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Development of electrochemically deposited surfaces based on copper and silver with bactericidal effect

PhD thesis

by

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and

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Preface

The work presented in this thesis is the result of an industrial PhD study at the Technical University of Denmark (DTU) under the scheme of industrial research set down by The Ministry of Higher Education and Science in Co-operation with Elplatek A/S. It marks the finale of a project which began on July 1st, 2016 and ended on June 30th, 2019.

The industrial PhD study has mainly been carried out at the Department of Biotechnology and Biomedicine DTU, under the supervision of Professor Lone Gram, and at Elplatek A/S.
The project was co-supervised by Professor Per Møller at the Department of Mechanical Engineering DTU, and Thomas Bjarnsholt at the Consterton Biofilm Centre (KU). It also included 2 months stay in Texas, U.S. for field-testing at the Southwest Regional Wound Care Center in Lubbock.

The project was financed by Innovation Fund of Denmark (case nr. 5189-00091B) and Elplatek A/S.

Nicole Ciacotich

Kgs. Lyngby, June 2019

Summary

Environmental surfaces play a major role in the transmission of nosocomial pathogens. Surfaces that prevent bacterial adhesion or exert a microbiocidal effect can be integrated into the existing disinfection practices to increase surface hygiene and reduce the incidence of healthcare-associated infections. The high antibacterial efficacy of copper alloy surfaces is due to the so-called contact killing, which is controlled by the redox activity of copper and the toxic action of copper ions. The redox activity of copper induces electrochemical reactions with the alloying elements and the surrounding environment, and this can, positively or negatively, influence the antibacterial activity of copper alloys. Bacterial-metal contact is crucial for establishing conditions of contact killing, so that the antibacterial efficacy of copper alloys can be optimized if the surface area to volume ratio is increased. These chemical and physical characteristics can be used to produce copper alloys with increased antibacterial efficacy. As such, an electroplated copper-silver alloy coating was developed with a high surface area to volume ratio, using the galvanic coupling of copper and silver to trigger electrochemical reactions, when in contact with bacterial cells.

The **purpose** of the present PhD project was to demonstrate the antibacterial activity of a newly developed copper-silver alloy coating obtained by electroplating, to explain its antibacterial properties with a materials science-based approach and to evaluate relevant conditions potentially influencing the efficacy. In the light of an increasing global demand for antimicrobial coatings to improve surface hygiene, this coating could contribute to reducing transmission of bacteria from surfaces, and the present work aimed to put it in the context of the copper-based antibacterial strategies.

The antibacterial and antiadhesive properties of the alloy coating were demonstrated in wet conditions (e.g. buffer and nutrient broth) and its antibacterial efficacy in dry conditions. In liquid environments, copper-silver alloy coated surfaces released copper ions in the bacterial suspension: copper dissolved rapidly due to the presence of silver in the alloy, according to the principle of galvanic corrosion. This explained the higher antiadhesive activity of the copper-silver alloy in buffer, even as compared to copper or silver alone. Bacterial numbers were reduced by 5-6 log units in suspension with copper-silver alloy coated surfaces in buffer. Nutrient broth neutralized copper ions in solution and protected bacterial cells, thus there was neither antibacterial nor antiadhesive efficacy in these conditions.

Under dry conditions, contact killing of bacteria on copper-silver alloy coated surfaces was evaluated using the U.S. EPA test methods for copper alloys. More than 99.9% reduction in numbers was achieved both after 2 hours exposure and over 24 hours of continuous bacterial contamination. A modified live/dead staining technique in combination with confocal laser scanning microscopy was applied in order to visualize *in situ* the killing of bacterial biofilms at the copper-silver alloy coated surfaces. *S. aureus* biofilm was inactivated more quickly than *P. aeruginosa* biofilm. Furthermore, *in situ* pH monitoring at the copper-silver alloy coated surfaces revealed a fast local pH raise due to the electrochemical reactions induced by potential differences between silver, copper and bacterial cells, when in contact with the alloy.

Chlorides and phosphates, commonly present in chemical detergents and disinfectants, can influence the antibacterial efficacy of copper alloy surfaces by reacting with copper and its alloying elements. Chlorides established conditions for active dissolution of copper and stabilized copper ions in solution, explaining the highest antiadhesive efficacy of the copper-silver alloy coating in chloride containing media. In contrast, copper did not dissolve in the suspension with only phosphates, therefore no antibacterial activity was observed in these conditions. Other indoor environmental factors, such as surface oxidation over time, build-up of organic material and abrasion of the surfaces can affect the antibacterial properties of copper alloy surfaces, and these factors are highly dependent on the specific application. Hence, two field tests of the copper-silver alloy as coating for door handles were carried out to evaluate the antibacterial performance and durability in clinical settings. Reference uncoated door handles had approx. twice as high microbial load as compared to the copper-silver alloy coated door handles. These data confirmed previous results of other copper and copper alloys-based antibacterial strategies. The lifetime of the copper-silver alloy coating was estimated to be at least one year in such applications, prior to re-coating interventions. Lastly, bacterial tolerance or resistance to the copper-silver alloy coating and cross-resistance to other antimicrobials did not appear as primary concern, as indicated by an adaptive laboratory evolution study in which *S. aureus* was exposed to the copper-silver alloy coating. The industrial scalability and production of the copper-silver alloy coating is possible, and it could be applied on already existing objects limiting the costs. Therefore, the copper-silver alloy coating qualifies as promising surface solution strategy to limit infectious diseases in healthcare settings, and microbial contamination in biopharmaceutical industry and food production environments.

Resumé (in Danish)

Miljøoverflader spiller en vigtig rolle i overførslen af nosokomielale infektioner. Overflader der forhindrer fastgørelsen af disse bakterier, eller har en mikrobiocid virkning, kan blive integreret i allerede eksisterende desinfektionspraksisser til at øge overfladehygiejnen, og reducere forekomsten af hospitalsinfektioner. Kobberlegeringer er overflader med en meget høj antibakteriel virkning, som skyldes den såkaldte ”kontaktdræbning” (contact-mediated killing), forårsaget af redox-aktivitet af kobber og toksisk virkning af kobber-ioner. Redox-aktiviteten skaber elektrokemiske reaktioner mellem legeringselementerne og omgivelserne, og således påvirkes den antibakterielle virkning af kobberlegeringer positivt eller negativt. Kontakten mellem bakterier og metallet er afgørende for at etablere kontaktdræbende betingelser. Derfor kan kobberlegeringernes antibakterielle effektivitet optimeres, hvis overflade til volumen forholdet forøges. Disse kemiske og fysiske egenskaber kan anvendes til fremstilling af kobberlegeringer med øget antibakteriel virkning. På den måde blev en galvanisk kobber-sølv legerings belægning udviklet med et højt overflade til volumen forhold, og den galvaniske kobling af kobber og sølv, der udløser elektrokemiske reaktioner, når bakteriecellerne er i kontakt med overfladeren.

Formålet med dette PhD studie var at demonstrere den antibakterielle aktivitet af den nyudviklede kobber-sølv legerings belægning opnået ved galvanisering, at kunne forklare dens antibakterielle egenskaber med en materialevidenskabsbaseret tilgang, og at vurdere relevante tilstande, der kan påvirke den antibakterielle effektivitet. I lyset af den stigende globale efterspørgsel på antimikrobielle belægninger for at forbedre overfladehygiejnen kan denne belægning bidrage til at reducere overførsel af bakterier fra overflader, og dette studie har til formål at sætte det ind i en sammenhæng med kobberbaserede antibakterielle strategier.

De antibakterielle og antiadhæsive egenskaber af belægningen med kobber-sølv legering blev demonstreret under våde betingelser (f.eks. buffer og næringsmedium), og den antibakterielle virkning under tørre omstændigheder. Under våde betingelser udløste kobber-sølv legeringen kobber-ioner i bakteriesuspensionen: kobber blev hurtigt opløst på grund af sølv i legeringen jf. det galvaniske korrosions princip. Dette forklarede den højere antiadhæsive aktivitet af kobber-sølv legeringens belægningen i buffer alene sammenlignet med kobber eller sølv alene. Antallet af bakterieceller i suspensionen med kobber-sølv legerings belægningen blev reduceret med 5-6 log enheder i buffer. Næringsmedium neutraliserede kobber-ionerne i

opløsningen og beskyttede bakterierne, og som en konsekvens af dette var der hverken antibakterielle eller antiadhæsive virkningerne under disse omstændigheder. Under tørre betingelser, blev U.S. EPA-testmetoder af kobberlegeringer brugt til at evaluere kontaktdræbningen af bakterier på kobber-sølv legerings belægningen. Mere end 99.9% bakterierne blev reduceret efter 2 timers eksponeringstid og i løbet af 24 timer med gentagne bakterielle kontamineringer. En modificeret ”live/dead” farvningsteknik i kombination med konfokal laser scanning mikroskopi blev brugt til at visualisere *in situ* drab af bakterielle biofilm på kobber-sølv legerings belægningen. *S. aureus* biofilm blev inaktiveret hurtigere end *P. aeruginosa* biofilm. Derudover viste *in situ* pH-overvågning på kobber-sølv legerings belægningen en hurtig øgning af pH som følge af de elektrokemiske reaktioner, som de forskellige elektrokemiske potentialer af sølv, kobber og bakterieceller inducerede, når de var i kontakt med overfladen.

