AgXX®.

2. Material and methods

2.1. Preparation and characteristics of the antimicrobial materials

Stainless steel gauze (V2A: DIN ISO 1.4301), 50 μm mesh, was used as base material for Ag and AgXX® coatings and as reference material. The wire diameter was 30 μm. Three types of samples were used in the tests: i) Uncoated stainless steel meshes, ii) Ag and iii) AgXX® plated stainless steel meshes with the same silver coating thickness. The Ag coating on the steel meshes was electroplated with a thickness of 3–5 μm from a commercially available cyanidic silver electrolyte. AgXX® coatings were also applied by electroplating processes, using commercial electrolytes for metal plating of silver and ruthenium. The silver coating thickness on the steel meshes was also 3–5 μm. The ruthenium was plated as a microporous coating onto the silver sublayer (<1 μm), thus free silver areas were accessible to the aqueous environment. As a result, the plated AgXX® coating was structured in a way that many micro galvanic cells were formed on the surface layer. Electrolytes and plating conditions have been modified to obtain the necessary composition and structure of the material: The samples were electroplated continuously in a reel-to-reel plating line. AgXX® plating on stainless steel meshes was performed in a continuous sequence of process steps: chemical and electrochemical cleaning of the stainless steel mesh surfaces, chemical and electrochemical activation of the stainless steel meshes, and silver and ruthenium plating. Between each process step adequate cleaning was performed by deionized water rinses. In a final process step the composite coating was conditioned by a vitamin derivative, then rinsed in deionized water and dried with a hot air-blower.

An EDX image of the AgXX® surface is shown in Fig. 1. It demonstrates the homogenous distribution of the silver (green) and ruthenium particles (red) on the surface.

Before use in the experiments the antimicrobial activity of AgXX® meshes was routinely checked by incubation with *Escherichia coli* DSM 498 at 37 °C for 18 h. Cytotoxicity of AgXX® was analyzed by neutral red assay using the human MRC-5 cell line (human lung fibroblast, ATCC® CCL-171) as described in [9–11]. AgXX® exhibited only slight

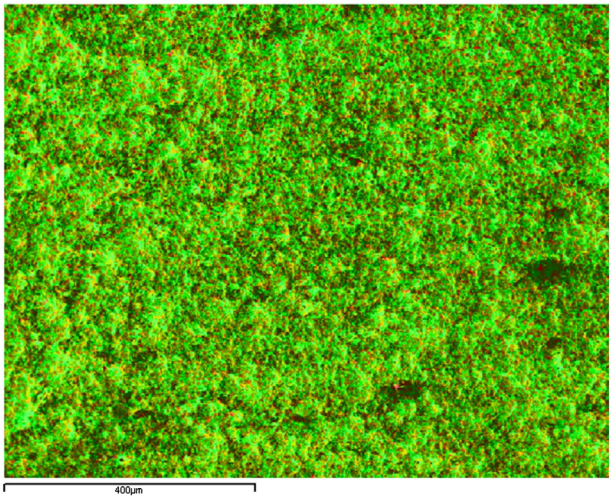

Fig. 1. EDX image of the AgXX® surface: The homogeneous distribution of silver (green) and ruthenium (red) on the surface is shown. The image was generated with a Philips XL 40 SEM with an Oxford EDX microanalysis probe at 125× magnification; working distance from cathode: 10.2 mm. The image was provided by D. Biermann, H. Abrahams (ISF, Dortmund, Germany) and T. Lisowsky (MultiBind biotec GmbH, Cologne, Germany). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Bacterial strains.

Strain	Genotype or relevant features[a]	Reference or source
E. coli		
RR1	HB101 *recA*	[18]
IMG 1711	DSM 498, K12 wild type	DSMZ[b], Braunschweig, Germany
E. faecalis		
T9	Clinical isolate, biofilm former, Cs^R, Sxt^R, Nal^R, Tri^R, Kan^R, Sm^R, Van^R, Tet^R, Rif^R, Fus^R	[19]
E. faecium		
E1162	Clinical isolate, biofilm former, Amp^R, Tet^R	[20]
L. erythra		
SE-32A-C8	DSM 17644, type strain	DSMZ, Braunschweig, Germany
S. aureus		
337423-1	Amc^R, Amx^R, Cfz^R, $Clox^R$, Lvx^R	Catheter
338550-1	Amc^R, Amx^R, Cfz^R, $Clox^R$, Erm^R, Lvx^R	Catheter
339031-2	Amc^R, Amx^R, Cfz^R, $Clox^R$, Erm^R, Lvx^R	Catheter
339056-2	Amc^R, Amx^R, Cfz^R, $Clox^R$, Erm^R, Lvx^R	Catheter
339300	Amc^R, Amx^R, Cfz^R, $Clox^R$, Lvx^R	Catheter
312042	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Erm^R, Lvx^R	Medical implant
410099	Amc^R, Amx^R, Cfz^R, $Clox^R$, Gen^R, Lvx^R, Mup^R	Medical implant
218154	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Erm^R, Lvx^R, Mup^R, Rif^R	Medical implant
339031	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Erm^R, Lvx^R	Catheter
215642	Amx^R	Medical implant
338503	Amx^R	Catheter
214967	Amx^R, Mup^R	Ulcer
S. capitis		
316479	Amc^R, Amx^R, Cfz^R, $Clox^R$, Gen^R, Lvx^R, Mup^R	Medical implant
S. epidermidis		
No. 52	CONCORDIA isolate, biofilm former, Van^R	[17]
No. 58	ISS isolate, biofilm former	[17]
RP62-A	Met^R, biofilm former	ATCC[c] 35984
338684	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Ctx^R, Gen^R, Lvx^R, Mup^R, Rif^R	Catheter
216663	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Erm^R, Gen^R, Rif^R	Medical implant
239879	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Erm^R, Gen^R, Mup^R, Tet^R	Catheter
239891	Amc^R, Amx^R, Cfz^R, $Clox^R$, Gen^R, Lvx^R, Mup^R, Rif^R	Catheter
213303	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Ctx^R, Erm^R, Gen^R, Lvx^R, Mup^R, Rif^R	Medical implant
214627-A	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Ctx^R, Erm^R, Gen^R, Lvx^R, Mup^R, Rif^R	Medical implant
310301-1	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Ctx^R, Erm^R, Gen^R, Lvx^R, Mup^R, Rif^R	Medical implant
338400-1	Amc^R, Amx^R, Cfz^R, $Clox^R$, Mup^R	Catheter
338515-1	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Ctx^R, Erm^R, Gen^R, Mup^R, Rif^R	Catheter
319622	Amx^R, Erm^R, Tet^R	Medical implant
219691	Amx^R, Erm^R, Lvx^R, Mup^R, Rif^R	Medical implant
S. hominis		
313732	Amc^R, Amx^R, Cfz^R, Cli^R, $Clox^R$, Ctx^R, Erm^R, Gen^R, Lvx^R, Mup^R	Medical implant

[a] Amp: ampicillin; Amc: amoxicillin + clavulanic acid; Amx: amoxicillin; Cfz: cefazoline; Cli: clindamycin; Clox: cloxacillin; CS: chitosan; Erm: erythromycin; Fus: fusidic acid; Gen: gentamicin; Kan: kanamycin; Lvx: levofloxacin; Met: methicillin; Mup: mupirocin; Nal: Nalidixic acid; Rif: rifampicin; Sm: streptomycin; Sxt: trimethoprim/sulfamethoxazole; Tet: tetracycline; Tri: trimethoprim; Van: Vancomycin.
[b] German collection of microorganisms and cell cultures.
[c] American type culture collection.

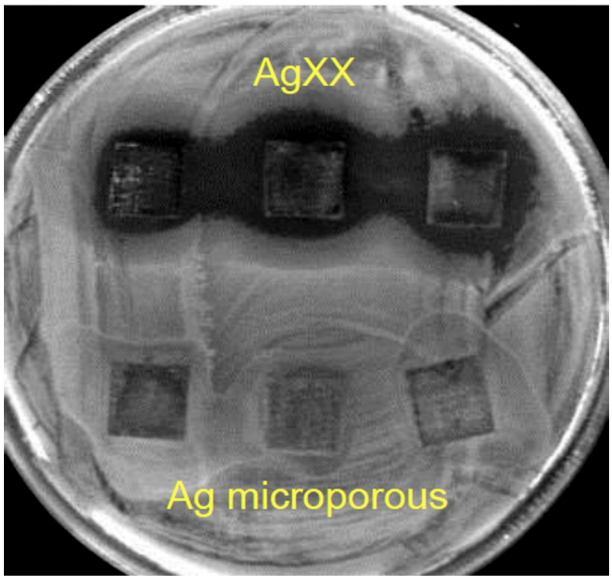

Fig. 2. AgXX®-mediated antimicrobial activity on *E. coli* RR1 on urine agar as compared to silver after incubation for 48 h at 37 °C (permission for republishing from [25] was granted by Leuze Publishing). The image was provided by T. Lisowsky, MultiBind biotec GmbH, Cologne, Germany.

cytotoxicity [12]. The assays were performed with AgXX® coated glass beads; the uncoated glass beads did not show any bactericidal or cytotoxic effect.

2.2. Cultivation of bacteria

All bacterial strains used in this work are listed in Table 1. Routinely, all *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis* and *Staphylococcus capitis* strains were grown in Tryptic Soy Broth (TSB) medium at 37 °C overnight. *E. faecium* E1162 [13], *E. faecalis* T9 [14], *E. faecalis* 12030 [15], *S. aureus* ATCC 29213 and *S. epidermidis* RP62-A [16] were from clinical origin, two isolates from extreme confined habitats, *S. epidermidis* No. 52 from the Antarctic research station CONCORDIA [17], *S. epidermidis* No. 58 from the International Space Station (ISS) [17] and 25 different *Staphylococcus* isolates were from patients of Hospital Universitario Donostia-IIS Biodonostia, San Sebastián, Spain. *Legionella erythra* DSM 17644 (DSMZ, Braunschweig, Germany) was cultivated on *Legionella* CYE agar base (Oxoid, Madrid, Spain) for 2 days at 37 °C, *E. coli* RR1 and *E. coli* DSM IMG 1711 were grown at 37 °C in LB medium overnight.

2.3. Growth inhibition on agar surface

2.3.1. Growth inhibition on Mueller–Hinton agar

Growth inhibition tests on agar surfaces were performed following CLSI (the Clinical and Laboratory Standards Institute) guidelines for disk diffusion susceptibility testing [21]. Bacteria were grown overnight in Mueller–Hinton (MH) medium or TSB at 37 °C. Turbidity of overnight cultures was measured in a Microscan turbidity meter (Dade Behring, West Sacramento, CA, USA) at 620 nm and adjusted to 1–2 × 10^8 CFU ml^{-1} by addition of MH medium. A sterile cotton swab was dipped into the culture and was streaked over the entire MH agar surface. This procedure was repeated twice, rotating the plate approximately 60° each time to ensure an even distribution of the culture. As a final step, the rim of the agar was swabbed. When the plates were dry, AgXX® (50 μm mesh), Ag (50 μm mesh) and steel reference mesh (50 μm mesh) (0.25 cm^2 each) were placed on the agar surface. The meshes were wetted with 10 μl sterile distilled water to establish good contact between the mesh and the agar surface. The cultures were incubated at 37 °C, inhibition zones around the meshes were monitored for 24 h, and the diameter of the inhibition zones was determined. The tests were performed in triplicate; mean values and standard deviation are given.

2.3.2. Growth inhibition on urine agar

25 μl of an overnight culture of *E. coli* RR1 (10^8 CFU ml^{-1}) were plated onto LB agar where water was replaced by human urine which was filtered through a 0.45 μm filter before use (= urine agar). 12 cm^2 AgXX® meshes and 12 cm^2 Ag meshes were placed on the agar. The meshes were wetted with 10 μl filtered (0.45 μm filter) human urine to establish good contact between the mesh and the agar surface and incubated at 37 °C for 48 h. Inhibition of bacterial growth was examined visually.

2.4. Growth inhibition in batch culture

Bacteria were grown overnight in MH medium at 37 °C. They were diluted in MH medium to $OD_{600} = 0.1$ and incubated for further 24 h at 37 °C in the presence of AgXX®, Ag, and V2A steel meshes. 8 cm^2 of each of the meshes were applied to 25 ml MH medium. OD_{600} of the cultures was monitored for 24 h with hourly measurements in the exponential phase; OD_{600} was plotted against the time. Additionally, at time points 3 h, 6 h, and 24 h representing early exponential, late exponential and stationary growth phase, respectively, CFU ml^{-1} of the cultures were determined. The experiments were performed in triplicate. Mean values and standard deviation are given.

2.5. Inhibition of adherence to surfaces

As proof of principle, the clinical staphylococcal isolate, *S. aureus* 215642, a strong biofilm former (S. Aguila-Arcos and I. Alkorta, personal communication), was selected. 96-well flat-bottom polystyrene plates containing 200 μl TSB medium per well amended with AgXX®, Ag,

Table 2
Inhibition zones (in cm) caused by AgXX® and Ag.[a]