Klorider og fosfater, der generelt findes i rengøringsmidler og desinfektionsmidler, kan påvirke den antibakterielle virkning af kobber-sølv legerings belægninger ved at reagere med kobber og de andre legeringselementer. Kloriderne etablerede betingelserne for aktiv opløsning af kobber, og stabiliserede kobber-ionerne i opløsningen, hvilket forklarede den højeste antiadhæsive virkning af kobber-sølv legerings belægningen i kloridopløsninger. Derimod opløstes kobber ikke i suspensionen, der kun indeholdt fosfater, og derfor blev den antibakteriel aktivitet ikke observeret under disse betingelser. Andre indendørs miljøfaktorer, f.eks. overfladeoxidation med tiden, opbygning af organisk stof og slid, kan påvirke de antibakterielle egenskaber af kobber-sølv legerings belægningen, og disse faktorer er stærkt afhængige af den specifikke anvendelse. Derfor blev to feltforsøg af kobber-sølv legeringen som belægningen på dørhåndtag udført til at evaluere den antibakterielle virkning og holdbarheden i kliniske omgivelser. Reference dørhåndtagene uden belægning havde en ca. dobbelt så høj bakteriemængde som de kobber-sølvbelagte. Disse data bekræftede tidligere resultater med andre kobber- og kobberlegeringsbaserede antibakterielle strategier. Kobber-sølv legerings belægningens levetid forventes at være mindst et år i lignende anvendelser, før det skal belægges igen. Endeligt forekom bakteriel tolerance eller modstandsdygtighed over for kobber-sølv legeringen og krydsresistens over for andre antimikrobielle stoffer ikke som en hovedbekymring. Det blev vist med et ”adaptive laboratory evolution” studie, hvor *S. aureus* blev udsat for kobber-sølv legeringen. Den industrielle skalerbarhed og produktion af kobber-sølv legerings belægningen er mulig, og den kan belægges på allerede eksisterende objekter, hvorved omkostningerne begrænses. Derfor kvalificerer kobber-sølv legerings

belægningen som lovende overfladeopløsningsstrategi til at begrænse smitsomme sygdomme i de sundhedsområder og mikrobiologisk kontaminering i den biofarmaceutiske industri og fødevareproduktionsmiljøer.

List of publications

This thesis is based on the following papers:

Paper 1:

Ciacotich N., Din U. R., Sloth J. J., Møller P. and Gram L. (2018). An electroplated copper–silver alloy as antibacterial coating on stainless steel. *Surface and Coating Technology* **345**, 96–104, https://doi.org/10.1016/j.surfcoat.2018.04.007

Paper 2:

Ciacotich N., Kilstrup M., Møller P. and Gram L. (2019). Influence of chlorides and phosphates on the anti-adhesive, antibacterial and electrochemical properties of an electroplated copper-silver alloy. *Biointerphases*, **Vol. 14**, No. 2, 021005, https://doi.org/10.1116/1.5088936

Paper 3:

Ciacotich N., Kragh N. K., Lichtenberg M., Tesdorpf J., Bjarnsholt T. and Gram L. (2019). *In situ* monitoring of the antibacterial activity of a copper-silver alloy using confocal laser scanning microscopy and pH microsensors. *Global Challenges*, 1900044, https://doi.org/10.1002/gch2.201900044

Paper 4:

Ciacotich N., Kvich L., Køhler S., Andersen A., Sanford N., Wolcott J., Bjarnsholt and Gram L. (2019). Copper-silver alloy coated door handles as a potential antibacterial strategy in real-life clinical settings. Manuscript in preparation.

Not included in this thesis:

Patent application:

Rasmussen J. B., **Ciacotich N.**, Gram L., Møller P. (2019). A method for electroplating antimicrobial coatings consisting of copper-silver alloys for highly and frequently bacterial contaminated surfaces in healthcare settings and food industry. Submitted.

Furthermore, during the PhD study, results have been presented at conferences as oral presentations to New Medical Products and Devices at Danish Technological Institute (2017), BIT's 15th Annual Congress (2017), Focus on medical innovation at IDA Ingeniørforeningen (2018), ATV-SEMAPP Antibacterial surfaces at DTU, AVS Symposium (2018), and as poster contribution at the Danish Microbiological (2017). The PhD project has also included attendance and presentations at meetings and events within the Materials Fast Track consortium (2017-2019).

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1. Introduction and outlook

"*Hospital-acquired infections are now killing more people every year in the United States than die from AIDS or cancer or car accidents combined - about 100,000"*

- Janine Benyus, Natural Sciences writer, TED Global 2009 conference.

In 2002, the Centre for Disease Prevention and Control estimated that each year nearly 1.7 million patients acquired infections in hospitals and 99,000 patients died [1]. Progress has been made in reducing healthcare-associated infections (HCAIs) since then, but there is still space for improvement in terms of prevention and reduction [2]. In EU/EEA countries, 3.8 million people annually acquired HCAIs in acute care hospitals between 2016 and 2017, and 90,000 people died each year [3,4]. HCAIs usually develop between 48 hours and 30 days, and are mainly (80-87%) caused by less than 20 different species of microorganisms, including *Staphylococcus aureus*, *Enterococcus* species, *Escherichia coli*, coagulase-negative staphylococci, *Pseudomonas aeruginosa*, and about 16-20% of these pathogens include multidrug-resistant (MDR) phenotypes [5]. The consequences of HCAIs are severe and closely interlinked; prolonged hospital stay, long-term disability, additional medical treatment, increased antimicrobial resistance and mortality rates [6]. Altogether, they have a dramatic effect on the economy of the healthcare system. In Europe and USA, the annual financial losses caused by their direct costs was estimated to approximately € 7 billion and US$ 6.5 billion in 2004, respectively [6]. HCAIs continue to escalate at an alarming rate and they remain a major safety concern for both patients and healthcare providers [5].

Environmental surfaces in hospitals play a major role in the transmission of nosocomial pathogens. Microorganisms have the ability to attach to both inert and biological surfaces and attached microorganisms, especially if allowed to form biofilms, are less sensitive to biocides, antibiotics and physical stress [7,8]. Although biofilms are generally associated with invasive medical devices, microbes can proliferate and survive on dry surfaces for extended periods of time, ranging from hours to years (Table I) [7,9].

Table I. Survival of human pathogens on dry hospital surfaces [10].

Organism	**Survival time**
Clostridium difficile (spores)	>5 months
Acinetobacter spp	3 days to 11 months
Enterococcus spp including VRE	5 days to >46 months
Pseudomonas aeruginosa	6 hours to 16 months
Klebsiella spp	2 hours to >30 months
Staphylococcus aureus, including MRSA	7 days to >12 months
Norovirus (and feline calicivirus)	8 hours to >2 weeks

Dry surfaces of a number of hospital furniture and environmental items, such as bedrails, bedtables, frames, door handles and IV poles are ideal sources of proliferation and transmission of pathogenic microorganisms. Objects in the vicinity of patients are more frequently contaminated due to their continuous exposure to patients and personnel, however, they were also less likely to experience a regular thorough cleaning compared to large open areas [10]. Object design can also influence the level of surface contamination: lever door handles display the highest levels of bacterial contamination as compared to pull handles and push plate designs, when adjusted for frequency of use [11]. Besides the "skin to object surface ratio", it is also likely that the regular daily cleaning fails to reach the hidden areas of more complex-shaped objects. From surfaces or via hand transfer from health care personnel, pathogenic microorganisms such as *Clostridium difficile*, methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus* (VRE), *Pseudomonas aeruginosa* and *Acinetobacter baumannii* can be directly transmitted from the previous to the next patient assigned to the room [10]. The risk of acquisition of VRE in the next room occupant is higher if the immediate prior occupant is VRE-infected or –colonized, and if a positive environmental VRE culture is obtained from the room prior to the admission [12]. Infection control policies and hand-washing are undoubtedly first-line strategies against HCAIs, however, hospital compliance, healthcare workers education and practical issues of implementation, especially in developing countries, pose major challenges [13]. Integrating surfaces that prevent bacterial adhesion or exert a microbiocidal effect to the existing disinfection practices can be a potential winning action on all sides. This second-line strategy has the advantage to be independent from human factors and provide a continuous antimicrobial activity extended to the whole object

surface. Clearly, the dual strategy has to be evaluated and designed considering the real-life applications and possible factors influencing its effectiveness.