Strain	AgXX®	Ag	Steel
E. faecalis 12030	0.74 ± 0.02	0.00	0.00
E. faecalis T9	0.58 ± 0.00	0.00	0.00
E. faecium 1162	0.75 ± 0.06	0.00	0.00
S. aureus 214967	0.75 ± 0.07	0.00	0.00
S. aureus 215642	0.80 ± 0.00	0.00	0.00
S. aureus 218154	0.65 ± 0.07	0.00	0.00
S. aureus 312042	1.10 ± 0.00	0.00	0.00
S. aureus 337423-1	0.70 ± 0.14	0.00	0.00
S. aureus 338503	0.65 ± 0.07	0.00	0.00
S. aureus 338550-1	0.85 ± 0.07	0.00	0.00
S. aureus 339031	0.80 ± 0.00	0.00	0.00
S. aureus 339031-2	0.80 ± 0.00	0.00	0.00
S. aureus 339056-2	0.85 ± 0.00	0.00	0.00
S. aureus 339300	0.85 ± 0.07	0.00	0.00
S. aureus 410099	0.90 ± 0.00	0.00	0.00
S. aureus ATCC 29213	0.87 ± 0.15	0.00	0.00
S. capitis 316479	0.90 ± 0.14	0.00	0.00
S. epidermidis 213303	1.05 ± 0.07	0.00	0.00
S. epidermidis 214627-A	1.20 ± 0.00	0.00	0.00
S. epidermidis 216663	1.15 ± 0.21	0.00	0.00
S. epidermidis 219691	1.00 ± 0.00	0.00	0.00
S. epidermidis 239879	1.10 ± 0.00	0.00	0.00
S. epidermidis 239891	1.20 ± 0.00	0.00	0.00
S. epidermidis 310301-1	1.00 ± 0.14	0.00	0.00
S. epidermidis 319622	1.10 ± 0.14	0.00	0.00
S. epidermidis 338400-1	1.00 ± 0.00	0.00	0.00
S. epidermidis 338515-1	0.85 ± 0.21	0.00	0.00
S. epidermidis 338684	1.00 ± 0.00	0.00	0.00
S. epidermidis No. 52	0.87 ± 0.05	0.53 ± 0.02	0.00
S. epidermidis No. 58	0.80 ± 0.10	0.55 ± 0.05	0.00
S. epidermidis RP62A	1.08 ± 0.25	0.60 ± 0.05	0.00
S. hominis 313732	0.70 ± 0.00	0.00	0.00

[a] Data represent mean values ± SD (n = 3).

and steel mesh (5 mm diameter each), respectively, were inoculated with 10 μl of a *S. aureus* 215642 overnight culture and grown at 37 °C for 24 h. The meshes were rinsed twice with distilled water to remove bacteria not attached to the surface, transferred to a new plate, fixed and processed as described in the Scanning electron microscopy (SEM) section.

For *L. erythra*, growth in the presence of AgXX® and steel meshes (10 cm^2 each) was investigated after incubation with agitation (100 rev min^{-1}) in BYE medium (10 g l^{-1} ACES buffer (Sigma-Aldrich), 10 g l^{-1} yeast extract, 2.4 g l^{-1} KOH, 0.4 g l^{-1} L-cysteine, and 0.25 g l^{-1} ferric pyrophosphate, pH adjusted to 6.85–6.95) at 37 °C. On days 0, 3, 11, 24, 30, 38, and 47, one culture each with AgXX® and steel mesh was analyzed for surface growth of *L. erythra*. Cells adhering to the meshes were detached with PBS (phosphate buffered saline) by vigorous shaking, the cell suspensions were serially diluted and applied to Hoechst 33258 (Molecular Probes Life Technologies) staining. Briefly, cells were incubated on poly-L-lysine coated slides at room temperature for 15 min, washed three times with PBS and stained with Hoechst 33258 (2 μg ml^{-1}) for 10 min. The cells were enumerated and images were taken using a Nikon Eclipse 90I microscope equipped with a camera with Software NIS Elements 3.0.

2.6. Scanning electron microscopy (SEM)

The localization of silver and ruthenium on AgXX® surfaces was performed in a Philips XL40 SEM using an EDX system (Oxford Instruments) at 10.2 mm working distance.

For the analysis of bacteria adhering to AgXX®, Ag and steel meshes, the following procedure was performed [22]. Samples were fixed in 2% glutaraldehyde in 0.1 mol l^{-1} Sörenson phosphate buffer (pH = 7.2) for

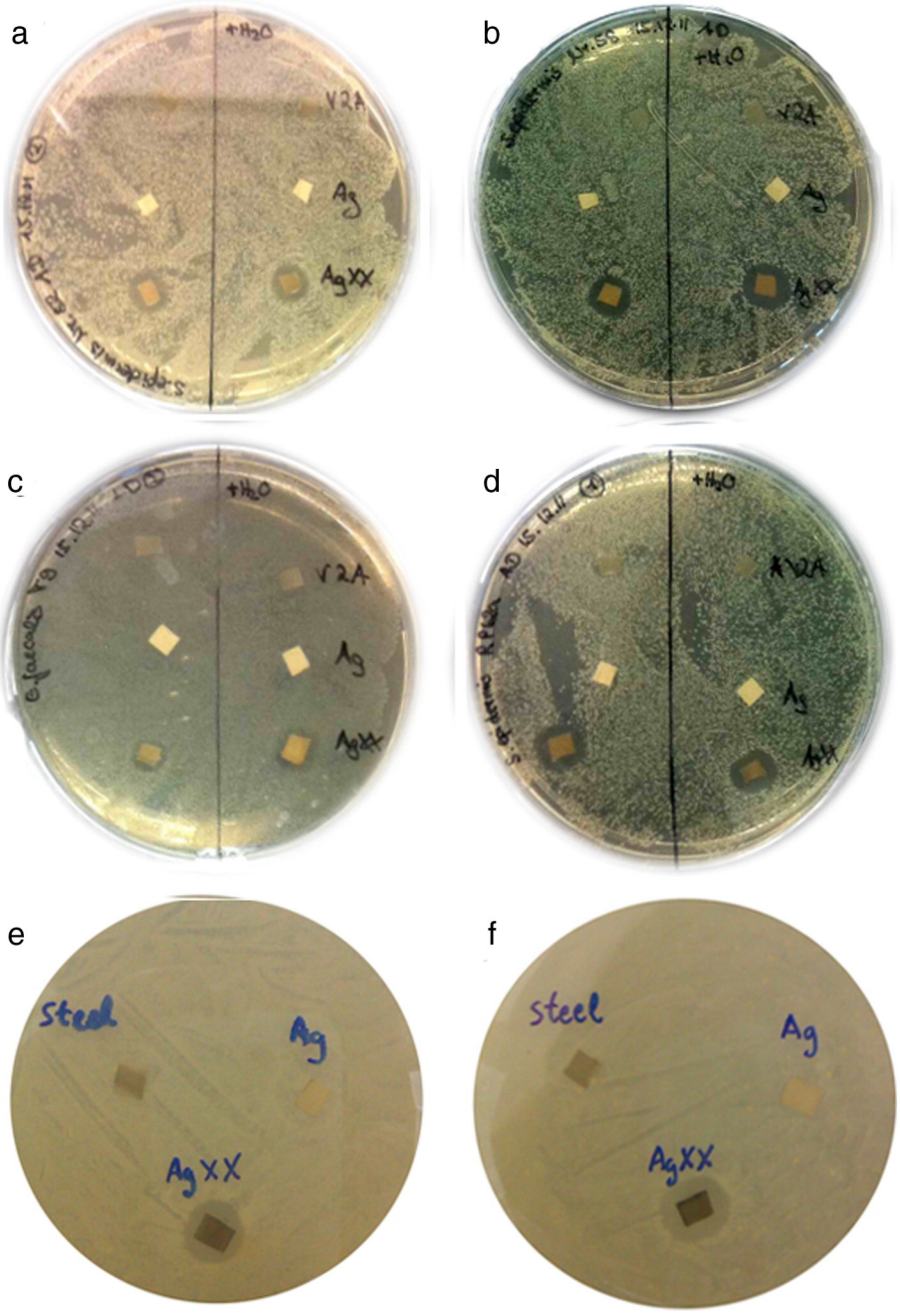

Fig. 3. AgXX®-mediated growth inhibition of Gram-positive pathogens as visualized on agar surface. (a) *S. epidermidis* No. 52, (b) *S. epidermidis* No. 58, (c) *E. faecalis* T9, (d) *S. epidermidis* RP62-A, (e) *S. epidermidis* 239891 and (f) *S. epidermidis* 214627-A.