Over the past decade, several studies have been dedicated to developing new effective antimicrobial strategies for surfaces, and copper-based antimicrobial solutions are among the best candidates due to the demonstrated superior efficacy of copper in comparison to other metals [14–16]. In this context, **the present thesis aims to demonstrate the antibacterial activity of a newly developed copper-silver alloy coating obtained by electroplating, to explain its antibacterial properties with a materials science-based approach and to evaluate relevant conditions potentially influencing the efficacy.**

The experimental work and results obtained during this project are summarized in four papers/manuscripts, while highlights of the results are included in this thesis. A patent application about the manufacturing method of the coating has been submitted, but it is not included in the present study. This thesis consists of six main chapters where the work on the copper-silver alloy coating is addressed within the state-of-art of the topic. Chapter 2 explores the antibacterial activity of copper with focus on copper alloys and surface properties involved in the bactericidal mechanism. An overview about antibacterial copper and copper alloy-based coatings and methods for determining their antibacterial activity are given in chapter 3 and 4. Relevant environmental factors potentially influencing the antibacterial activity of copper alloys in real-life applications are discussed in chapter 5. Chapter 6 presents the results of field-testing of copper alloy surfaces in clinical settings, and chapter 7 evaluates the possibility of bacterial tolerance or resistance to copper and cross-resistance to other antimicrobials in clinical settings.

2. The antibacterial activity of copper ions and copper alloy surfaces

Since ancient times, copper and copper compounds have been used with medical and hygiene purposes [15]. The use of copper as an antimicrobial agent has recently been rediscovered as an alternative strategy due to the selective pressure and development of bacterial resistance caused by antibiotics [15,17]. Copper in its metallic bulk state (99.99% pure or alloyed), as nanoparticles, as cuprous (Cu(I)) and cupric (Cu(II)) ions, exhibits antibacterial, antifungal and antiviral activity [15,17].

In the present study, attention has been given to copper alloys as antibacterial agents due to the number of clinically relevant bacteria causing HCAIs. Metallic bulk state and ionic form of copper have been considered, since these are the possible copper states that bacteria encounter, when exposed to copper alloys.

The mechanism of action of copper alloy surfaces is essentially different from that of copper ions in solution [18]. Bacteria are rapidly killed by the contact with dry metallic copper surfaces and by the very high concentration of copper ions that is reached locally at the interface [15,18]. [15,18]. If immersed in a bacterial suspension, copper alloys can release cuprous and cupric ions and the excess of copper ions challenges the bacterial homeostatic mechanisms [18]. However, the concentration of copper ions in a bacterial suspension is considerably lower than that in the aqueous interface on a dry copper alloy surfaces. This and the bacterial-metal contact make contact killing different and more effective than killing by copper ions in solution [18]. The toxicity of copper ions and synergism with other antimicrobial agents, such as chloride-containing compounds and silver, is discussed in section 2.1, whereas section 2.2 presents contact killing of copper alloy surfaces. The role of surface properties in the bacterial-metal contact and the antibacterial activity of copper alloy surfaces is examined in section 2.3.

2.1 Toxicity of copper ions to bacteria

Among all the noble metals, copper is the only essential trace element necessary for a number of biological processes in most living organisms [14]. Lysyl oxidase, dopamine β-hydroxylase, cytochrome *c* oxidase and superoxide dismutase are just few examples of more than 30 types of copper-containing proteins that are known so far [15]. Copper acts as an electron donor or

acceptor in these enzymes by redox cycling between Cu(I) and Cu(II) ions [15]. Copper has standard reduction potentials of 342 and 521 mV (SHE) for the redox couples Cu/Cu(II) and Cu/Cu(I), respectively, which are in the range of biologically relevant redox potentials [19,20]. The ability of cycling between its oxidation states at these potentials also makes copper a potential source of cell damage [15]. All cells regulate the levels of intracellular copper through copper homeostasis, however, this system can be overloaded by high copper concentrations, and intracellular copper can reach toxic levels [18]. The replacement of iron cofactor in iron sulfur cluster proteins is currently believed to be the main toxic action of copper [18] (Figure 1).

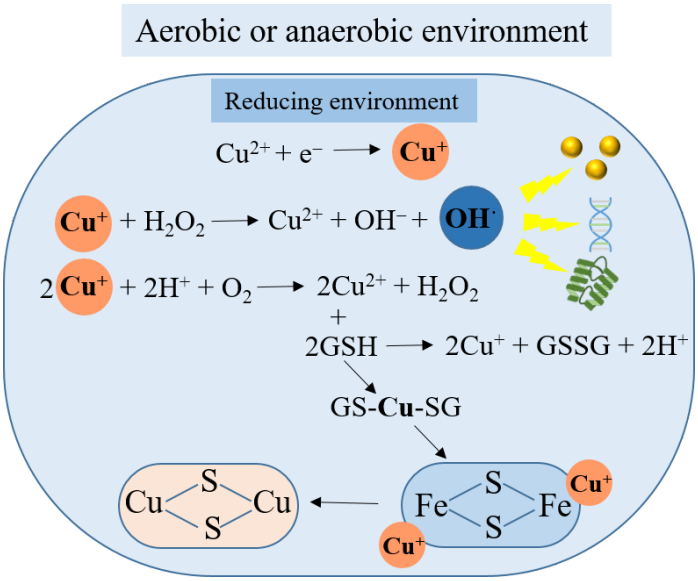

Figure 1. Mechanism of copper toxicity. When Cu(II) has entered the bacterial cell, it is reduced to Cu(I) in the reducing conditions of the cytoplasm. Cu(I) can then participate to Fenton-type reactions and produce highly reactive hydroxyl radicals. ROS react with lipids, proteins and nucleic acid. Cu(I) can also cause depletion of cellular thiols, i.e. by oxidizing GSH to GSSG. Under anaerobic conditions, gluthatione-copper complexes (GS-Cu-GS) can act as copper-donor for metalloenzymes, e.g. iron-sulfur cluster proteins, where Cu(I) displaces iron from the cluster. Modified from Solioz [18].

Cu(I) is Fenton active and therefore generates reactive oxygen species (ROS) when it is oxidized to Cu(II). ROS can inhibit respiration, lipid peroxidation and oxidative damage of proteins [18]. Copper can also lead to depletion of glutathione (GSH) that protects the cell against heavy metal toxicity [18]. Cu(I) is considerably more toxic than Cu(II) due to its higher thiophilicity, and because the cytoplasmic membrane is more permeable to Cu(I) [18].

The antibacterial efficacy of copper ions can be enhanced by the presence of other antimicrobial agents, such as chloride-containing compounds or silver. Copper ions Cu(II) worked synergistically, but likely through independent biochemical routes, with quaternary ammonium compounds (e.g. benzalkonium chloride) to kill *P. aeruginosa* biofilms [21]. A concentration 128-fold lower than either agent alone was enough to reduce the number of surviving *P. aeruginosa* cells to below the detection level after 24 hours exposure [21]. Combination of copper Cu(II) and silver ions Ag(I) were effective in controlling *Legionella* spp. in water disinfection system [22]. Silver has well recognized antibacterial properties on its own and Ag(I) can deactivate membrane proteins by binding to their thiol groups [14]. Silver also has a standard redox potential (799 mV (SHE) for the Ag/Ag(I) redox couple) in the range of biological reduction potentials, although it cannot cycle between two oxidative states [20]. Concentrations of 0.04 mg/L Cu(II) and Ag(I) completely inactivated *Legionella* after 1.6 hours, whereas the same concentration of Cu(II) alone was similarly effective only after 24 hours, and a double concentration of Ag(I) was effective after more than 24 hours [22]. In addition, the synergistic antibacterial activity of copper and silver ions against *Legionella pneumophila* was enhanced in presence of free chlorine [23]. In the present study, *S. aureus* in suspension with a copper-silver alloy coated surface at an initial cell concentration of 10^6 and 10^8 CFU/mL in phosphate-buffered saline (PBS) solution, was reduced dramatically to levels of 10 and 10^2 CFU/mL, respectively, after 24 hours (p=0.042) [Paper 1] (Figure 2a). When in a suspension with a stainless steel surface, the levels of *S. aureus* were unchanged after 24 hours, remaining at 10^6 and 10^8 CFU/ml.

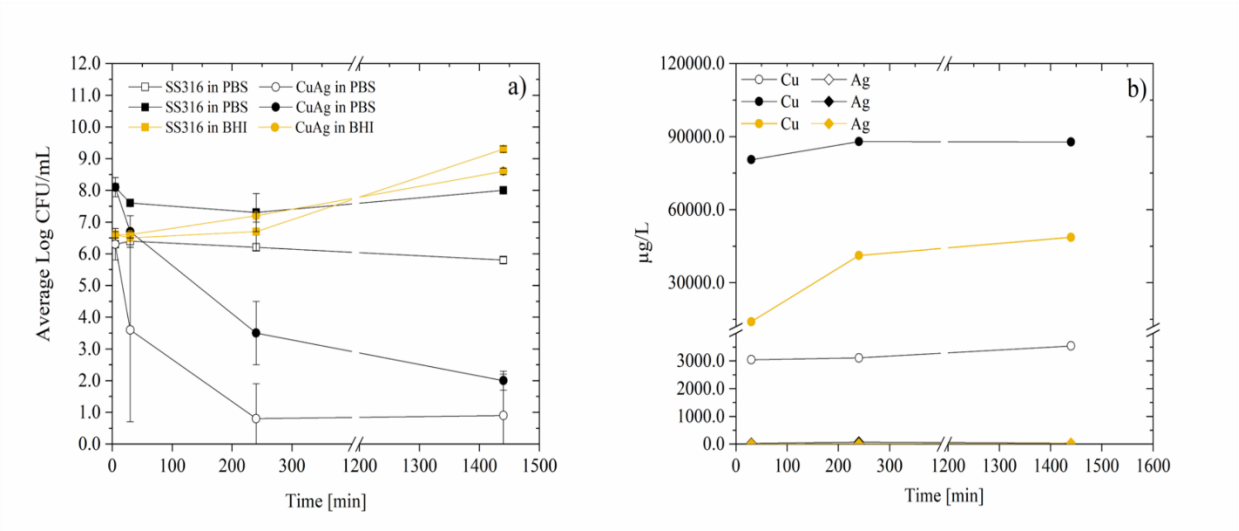

Figure 2. Survival of *S. aureus* 8325 at an initial concentration of 10^6 CFU/mL in PBS (○) and BHI (■), and 10^8 CFU/mL in PBS (●) suspended with copper-silver alloy coated and uncoated stainless steel surfaces after 30 minutes, 4 and 24 hours. Error bars indicate the standard deviation among the experimental replicates (a). Concentrations of copper and silver ions was measured in selected corresponding test suspensions of diluted (○) and concentrated (●) *S. aureus* culture in PBS and in BHI (●) with the copper-silver alloy coated surfaces (b) [Paper 1].