1 to 4 h, washed three times in iso-osmolar phosphate–sucrose buffer and post-fixed in 1% osmium tetroxide in Sörenson phosphate buffer. After washing in distilled water, samples were dehydrated by an ethanol series (10 min each in 30%, 50%, 70%, 90%, 96% and 100% ethanol), washed twice in 100% ethanol and three times (10 min each) in hexamethyldisilazane before air-drying. Samples were mounted onto SEM stubs, gold coated in an Emitech K550X sputter coater, visualized and micrographed using a Hitachi S-4800 SEM at 15 kV accelerating voltage or a Hitachi S3400N SEM at 25 kV.

2.7. Tap-water device to study the growth of L. erythra

To investigate the possible application of AgXX® meshes in drinking water pipes we inoculated drinking water with *L. erythra* DSM 17644, an apathogenic *Legionella* species, at a concentration of 3×10^6 CFU ml^{-1}, a concentration of *Legionella* commonly found in legionellosis outbreaks [23,24]. Drinking water was pumped through a silicone hose (inner diameter, 3.2 mm; outer diameter, 6.4 mm, Thermofisher) from a storage vessel with a peristaltic pump (PumpDrive 5101, Heidolph Instruments) at a flow rate of 35 ml min^{-1}. In the outlet of the hose where the water is extracted, a 1 cm^2 mesh of AgXX®, Ag, or steel was inserted. The meshes were rolled up to ensure that all the water passes through the mesh. 1.5 ml water samples were collected at various time points (0 h, 2 h, 6 h, 1 day, 4 days, 6 days and 7 days) and analyzed. Serial dilutions of the samples (10^{-1} to 10^{-3}) were plated onto *Legionella* BCYE agar plates and incubated at 37 °C for 48 h. *L. erythra* colonies were enumerated as CFU ml^{-1}. At the end of the experiment (day 7) the meshes were removed and analyzed for bacteria attached to them in a Hitachi S-4800 scanning electron microscope at 15 kV accelerating voltage as described above.

3. Results

3.1. AgXX® has much higher disinfection capacity in urine than Ag

Disinfection capacity of AgXX® and silver was compared in urine. Urine contains numerous silver complex forming proteins and chlorides. 2.5×10^6 cells of an overnight *E. coli* RR1 culture were plated on urine agar. AgXX® meshes and Ag coated meshes of the same size and silver coating thickness were placed onto the urine agar surface. After 48 h at 37 °C the plates were examined for bacterial growth inhibition: Silver exhibited no antimicrobial effect. In contrast, the AgXX® coating showed very strong antimicrobial activity (Fig. 2).

3.2. AgXX®-mediated growth inhibition of Gram-positive pathogenic bacteria

To test the antimicrobial activity of AgXX® and Ag on Gram-positive bacteria, growth inhibition tests on agar surfaces were performed. Uncoated steel meshes were included as negative controls. The growth of all tested bacteria (in total 30, among them 28 clinical isolates) was inhibited in the presence of AgXX® (Table 2), the strongest effect was observed for the clinical *Staphylococcus* isolates, *S. epidermidis* 239891 and *S. epidermidis* 214627-A with 1.2 cm inhibition area after 24 h. Ag showed only a minor inhibition effect on some of the bacteria, steel without coating had no effect on bacterial growth, as expected (Table 2). In Fig. 3, exemplarily, agar plates showing the inhibition areas of the two most strongly impacted strains, *S. epidermidis* 239891 and *S. epidermidis* 214627-A, three other strongly inhibited *S. epidermidis* strains and of *E. faecalis* T9 which was inhibited to a lower extent are presented.

Additionally, for *S. epidermidis* RP62-A and *S. epidermidis* No. 58, the effect of the two different antimicrobial coatings on growth in batch culture was investigated (Fig. 4). For *S. epidermidis* RP62-A, the inhibitory effect of AgXX® was very strong. In MH medium with uncoated steel the strain reached an OD_{600} of 1.69 ± 0.12 after 24 h, in the presence of Ag it reached an OD_{600} of 1.47 ± 0.05, whereas growth in the presence of AgXX® was completely inhibited (maximum $OD_{600} = 0.09 \pm 0.06$). For the ISS isolate, *S. epidermidis* No. 58, the inhibitory effect of AgXX® was less pronounced. The control culture reached an OD_{600} of 3.04 ± 0.10, Ag had no effect (OD_{600} of 2.99 ± 0.12), the culture with AgXX® reached an OD_{600} of 2.07 ± 0.81 after 24 h of growth.

Interestingly, with regard to the culturable fraction, *S. epidermidis* RP62-A showed nearly identical CFU ml^{-1} values after 3 h of growth (early exponential growth phase), irrespective of the addition of AgXX®, Ag, or steel. After 6 h of growth the CFU ml^{-1} values for the cultures with AgXX® and Ag were still nearly the same. After 24 h the culturable fraction of bacteria in the culture with AgXX® had drastically decreased to $1.43 \times 10^8 \pm 1.62 \times 10^8$, whereas the cultures with Ag and