The presence of silver in the copper-silver alloy coating triggered a continuous release of copper ions in the test solutions, and the release was further enhanced by the more concentrated bacterial suspension, as shown in Figure 2b. In this respect, copper and silver in the alloy had a synergistic effect in the chloride-containing solutions, although there was virtually no silver release from the alloy coating (Figure 2b).

S. aureus at 10^6 CFU/mL in suspension with copper-silver alloy coated surface in Brain-Heart Infusion (BHI) medium reached approx. 10^9 CFU/mL after 24 hours [Paper 1] (Figure 2a). The same level was reached in suspension with uncoated stainless steel, indicating that copper ions were neutralized by the rich medium. Copper ion release was increased in the presence of growth media, however, the sequestering action of the nutrients reduced the available copper ions in solution, and this was mirrored by the lack of killing activity (Figure 2a and b). In addition, bacteria under growth conditions could efficiently regulate the uptake of copper ions and counteract their toxic action [18]. In this test setup, bacterial cells were exposed to both copper ions and contact with the alloy surface, so it cannot be excluded that the latter had some effect. The contact with a copper alloy surface is, however, crucial in conditions of dry exposure, as explained in section 2.2 [Paper 1].

2.2 Contact killing by copper alloy surfaces

The contact-mediated microcidal activity of dry copper alloy surfaces is called contact killing, and it is a rapid and effective phenomenon responsible for the recent interest in these surfaces [15]. The copper alloy surfaces release a high concentration of copper ions that accumulate at the aqueous interface with the bacterial cells [15] (Figure 3). This causes severe membrane damage and overload of toxic copper ions in the cytoplasm leading to inhibition of metabolic activities, nucleic acid degradation and bacterial death [15,18].

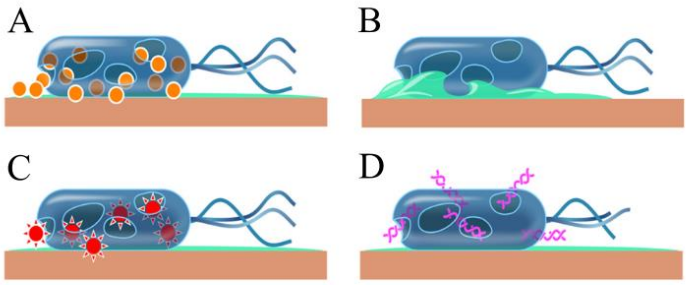

Figure 3. Events in contact killing. (A) Dissolved copper from the surface causes cell damage. (B) Cell membrane ruptures and cytoplasmic content is lost because of copper and other stress phenomena. (C) Copper ions induce the generation of reactive oxygen species, which cause further cell damage. (D) Genomic and plasmid DNA becomes degraded [15].

Bacterial cell membranes mainly consist of phospholipids, polar head groups covalently bonded to two long-chain fatty acids containing saturated and unsaturated double bonds [24]. Unsaturated fatty acids in the bacterial cell membranes can be oxidized by hydroxyl radicals, and released copper (Cu(I)) catalyzes their formation via the Fenton- like reaction below:

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + \cdot OH + OH^- \quad (1)$$

As a consequence, the distorted phospholipid bilayer can no more absolve its function, the membrane loses integrity, and its permeability and the regulating activity of integral membrane proteins are affected [24]. Thus, toxic copper ions have easy entry and overload the bacterial cell, inhibit metabolic activities and degrade nucleic acid. An *E. coli* mutant strain with higher levels of unsaturated fatty acids exhibited an earlier rise in lipid peroxidation, higher sensitivity to contact killing, and an earlier onset of DNA degradation [19,24]. The reactive oxygen

species (ROS) generated by the catalytic activity of Cu(I) can also inhibit the respiratory chain or divert electrons from it, leading to further ROS production [19].

The contact between the metallic surface and the bacteria is crucial in contact killing, since it damages the cell envelope allowing access of copper ions in the cytoplasm, where further damage ensues [19]. There was essentially no killing of an *Enterococcus hirae*, when the contact between bacteria and the copper surface was prevented, but not the ionic release [19]. Interestingly, *E. hirae* cells exposed to a metallic iron surface in presence of 4 mM $CuSO_4$ were killed in 100 minutes [19]. Iron is also a redox active metal, (Fe(II)/Fe(III) has a standard redox potential of 770 mV (SHE), but has no antibacterial activity by itself. Therefore, ionic copper and metallic iron surfaces acted synergistically to cause copper ions-mediated contact killing of *E. hirae* on iron surfaces [19]. This clearly indicates that both the metal-bacterial contact and presence of toxic ions is required for efficient contact killing.

2.3 The role of surface properties in contact killing by copper alloys

As mentioned above, bacterial-metal contact is a crucial factor for contact killing by copper surfaces. The metallic surface topography, its composition and electrochemical reactivity towards the surrounding environment can influence the exposed area, surface chemistry and ionic release. The state of a copper alloy surface is therefore decisive for the efficacy of contact killing. Three key physicochemical aspects summarize the surface properties involved in contact killing by antibacterial metallic materials, and in particular by copper alloys (Figure 4).

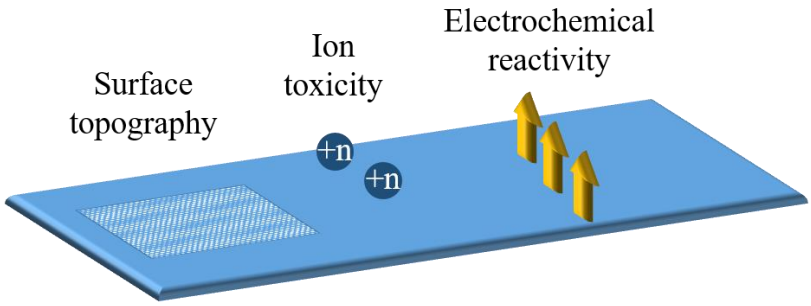

Figure 4. Ion toxicity, surface topography and electrochemical reactivity are key factors influencing the contact killing by an antibacterial metallic surface. Modified from Hans et al. [16].

In subsections 2.3.1 and 2.3.2, surface topography and electrochemical reactivity are discussed, whereas the understanding and use of electrochemical properties of copper to obtain copper alloys with enhanced antibacterial activity is presented in 2.3.3.

2.3.1 Surface topography of copper alloys

In the context of surface hygiene and cleanliness, the relationship between surface roughness and bacterial adhesion currently remains a controversial aspect [25]. Adhesion forces increased with greater surface roughness (at the µm-scale), and bacterial adhesion was enhanced in the presence of rough inert, such as borosilicate glass, surfaces [25,26]. However, the surface finish of stainless steel (2B finishing, grit 80, grit 120, grit 4000 polishing and electropolishing) influenced the corrosion properties of the metal but not the attachment and removal of *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Candida lipolytica* in a flow system [27]. In addition to surface charge and wettability, static or flow test conditions and size of bacteria in comparison to micro scratches and grooves can explain the contrasting results [25,28]. Surface roughness is, however, a key determinant of antibacterial activity of copper alloys. Under wet plating test conditions, electroplated copper surfaces were able to kill *E. coli* more rapidly (i.e. 60 minutes vs. 100 minutes-exposure for 8-log reduction) than surfaces with a smoother finish (rolled or polished) [29]. The rougher copper surface had a greater exposed area, thus ionic release and bacteria-metal contact were enhanced [29] (Figure 5).