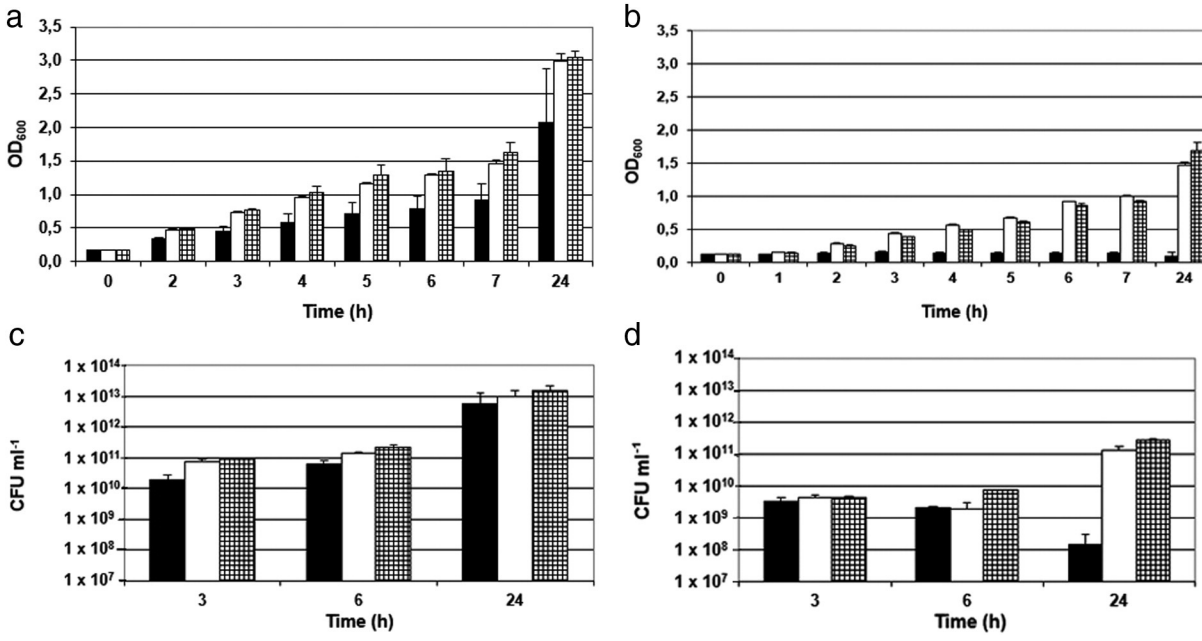

Fig. 4. AgXX®-mediated growth inhibition in batch culture: (a) and (b) as OD_{600} values of *S. epidermidis* No. 58 (ISS) and *S. epidermidis* RP62-A, respectively, (c) and (d) as culturable cells (CFU ml^{-1}) of *S. epidermidis* No. 58 (ISS) and *S. epidermidis* RP62-A, respectively. Error bars denote standard deviation. (■) AgXX®; (□) Ag; (▦) steel.

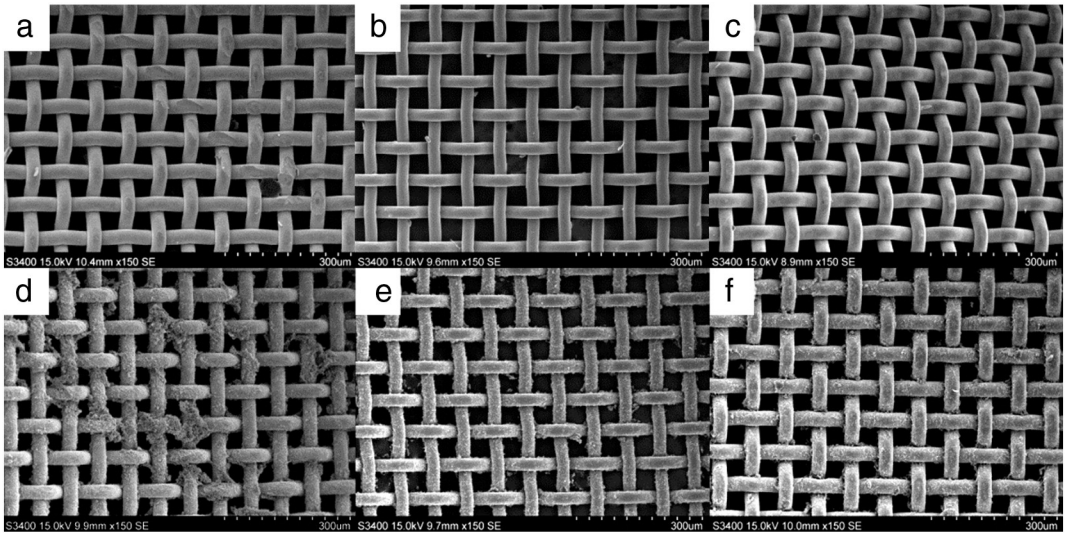

Fig. 5. *S. aureus* 215642 growth was strongly reduced by AgXX®, reduced by Ag, no effect was observed for uncoated steel. SEM images were taken after incubation in presence of the bacteria for 24 h at 37 °C. Upper panel without bacteria: (a) steel, (b) Ag, (c) AgXX®, lower panel with *S. aureus* 215642: (d) steel, (e) Ag, (f) AgXX®.

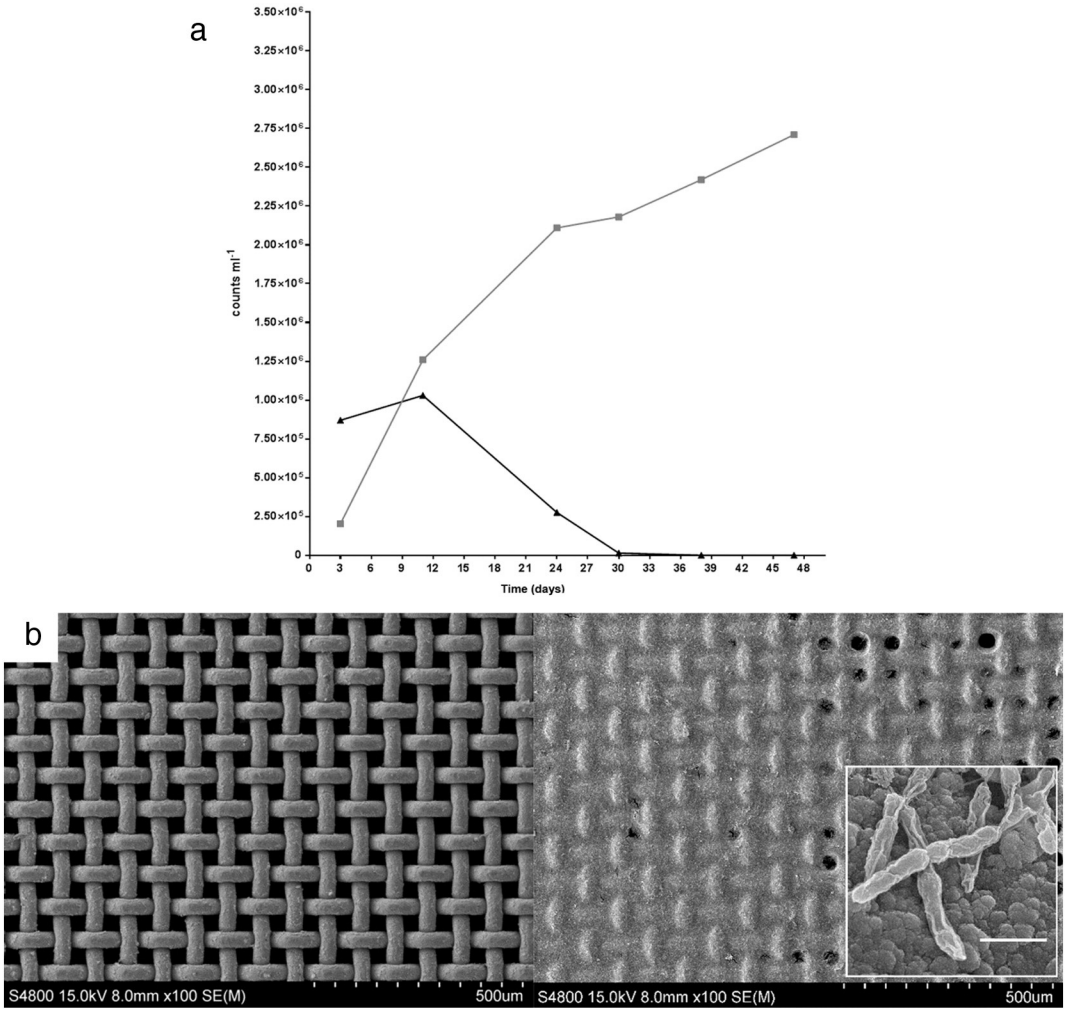

Fig. 6. Effect of AgXX® on growth of *Legionella erythra*, (a) given as total Hoechst-stainable counts (mean values of 15 counts each per sample). (▲) AgXX®; (■) steel. (b) Inhibition of *Legionella* surface growth by AgXX®. Left panel, AgXX® mesh after 47 days in CYE medium with *Legionella*, right panel, steel mesh after 47 days in CYE medium with *Legionella*. The inset in the right panel shows *Legionella* cells on the steel mesh (scale bar 1 μm).

steel showed very similar CFU ml^{-1} values, of $1.33 \times 10^{11} \pm 0.40 \times 10^{11}$ (Ag) and $2.9 \times 10^{11} \pm 0.22 \times 10^{11}$ (steel) (Fig. 4d). In contrast, the effect on the ISS isolate, *S. epidermidis* No. 58 was less pronounced. The number of culturable bacteria in the presence of AgXX® was significantly smaller from the beginning of the experiment (after 3 h $1.93 \times 10^{10} \pm 0.86 \times 10^{10}$ CFU ml^{-1}, in contrast to $7.73 \times 10^{10} \pm 0.96 \times 10^{10}$ CFU ml^{-1} for the culture with Ag). After 6 h the culture with AgXX® had reached a number of $6.20 \times 10^{10} \pm 2.20 \times 10^{10}$ CFU ml^{-1}, whereas the culture with Ag amounted to $1.37 \times 10^{11} \pm 0.23 \times 10^{11}$ CFU ml^{-1} (Fig. 4c). After 24 h the CFU ml^{-1} in the culture with AgXX® increased to $5.61 \times 10^{12} \pm 7.56 \times 10^{12}$ CFU ml^{-1}. However, bacterial growth was less pronounced in comparison to Ag with $9.53 \times 10^{12} \pm 5.35 \times 10^{12}$ CFU ml^{-1} and steel with $1.53 \times 10^{13} \pm 6.21 \times 10^{12}$ CFU ml^{-1}.

Moreover, for *S. aureus* 215642, a medical implant isolate, surface growth on AgXX®, Ag and uncoated steel was studied. After 24 h incubation the meshes were analyzed by SEM. While on the steel and the Ag mesh bacterial cells were visible, the AgXX® mesh was almost blank (Fig. 5).

3.3. AgXX®-mediated growth inhibition of Gram-negative waterborne pathogens

To study the growth effect of AgXX® on a waterborne pathogen, *L. erythra* was grown in batch cultures amended with AgXX® at 37 °C, in parallel with cultures amended with the uncoated steel mesh. Cultures were analyzed at different time points: Cells adhering to the meshes were detached and enumerated by fluorescence microscopy after Hoechst 33258 staining. The bacterial counts represent the median of 15 counts performed with each sample. Similar slow growth of *Legionella* in the AgXX® and steel mesh amended cultures was observed until day 11 (Fig. 6a). Then a remarkable decrease of Hoechst 33258-stainable bacteria in the culture with AgXX® was observed, at day 24 the difference to the reference culture was one log, at day 47 no cells stainable with Hoechst 33258 were detached from the AgXX® mesh, whereas cell counts on the reference mesh amounted to 2.71×10^6 ml^{-1} (Fig. 6a). These results coincide well with the data obtained by SEM after 47 days of growth. The steel mesh was densely covered by *Legionella* whereas the AgXX® mesh was completely blank (Fig. 6b).

3.4. AgXX®-mediated reduction of Legionella growth in tap-water pipes

To study the effect of AgXX® on growth of the waterborne pathogen *Legionella* in drinking water, a tap-water pipe was simulated under laboratory conditions (Fig. 7a): Drinking water was inoculated with 3×10^6 CFU ml^{-1} of *L. erythra*, a common concentration of *Legionella* in legionellosis outbreaks. *L. erythra* growth was followed for seven days by measuring culturable cells (CFU ml^{-1}) in set-ups with AgXX®, Ag or steel mesh in the outlet of the water hose (Table 3). In the hose with the steel and the Ag mesh *L. erythra* CFU ml^{-1} decreased only slightly in seven days, namely by approximately 0.5 log, whereas the reduction in the presence of AgXX® was 1.1 log (Table 3). A significant difference in culturable bacteria for the set-up with AgXX® compared to Ag and steel was visible from day 4 on. At the end of the experiment (on day 7) the meshes were analyzed by SEM for adherence of *L. erythra* cells (Fig. 7b). We observed that the steel and the silver mesh were covered by *Legionella* cells, the steel mesh more densely than the silver mesh whereas the AgXX® mesh was almost blank.

4. Discussion

In this study we compared the antimicrobial activity of a conventional silver coating generated by the galvanic method with that of AgXX®, a specifically structured and coated silver/ruthenium surface which is up-graded and conditioned by a special post-treatment. Our data demonstrate that the antimicrobial effect of AgXX® is superior to that of conventional silver coatings with similar silver content with respect to growth inhibition of pathogenic Gram-positive and Gram-negative bacteria including multiple antibiotic resistant clinical *Staphylococcus* and *Enterococcus* isolates.