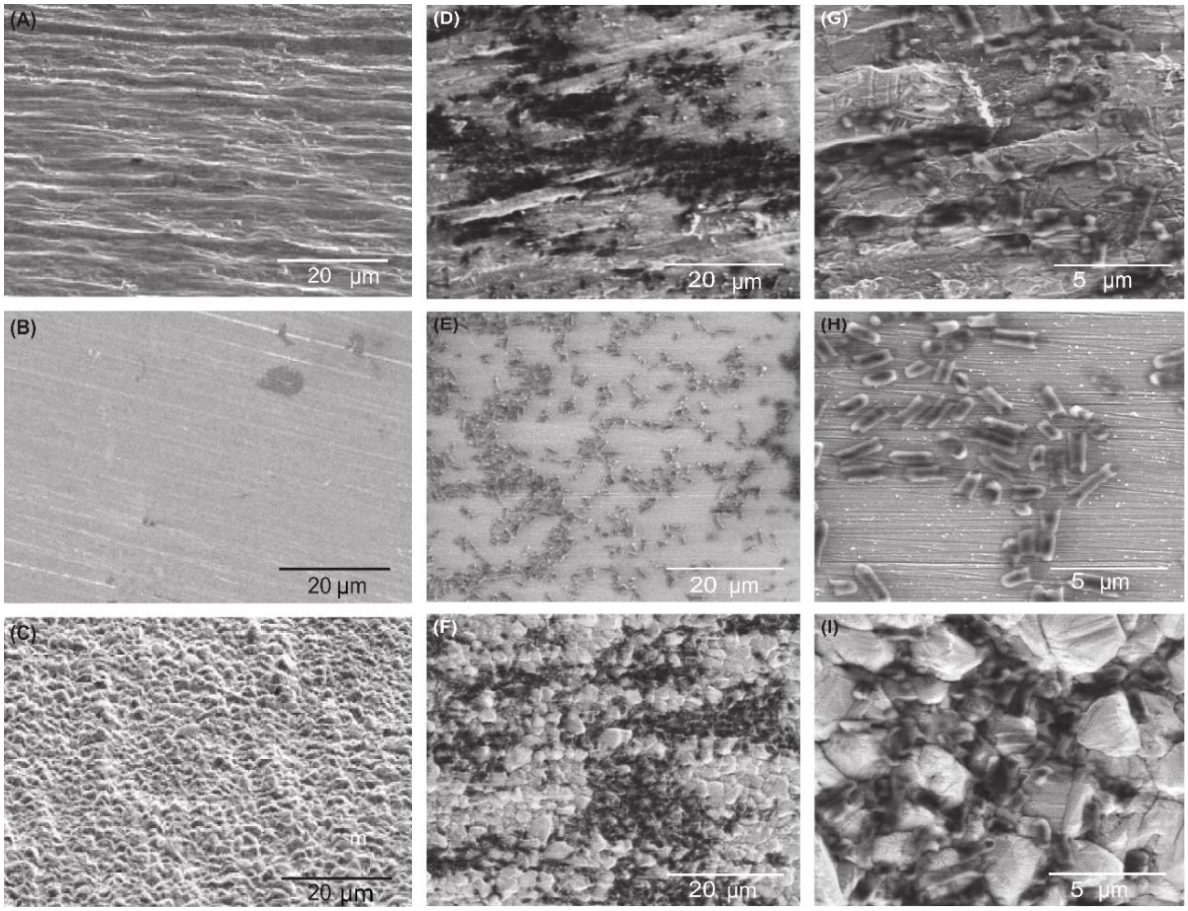

Figure 5. Scanning electron micrographs of industrial rolled copper (A), polished copper (B) and electroplated copper (C) surfaces. Scanning electron micrographs of *E. coli* on rolled copper surfaces (D, G), polished copper (E, H) and electroplated copper surfaces (F, I) surfaces [29].

In the present study, the copper-silver alloy coated surface obtained by electroplating on stainless steel, was also characterized by high surface roughness, and it had approx. 25% more exposed area, as compared to the 2B finished AISI 316 stainless steel substrate (Figure 6).

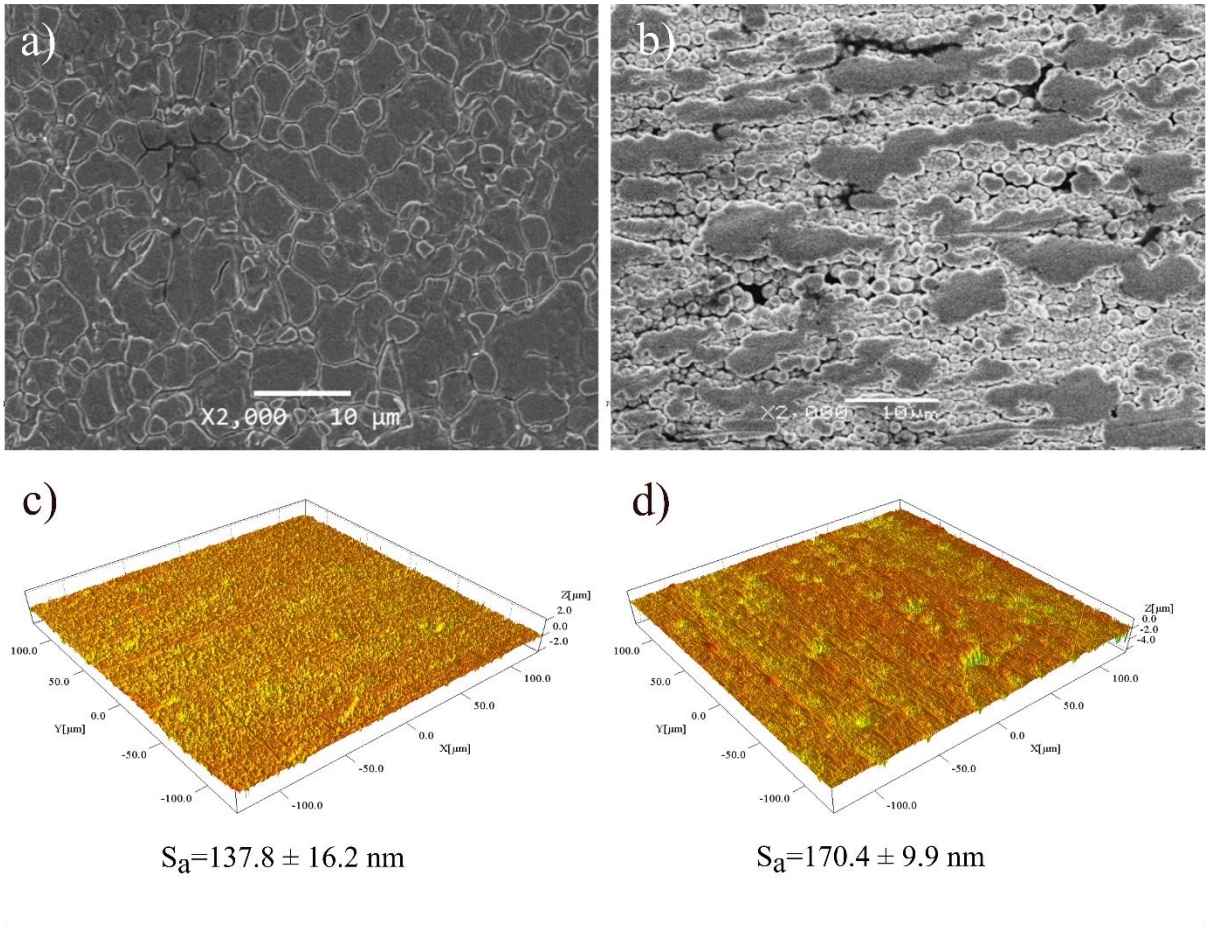

Figure 6. Scanning electron microscopy (SEM) images of uncoated (a) and copper-silver alloy coated (b) AISI 316. Surface mapping of uncoated (c) and copper-silver alloy coated (d) AISI 316 was done using a confocal microscope LEXT® OLS 4100, Olympus, Tokyo, Japan and SPIP software (Image Metrology,Hørsholm, Denmark). The areal roughness values of both surfaces was calculated as the average (±standard deviation) of 5-point measurements at 50× magnification [Modified from Paper 1].

The levels of attached *S. aureus* recovered from electroplated pure copper and copper-silver alloy coatings were approx. 2 and 4.5 logs reduced in comparison to 2B finished AISI 316L stainless steel after 30 minutes exposure in PBS [Paper 1] (Table II).

Table II. Attachment of *S. aureus* 8325 to coated and uncoated stainless steel AISI 316L. Coatings were obtained by electroplating AISI 316L with pure copper, pure silver, and copper-silver alloy. Numbers are mean values ± standard deviations of three biological replicates [Modified from Paper 1].

Initial cell concentration Log (CFU cm^{-2})	**Time (h)**	**Attachment (Log (CFU cm^{-2})) of *S. aureus* 8325**			
		AISI 316L	**copper**	**silver**	**copper-silver alloy**
7.0	0.5	4.7 ± 0.1	2.5 ± 0.7	4.9 ± 0.1	0.1 ± 0.1

In these test conditions, bacteria cells were suspended with copper ions releasing electroplated metallic surfaces, thus exposed to both surface contact and toxic ions. The surface topography and composition of the electroplated copper-silver alloy enhanced its antibacterial activity, resulting in the lowest number of live attached bacteria recovered from the surfaces. Electroplated pure silver surfaces had a comparable roughness with electroplated copper and copper-silver alloy, however, silver did not chemically interact (i.e. no release of toxic ions) when immersed in PBS [Paper 1, 2]. Thus, the electroplated silver surfaces simply offered a greater available area for the bacterial attachment, resulting in the highest number of live attached bacteria recovered from the test surfaces (Table II). It follows that roughness is not a stand-alone parameter to determine the antibacterial properties of a metallic surface, but it is interlinked with the reactivity of the material in the test conditions.