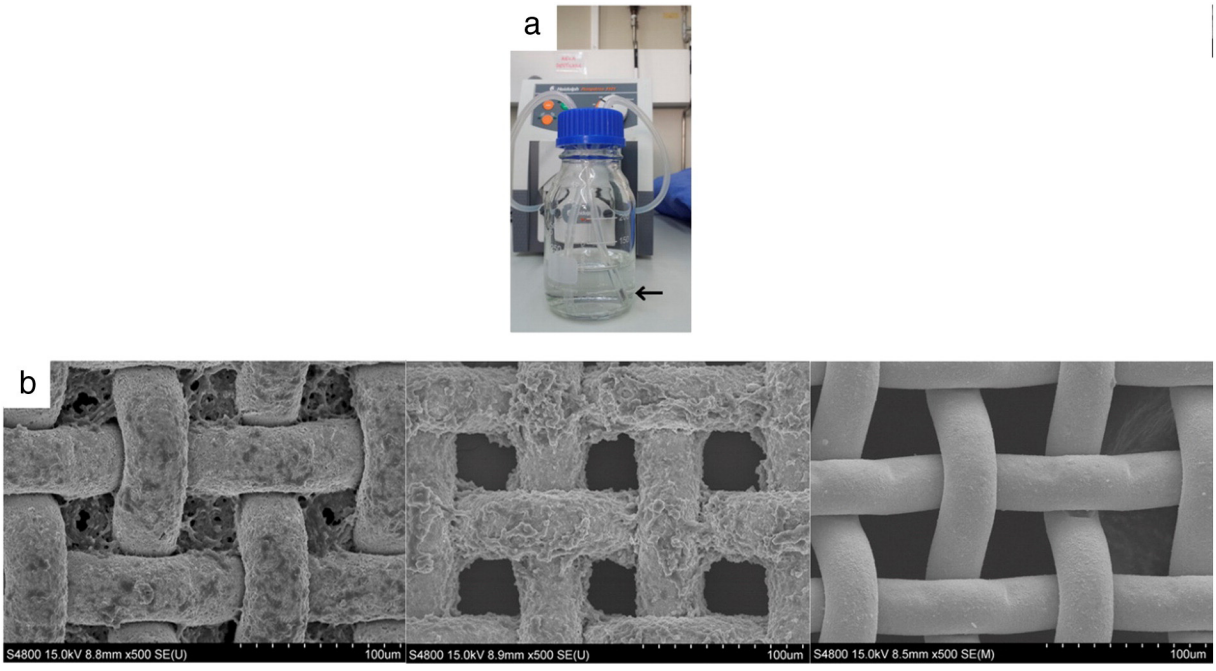

Fig. 7. *Legionella* tap-water experiment. (a) Setup of the experiment. The AgXX® mesh in the outlet of the silicone hose is marked with an arrow. (b) AgXX®- and Ag-mediated reduction of *Legionella* surface growth: steel (left panel), Ag (center), AgXX® (right panel), in tap water inoculated with *L. erythra* after seven days.

Table 3
Legionella erythra CFU ml^{-1} tap water.[a]

Time (h)	Ag (CFU ml^{-1})	Steel (CFU ml^{-1})	AgXX® (CFU ml^{-1})
0	3.12×10^6	3.05×10^6	3.12×10^6
2	3.26×10^6	3.33×10^6	3.12×10^6
4	3.40×10^6	2.41×10^6	2.84×10^6
24	2.92×10^6	1.99×10^6	1.29×10^6
96	1.32×10^6	1.42×10^6	6.95×10^5
144	7.07×10^5	1.48×10^6	1.71×10^5
168	8.51×10^5	7.95×10^5	1.71×10^5

[a] Data represent mean values (n = 2).

AgXX® also proved to be effective against different strains of *E. coli*, including the highly pathogenic Shiga toxin-producing *E. coli* O104:H4 strain (E. Grohmann and I. Alkorta, unpublished data) which caused an outbreak in Germany in 2011 with 3842 people who developed clinical illness [26,27]. Thus, AgXX® inhibits the growth of multiple antibiotic resistant bacteria commonly causing serious infections in hospitals and in the community, namely *E. coli* and *S. aureus* [1].

AgXX® only releases small amounts of silver ions in chloride free solutions as compared to conventional silver technologies; a maximum of 0.1–0.2 mg l^{-1} was measured after 12 weeks exposure of AgXX® in distilled water. The silver ion concentration in chloride containing solutions is even lower, thus in German drinking water with chloride concentrations varying from 16–250 mg l^{-1}, according to the AgCl solubility product, the free silver ion concentration is only in the range of 42.3 μg l^{-1} to 2.7 μg l^{-1}. In physiological solutions with high chloride concentrations, e.g., in Dulbecco's Modified Eagles Medium (DMEM with 25 mmol l^{-1} Hepes, Biochrom, Berlin, Germany) which is used for cultivation of cell lines, AgXX® showed high antimicrobial activity. By mass spectrometry analysis, no silver ions were detected in the DMEM solution that had been in contact with AgXX® for 24 h (U. Landau, C. Meyer, Largentec GmbH, E. Ehrentreich-Förster, M. Grießner, Fraunhofer Institute IBMT Golm/Potsdam, Germany, data to be published).

The strong antimicrobial activity of AgXX® is caused by the specific composition and structure of the AgXX® coating and is not dependent on the release of silver ions. The minimum silver ion concentration that was required to kill *E. coli* IMG 1711 in growth inhibition tests on agar surfaces with conventional silver coatings (T. Lisowsky, MultiBind biotec GmbH, Cologne, Germany, personal communication) was found to be 6.4 mg l^{-1}. This silver ion concentration is 32–64 times higher than the highest silver ion concentrations of 0.1–0.2 mg l^{-1} that we found after 12 weeks storage of AgXX® in distilled water (C. Meyer, personal communication).

We postulate the following mechanism for the antimicrobial activity of AgXX®. Micro galvanic elements on the AgXX® surface mainly consisting in silver and ruthenium generate an electric field interfering with the charged bacterial cell membranes. At the ruthenium micro cathodes of the galvanic cells catalytically supported redox reactions generate reactive oxygen species (ROS), e.g., diffusible molecules such as hydrogen peroxide which kill the microorganisms and cause the formation of inhibition areas around AgXX® meshes on agar plates as shown in Figs. 2 and 3. At the silver/silver chloride micro anodes of the galvanic cell microorganisms are oxidized by electron shuttle from the microbes to the semiconducting anode surface.

As the antimicrobial effect of the thin AgXX® coatings (3–5 μm) is not dependent on any substance released from the coating material, the lifetime of AgXX® is only limited by mechanical destruction of the coating. In industrial water disinfection, AgXX® coated meshes (Raschig rings) were found to be antimicrobially active even after more than five years (Fig. 8).

Due to its "physical-catalytic" action and its composition, the antimicrobial AgXX® coating complies with all the requirements of a sustainable technology: AgXX® finishes are non-toxic as their efficiency is not dependent on the release of silver ions or toxic substances as it is the case with biocides or disinfectants and therefore may be claimed as an environmentally friendly technique. As AgXX® is based on metals it is recyclable. Industrial applications of only a few μm thick AgXX® coatings worked out to be long lasting. As AgXX® coatings consist of a multitude of micro galvanic cells AgXX® is operated without supply of external energy in contrast to disinfection technologies such as e.g., UV- or ultrasonic-systems. Thus, AgXX® is easy to use and has already found its way into industrial water disinfection [28]. Further applications will develop in medical technology. The antimicrobial activity of AgXX® can also be introduced into all kinds of consumer products. A new plating technique for the generation of AgXX® coatings has been successfully applied onto different plastic base materials used in medical and consumer applications. Another area of application under investigation is the preparation of AgXX® based antimicrobial lacquers.

In the presence of AgXX®, a clear inhibition of surface growth of all pathogenic bacteria tested was observed (Figs. 5, 6, and 7b). For the waterborne pathogen *Legionella*, the growth inhibitory effect of AgXX® was clearly evident from day 11 of the assay. This observation is in contrast to what was observed for the steel mesh, where surface growth significantly increased from the beginning and continued until the end of the experiment (Fig. 6a). After day 11 the number of Hoechst-stainable bacteria on AgXX® decreased significantly, after 30 days no *Legionella* cells were detectable any more (Fig. 6a).