2.3.2 Electrochemical reactivity of copper alloys

Copper is a malleable and ductile metal with a good thermal and electrical conductivity, and thanks to its properties it is widely found, as pure metal, alloys and coatings, in many applications, such as electrical wiring, pipes, valves, fittings, coins, furniture and building material, not to mention the use of chemical copper compounds [30]. Copper is, however, prone to atmospheric corrosion, i.e. the electrochemical process leading to surface oxidation and modification of the material properties. Under environmental conditions, humidity, pH, oxygen availability, presence of oxidizing agents or complexing compounds strongly affect the electrochemical behavior of copper [30].

Copper has two main oxidation states (+1 and +2), therefore it can exist as Cu(I) and Cu(II) ions in aqueous environment. Cu(I) ion is a soft acid and is stabilized by the presence of soft bases, whereas Cu(II) ion is a borderline acid and water (hard base) stabilizes it, according to the hard and soft acids and bases principle [31]. Also, Cu(I) ion undergoes a disproportionation reaction in aqueous media, which means that the formation of Cu(II) ion and metallic copper is thermodynamically favored (2). The net reaction from the reduction (ii) and oxidation (iii) is

$$2Cu^{+} \leftrightarrow Cu^{2+} + Cu \quad (2)$$

with a redox potential of (521−153=368 mV) (Table III).

Table III. Equilibrium reactions and standard redox potentials for copper and silver calculated against the standard hydrogen electrode (SHE) [20].

	Equilibrium reaction	**Standard redox potentials (E^0) values vs. SHE**
(i)	$Cu^{2+} + 2e^{-} \leftrightarrow Cu$	341 mV
(ii)	$Cu^{+} + e^{-} \leftrightarrow Cu$	521 mV
(iii)	$Cu^{2+} + e^{-} \leftrightarrow Cu^{+}$	153 mV
(iv)	$Ag^{+} + e^{-} \leftrightarrow Ag$	799 mV

However, Cu(I) ion can be stabilized by the presence of soft or borderline bases, such as RS^{-} (R stands for alkyl or aryl group) and Cl^{-}, when they are also present in the aqueous environment [31]. The Pourbaix diagrams also show that Cu(II) ion is the predominant state up to pH 6 in pure water (Figure 7). Cuprous oxide (Cu(I) oxide or Cu_2O) can form from pH 4.5 to 12, but in most instances Cu(I) ion is subsequently oxidized to Cu(II) ion, and cupric oxide (Cu(II) oxide or CuO) and hydroxide are formed above pH 6 in the stability region of water (Figure 7).

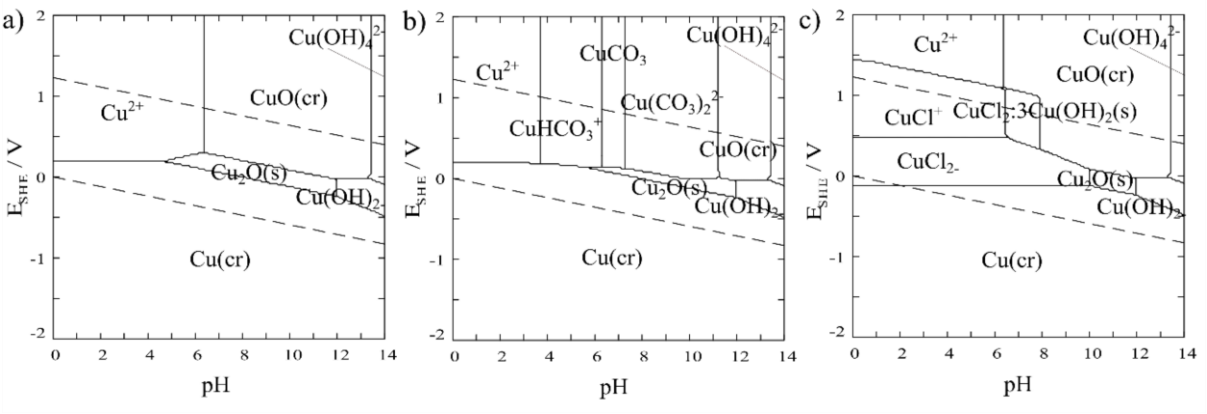

Figure 7. Predominance (Pourbaix) diagrams (E-pH) of copper ([Cu+] = 10^{-5}M) in pure water (a), presence of carbonates ([CO_3^{2-}]= 1M) (b) and chlorides ([Cl^-]= 1M) (c). The stability region for water is indicated by the dotted lines. Medusa software is used for the calculation [32].

In the presence of carbonates, Cu(II) ion forms $CuCO_3$ and $[Cu(CO_3)_2]^{2-}$ at pH 6-11, and stable Cu(I) chloride complexes can form in chloride-containing media [30] (Figure 7).

Under atmospheric conditions, metallic copper surfaces naturally tend to oxidize and this may affect their antibacterial properties. In a dry atmosphere, cuprous oxide preferably forms, whereas the formation of cupric oxide is favored in a humid atmosphere [16]. Thus, in ambient air and humidity, a copper oxide layer can consist of both cuprous and cupric oxides in varying proportion depending on oxidizing conditions and aging [16,33]. Oxidizing conditions and acidic pH induce dissolution of copper oxides to Cu(II) ions, whilst Cu(I) ions are released and cuprous oxide is formed at more alkaline pH in the presence of chlorides [16] (Figure 7). Cu(I) ion is more toxic against bacteria than Cu(II) ion, and it can be released from metallic copper and cuprous oxide [33]. The influence of chlorides on copper alloy surfaces will be further elaborated in chapter 5.

Cupric oxide predominantly formed in presence of PBS and Tris-Cl buffer solutions under wet plating test conditions, and had less antibacterial activity against *E. hirae* (4 logs reduction) with respect to cuprous oxide or metallic copper (7 logs reduction) after 300 minutes of exposure [33]. The lower solubility of cupric oxide (pK_s of -23.5) and release of less toxic Cu(II) ion explain the reduced antibacterial activity, as compared to cuprous oxide (pK_s of -9.0) and metallic copper [16]. Silver oxide (Ag(I) oxide or AgO) has an even higher solubility (pK_s of -7.7) than cuprous oxide, but metallic silver surfaces do not readily oxidize under environmental condition, due to the nobility (more positive reduction potential) of silver, so they have no antibacterial effect [16] (Table III). Therefore, the electrochemical reactivity of a

metallic surface, intended as reducing/oxidizing activity and behavior in the surrounding environment, is important to determine and evaluate its antibacterial properties.

2.3.3 Use of electrochemical properties of copper to produce antibacterial surfaces

Knowledge about the electrochemical reactivity of metals and electrochemical mechanisms of corrosion can be used to accurately engineer surfaces with enhanced antibacterial properties. By combining two metals with different reduction potentials, the selective oxidation of the less noble metal (less positive potential) is achieved. This is the principle of galvanic or bimetallic corrosion that is generally an unwanted phenomenon, especially in construction and connector materials. However, the antibacterial properties of copper can be enhanced by coupling with a more noble metal, e.g. silver, in principle because the release of toxic copper ions is increased as a result of the galvanic corrosion. This was the idea behind the design of a copper-silver (90-10 wt%) alloy laser-clad coating for stainless steel [34]. A 28-times higher release of copper ions was obtained from this copper-silver alloy in comparison to pure copper, and this corresponded to a superior antibacterial efficacy of the alloy against *E. coli* [34]. The "sacrificial" dissolution of copper also maintained the level of silver ions low [34]. However, the release of copper ions, i.e. the oxidation reaction, is not the full picture of the galvanic redox process.

A copper-silver alloy coated surface is electrochemically active, which means that a galvanic cell is established and electrons move from the anode to the cathode in presence of an electrolyte. Copper has a lower electrochemical potential than silver (Table III), so it oxides to copper ions and electrons. Electrons move to the cathode (silver) and in presence of an aqueous surface layer, the reduction reaction

$$O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \quad (3)$$

takes simultaneously place. In the present study, an almost instant local pH raise to values of approx. 9.5 was measured, followed by a slower decrease due to Cu_2O formation (in presence of chloride) at the surface of the alloy [Paper 3] (Figure 8b).

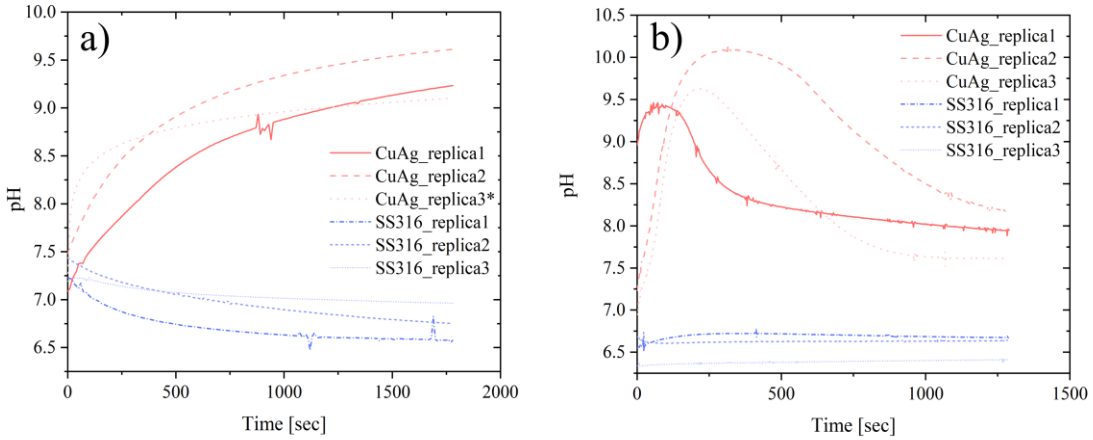

Figure 8. pH monitoring at copper-silver alloy coated and uncoated SS316 surfaces with 0.15 M NaCl 0.5% agarose matrix loaded with *Staphylococcus aureus* 8325 suspension (a) and unloaded (b). *the replicate was fitted with a model that allowed extrapolation of its initial pH rise, due to a slower positioning of the sensor [Paper 3].

The pH rapidly increased and remained at values of approx. 9.0, when a *S. aureus* suspension was present at the interface [Paper 3] (Figure 8a). In these conditions, a new galvanic series was established: silver, holding the highest (most positive) reduction potential, followed by copper and bacteria [Paper 3]. Bacteria are reducing agents, i.e. the preferred site for the oxidation reaction to occur, in this three-element system. Therefore, it was hypothesized that the galvanic coupling of copper and silver induced the oxidation of bacterial cells in contact with the alloy, since they had the lowest reduction potential, and the reduction reaction occurred at the metal sites. At the same time, copper was oxidized to copper ions and the reduction reaction generated OH^- on silver in the areas not occupied by bacteria cells [Paper 3] (Figure 9).

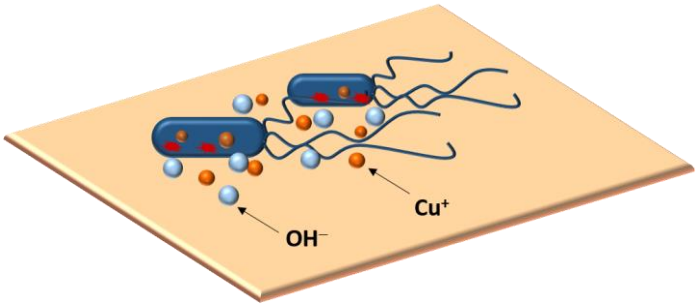

Figure 9. The antibacterial activity of the electroplated copper-silver alloy is due to a redox reaction, induced by the galvanic coupling of the metals and by bacteria in contact with the alloy. Oxidation of bacterial cells, release of copper ions and local pH raise under environmental conditions can ensure antibacterial activity of this alloy in the intended applications [Paper 3].

2.4 Conclusions on the antibacterial activity of copper ions and copper alloy surfaces

Copper alloy surfaces are efficient in contact killing because of:

i. The redox activity of copper
ii. Bacterial intracellular damage caused by toxic copper ions

The amount of copper ions available per bacterium makes contact killing essentially different from copper ions toxicity in a bacterial suspension. Once the contact with a copper alloy surface is established, copper ions start dissolving and the portion of bacteria laying on the surface is rapidly soaked in very high (mM) copper concentrations [18]. The copper ions concentration is particularly high also due to the absence of copper binding agents, such as buffer or media components, which can instead be present in bacterial suspensions. In the latter case, bacteria are exposed to an actual concentration in the range of µM, and they are protected by the nutrient components [18]. Copper homeostasis mechanisms can intervene and efflux copper ions out of the bacterial cell counteracting their toxic action. An enlarged exposed area increases both the bacterial-metal contact and the ionic release, hence rougher copper alloy surfaces have higher antibacterial activity. Since copper is a redox active metal, copper alloy surfaces react with the surrounding environment. Humidity, pH and oxidizing conditions can induce the dissolution of copper and the formation of copper oxides, which may influence the antibacterial activity of copper alloy surfaces. However, the understanding of the electrochemical properties of copper can be used to obtain copper alloy surfaces with superior antibacterial activity.

3. Copper alloy and copper-based coatings and their potential as antibacterial strategies

Brass (copper-zinc) and bronze (copper-tin) are the best-known copper-containing alloys that have been used for centuries in different applications, from decorative to low friction materials. Copper alloys have been receiving increasing research interest, since one study demonstrated the antibacterial activity of brass doorknobs against *Escherichia coli* in a hospital [35]. In 2008, the U.S. Environmental Protection Agency (EPA) registered five groups of alloys (later updated to six) containing from 60 to 99.99% of copper, as antibacterial agents [16,36,37] (Table IV).

Table IV. Nominal alloy composition (weight %) of six registered copper alloys (Antimicrobial Copper Cu^+ alloys) [36,37].

Alloy UNS Number	Cu	Zn	Sn	Ni	P
C11000: Copper	99.9				
C26000: Brass	70	30			
C51000: Bronze	94.8		5		0.2
C70600: Cu-Ni	88.6			11.4	
C75200: Cu-Ni-Zn	65	17		18	
C28000: Brass	60	40			

Doorknobs, bedrails, bathroom fixtures, tables, armrests, IV poles made of such antimicrobial copper alloys are already commercially available, and have been installed in hospital wards [38–40]. Copper is a very versatile material, it is ductile, 100% recyclable and easy to process. More than 400 copper alloys can be produced by metal casting processes only [41].

Hence, there is a great potential also for other manufacturing processes to offer alternative copper alloys or new combinations that can suit the requirements in antibacterial applications. Surface coatings and films are particularly attractive solutions, since they can impart the desired antibacterial functional characteristics to the surface of a bulk material characterized by other properties, e.g. mechanical strength and low-cost [42]. The surface technology sector is one of the most significant cross-sectoral manufacturing branches in the European economy, although smaller when compared with the whole mechanical engineering sector [43]. Electroplating takes up one third of the surface technology sector in Europe, painting industry another third, and the last third includes chemical and physical vapor deposition, plasma technologies, metal spraying and their combination [43]. There is a global demand for improved solutions against transmission of life-threatening diseases, and surface engineering

techniques can be used to produce antibacterial copper-based coatings, thus increasing surface hygiene.

Section 3.1 presents a few examples of antibacterial copper-based coatings, commercially available or under development. Electroplating, as an industrial process to obtain the antibacterial copper-silver alloy coating, and antimicrobial coatings and sustainable development is discussed in 3.2 and 3.3.

3.1 Antibacterial copper alloy and copper-based coatings

Antibacterial coatings can provide cost-effective and tailored solutions meeting the demands of specific applications. Copper-based coatings have natural limitations and their chances of success in the intended use and applications increase, if the strategies of design and implementation are attentively evaluated. Coatings that can be applied on already existing objects are particularly advantageous, since this can limit the costs. The industrial performance and scalability of the manufacturing process are major factors affecting the commercial success of laboratory-developed production techniques. Table V presents a few examples of antibacterial copper-based coatings.

Copper oxide impregnated polymeric solid surfaces (Cupron Enhanced EOS Surfaces) and copper particles-methyl methacrylate resin composite coating (Copper Armour™) are already commercial product and have been used in clinical trials (Table V). In a hospital in Santiago (Chile), the level of microbial contamination was reduced in intensive care unit rooms where Copper Armour™ coated bed rails, IV poles, overbed and bedside tables were installed [44] (Table V). Catheters coated with silver-copper nanoparticles efficiently prevented the adherence of *S. aureus* MRSA *in vitro* (0 to 12% colonization) and catheter infections *in vivo* (0 to 20% colonization), compared to uncoated catheters (50 to 100% colonization *in vitro*; 83 to 100% *in vivo*) [45] (Table V). However, the adsorption of plasma proteins on the catheter surface generated a sheath hindering the contact of the Ag/Cu film and limiting its activity [45].

Table V. Antibacterial copper-based coatings are listed according the manufacturing process, composition, applications and antibacterial efficacy. Main advantages and disadvantages for each solution are also reported. Antibacterial efficacy was assessed using *a wet plating method, ** U.S. EPA copper test protocols, *** a wet plating method, † a dry plating method, †† immersion testing, ††† adhesion testing (see references for an extensive explanation of the assessments).