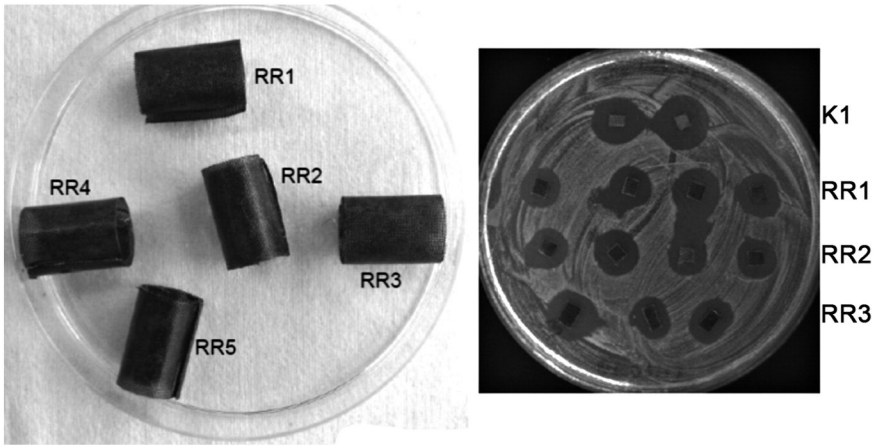

Fig. 8. Long-term activity of AgXX® Left panel: AgXX® Raschig rings (RR 1 to 5) after five years in cooling water for tools in plastics molding. Right panel: RR 1 to RR 3 still showed high antimicrobial activity on *E. coli* DSM 498 incubated on LB agar for 18 h at 37 °C. K1: AgXX® reference mesh with high antimicrobial activity as positive control. The image was provided by T. Lisowsky, MultiBind biotec GmbH, Cologne, Germany.

A strong inhibitory effect of AgXX® was also observed in solutions with silver complex forming substances or silver precipitating anions (generation of insoluble silver salts), thus under environmental conditions where conventional silver technologies are limited or completely hindered, as shown for urine in Fig. 2. Hence, the toxicity problems that often occur at higher silver ion concentrations [29–31] can be avoided by using AgXX®-coated surfaces.

Silver is one of the best studied bactericidal agents in water supplies [32–36]. To investigate the possible application of AgXX® in water distribution and supply systems, a tap-water pipe was simulated to compare the bactericidal effect of conventional Ag technology with AgXX®. Tap water inoculated with *L. erythra* showed a more than one log decrease of *L. erythra* CFU ml^{-1} after seven days in the presence of AgXX® (Table 3). This observation was confirmed by SEM, on both Ag and steel meshes abundant *L. erythra* cells were observed, whereas the AgXX® meshes were devoid of bacteria (Fig. 7b).

To better understand the antimicrobial activity of AgXX® it is important to elucidate the microbial response upon contact with AgXX® on molecular level. Experiments in this direction are in progress in our laboratories.

5. Conclusion

The novel antimicrobial surface coating proved to be efficient against a variety of pathogenic Gram-positive and Gram-negative bacteria including strong biofilm formers such as *S. aureus*, *S. epidermidis*, Shiga toxin-producing *E. coli* and *Legionella*. The antimicrobial action was demonstrated to be independent of the release of silver ions, but AgXX® seems to be rather active via diffusible hydrogen peroxide molecules that are generated at the ruthenium cathode via the reduction of oxygen. Thus, the material is durable, recyclable and only slightly cytotoxic opening a broad spectrum of applications in water technology, medical technology and consumer products.

Conflict of interest

No conflict of interest declared.

Acknowledgments

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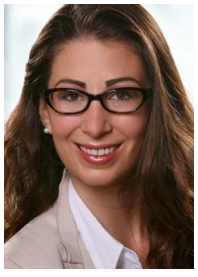

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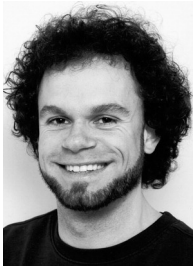
Wilhelm Schmieder is a the University of Freiburg, Germany. He graduated in Microbiology (B.Sc.) on the carbon dioxide fixation cycle of a thaumarchaeon. He performed his Master's thesis in the laboratory of Elisabeth Grohmann. He examined resistance development and physiological response of staphylococci upon long-term contact with silver-coated antimicrobial materials, such as AgXX®. His research interests are in the analysis of new antimicrobial agents and their clinical applications.



Emanuel Clauss-Lendzian has been trained at the University of Freiburg, Germany, in Virology, Cell Biology, Molecular Genetics and Microbiology. He currently works in the laboratory of Elisabeth Grohmann. In his research, he focuses on the gene expression of the nosocomial pathogen *Enterococcus faecalis* subjected to metal stress by antimicrobial materials such as AgXX® with special interest in next generation sequencing technology.

Tuerkan Sakinc-Gueler trained at the Ruhr University Bochum, Germany, and presently holds a position as Assistant Professor of Infectious Diseases at the University Medical Centre Freiburg, Germany. She is a Molecular Biologist with special interest in staphylococcal virulence mechanisms. Her research focuses on urinary-tract-infections caused by Gram-positive pathogens.

Ricardo Andrade received his Ph.D. in Biology from the University of the Basque Country, Spain. His research focused on nucleocytoplasmic protein import and membrane permeability during programmed cell death. For the last two decades, he has collaborated in the development of the Microscopy Facility in the Department of Biomedicine at the University of the Basque Country. At present, he is in charge of this Facility, which covers both light and electron microscopy techniques.

Itziar Alkorta received her Ph.D. from the University of the Basque Country, Spain and completed postdoctoral research at the University of California, Berkeley. Currently Dr Alkorta is Professor of Biochemistry at the University of the Basque Country. She is a Biochemist interested in the molecular mechanisms of bacterial conjugation. In particular her research focuses on the study of conjugative coupling proteins as a way of controlling antibiotic resistance spread among bacteria.

Carsten Meyer studied Chemistry at the Free University of Berlin, Germany, followed by a Ph.D. in Electrochemistry. He is employed by the Largentec GmbH responsible for research and development on AgXX® technologies. Additionally he is teaching Chemistry at the University of Applied Sciences in Berlin.

Uwe Landau educated in Metallurgy at the Technical University of Clausthal and Technical University of Berlin, Germany, and has a Ph.D. in Electrochemistry. He was Professor of Surface Technology at Technical University of Berlin and led his own company in the field of electroplating for electronic industry for over 20 years. After selling his company he founded Largentec where he developed and industrialized AgXX®-technology. He is an owner of many patents in surface technology and metallurgy and is active in a leading position in the German Electroplaters Association (DGO) and industrial research institution for Precious Metals and Metal Chemistry (fem).

Elisabeth Grohmann trained at the Technical University of Graz, Austria in Biochemistry and Molecular Biology and presently holds a position as Professor of Molecular Biology at the University Medical Centre Freiburg, Germany. She is a Molecular Biologist with special interest in antibiotic resistance transfer among Gram-positive pathogens. Her research focuses on the anthropogenic impact on the dissemination of antibiotic resistance genes and its prevention as well as on unraveling the mechanisms of conjugative type IV secretion machines in *Enterococcus*.