Manufacturing technique/copper incorporation	**Composition and copper state**	**Applications**	**Antibacterial efficacy**	**Advantages**	**Disadvantages**	**Ref.**
Atmospheric pressure jet plasma	Cu(II) oxide (0.4-7.5 at%) thin (nm) coating on ABS	Polymer surfaces (e.g. textiles and weavings)	Approx. 2-log reduction *S. aureus* after 2 h*	Low temperature, and no vacuum required.	Low antibacterial efficacy. Process dependent-composition and size limited.	[46]
Polymer and copper oxide blend are mixed, heated and cured in mold	Cu(I) oxide (16 wt%) in blend with acrylic and polyester resins	Wide range of base materials (e.g. table countertops)	Approx. 5-6-log reduction *S. aureus* (MSSA and MRSA), *E. aerogenes*, *P. aeruginosa* and *E. coli* after 2 and 24 h*	No size limitation. Can be cut and shaped to produce a final product.	Multi-step process. Mechanical durability.	[47]
Aerosol assisted chemical vapor deposition	Cu-nanoparticles incorporated in polydimethylsiloxane	Potentially air filters and touch surfaces	Approx. 4-log reduction *E. coli* and *S. aureus* after 15 min and 1 h***	Superhydrophobicity preventing bacterial adhesion.	High temperature and possibly object size limited. Mechanical durability.	[48]
Polymeric matrix, copper particles and hardener are homogenized and solidified	Copper particles of various size and shape embedded in methyl methacrylate resin (60/40)	Bed rails, IV poles, overbed and bedside tables	Approx. 5-6-log reduction *S. aureus*, *P. aeruginosa*, *E. coli* and *L. monocytogenes* after 1 and 24 h**	Can be used to modify existing hospital surfaces.	Separated formulations of components and multi-step process.	[44]
Sequential magnetron sputtering	Multilayer Ag-Cu surface films (5 nm)	Medical devices	Approx. 5-log reduction *S. epidermidis* after 10 h†	Enhance the efficacy of silver or copper single layer films.	Process requires high vacuum conditions.	[49]
Direct-current magnetron sputtering of silver-copper nanoparticles	Ag/Cu (67-33 at%) film (80 nm)	Catheters	Approx. 5-log reduction *S. aureus* MRSA after 90 min††	No skin toxicity in *ex vivo* human model.	Process requires high vacuum conditions. Formation of fibrin sheath *in vivo*.	[45]
Laser cladding	Cu-Ag (90-10 wt%) alloy coating	Stainless steel in healthcare settings	6-log reduction *E. coli* ***	Applicable on existing objects.	Time-consuming and no composition uniformity.	[34]
Electroplating	Cu-Ag (60-40 wt%) alloy coating (2-10 µm)	Metals in e.g. healthcare settings and food production equipment	Approx. 7-log reduction *S. aureus* and 5-log reduction *E. coli* ††† Approx. 5-6-log reduction *S. aureus* MSSA and MRSA, *P. aeruginosa*, *E. aerogenes***	Not limited by the substrate shape, size and material. Applicable on existing objects.	Multi-step process for thick coatings. Polymers need specific pretreatment.	[Papers 1, 3, 4]

3.2 Electroplating and the potential of a copper-silver alloy coating

Electroplating is versatile technique and especially suitable for large-scale production; metallic, plastic, ceramic or composite substrates are all virtually suitable to electroplating after the appropriate pretreatment. Moreover, size and shape complexity of the substrate are not major restraints.

The copper-silver (60-40 wt%) alloy coating can be electroplated on solid materials, such as stainless steel, that can have a complex shape, thanks to the versatility of this deposition technique (Table V) [Patent application]. As such, the surface of an item is endowed with high antibacterial properties, maintaining the bulk properties of the base material. Considering an item with a surface area of approx. 1 dm^2 (e.g. a door handle), the production cost of electroplating the item with the copper-silver alloy coating (2 μm) is approx. € 5. The material cost (considering the current quotation of silver, the most expensive component, to be approx. 430 €/kg), average labor (approx. 40 €/h) and equipment cost (approx. 5 €/h) in a medium-low production volume (~100 parts) is used for giving an idea of the expected costs associated with this process, according to the source [50]. The online price of a set of stainless steel door handles (Ruko Assa Abloy) is approx. € 10, if coated with the copper-silver alloy the total price will be € 20, which is still considerably lower than the price of an equivalent brass door handle set from the same manufacturer (€ 67).

Therefore, considering its antibacterial properties and potential industrial feasibility, the copper-silver alloy electroplated coating qualifies as promising antibacterial coating solution. It can be implemented as target intervention, alone or in combination with other technologies, on already installed or new objects in hotspot areas for bacterial contamination, according to the customers' requirements.

Additional parameters need also to be taken into account in an industrial production. Wastewater management is of prime importance in the electroplating industry and determines its environmental footprint and process cost. Metal recovery and recycling from exhausted baths, and stripping (removal) of old coatings are necessary to maintain the electroplating process a competitive technique. This is important also in the light of a regenerative design approach, where the items can be re-coated once the copper-silver alloy coating would have reached the end of its lifetime (approx. 12 months depending on the application). This will ensure a regular quality check and high performances in terms of antibacterial efficacy.

According to the Centers for Disease and Prevention, the average annual direct cost to treat hospital acquired infections per patient is between US$ 20,549 and 25,903 (approx. € 18,400-23,100) [51]. Therefore, the re-coating costs (approx. the same order of magnitude of the coating costs) will be amply redeemed if infections will be prevented.

3.3 Antimicrobial coatings and sustainable development

Global-warming and population ageing are putting pressure on healthcare systems, increasing the demand for care, services and technologies to prevent and treat diseases [52,53]. The increasing concern of bacterial infection and transmission of life-threatening diseases have raised emphasis on environmental hygiene [54]. In this context, the global demand for antimicrobial coatings has been increasing, and in turn, the market size of antimicrobial coatings has growth. The global antimicrobial coatings market produced more than US$2.5 billion revenue in 2015 with a forecasted market growth of 10.1% CAGR (compound annual growth rate) by 2024, according to the latest report (May 2019) from MarketWatch and Comtex News Network. It comprises not only the medical and healthcare sector, but also indoor air quality, food application, antimicrobial textile, mold remediation application and construction [55]. Antimicrobial coatings can contribute to reach at least two of the global goals for sustainable development, i.e. good health and clean water and sanitization (Figure 10).

Figure 10. The 17 global goals for a better world by 2030 (https://www.globalgoals.org/).

The solution for preventing environmental surfaces, such as door handles, to spread pathogens and infectious diseases is at hand.

3.4 Conclusions on copper alloy and copper-based coating and their potential as antibacterial strategies

Antibacterial solutions based on copper alloys are already commercially available. Antibacterial copper-based coatings can offer more suitable alternatives as target intervention in some specific applications, but can also be used in combination with the existing technologies to achieve an all-round protection against microbial contamination in healthcare settings, biopharmaceutical industry and food production environments. The global demand of antimicrobial coatings has been increasing to prevent spread of life-threatening diseases, and the copper-silver alloy electroplated coating has the potential to contribute to the fight against transmission of pathogens.

4. Methods for determining the antibacterial activity of copper alloy and copper-based surfaces

Regulatory agencies such as ASTM International, the European and International Organization for Standardization (ISO), the United States Environmental Protection Agency (U.S. EPA) and the Japanese Industrial Standards (JIS) Committee provide different test methods for the assessment of the antimicrobial activity of materials. However, there is currently no universal standardized test method for the determination of biocidal efficacy of hard surfaces [56]. Investigators have the freedom to choose the preferred test method according to the testing material and modify test protocol as long as a rationale is provided [56]. This has, per contra, the drawback that comparing the performances of antimicrobial hard surfaces from different studies is very difficult. Another important issue to consider is the resemblance of the testing to real-life conditions. Test methods need to provide an estimation of the antibacterial performances taking into account as many as possible relevant environmental parameters, while ensuring reproducibility [56]. However, test methods cannot yet provide accurate information about the long-term performances of antibacterial surfaces and in addition, the insufficient time devoted in the testing and analysis of the active antibacterial agents has limited the spread of currently available products [42].

In section 4.1, enumeration-based test methods (official and from literature) for assessing the antibacterial activity and efficacy of surfaces are presented, and compared in the light of their applicability to copper alloys and in particular to the copper-silver alloy coating, and relevance to the intended application. Section 4.2 discusses the alternative use of microscopy combined with live/dead staining techniques on copper alloys, and presents an experimental setup that allows *in situ* visualization, monitoring and quantification of contact killing of bacterial biofilm on the copper-silver alloy coating [Paper 3].

4.1 Enumeration-based methods for assessing the antibacterial activity of surfaces

Quantitative tests aim at determining the actual level of bacterial reduction, i.e. antimicrobial activity, of a test material as compared to a control material/suspension after a certain exposure time. Whilst quantifying bacterial survival in suspension is relatively straightforward, bacteria exposed to a surface attach, and need to be removed prior to the quantification of viable cells.

Most of the official protocol use sonicating, vortexing or a stomacher to detach bacteria from the test surfaces, after immersion in a neutralizer medium that stops their antibacterial activity. After that, the suspension is serially diluted and plated on agar. Viable cell counting is usually performed after 24 or 48 h. Some protocols use a direct inoculation technique, i.e. the inoculum (10-400 µl) is applied and spread on the surface, while others require the immersion of test samples in a bacterial suspension. The available test methods for antibacterial surfaces and their most relevant features are summarized for comparison in Table VI.

Table VI. Standardized test methods to determine the antibacterial activity of surfaces.

Test name	Test organisms	Inoculum level and volume	Test conditions	Cell recovering	Acceptance threshold for antibacterial activity
Direct inoculation methods					
JIS Z-2801/ ISO 22196	– *S. aureus* ATCC 6538P – *E. coli* ATCC 8739	– 2.5-10×10^5 CFU/mL – 400 µL on 50×50 mm samples	RH ≥ 90% (35±1)°C	Using 10 mL neutralizer (SCDLP broth) and e.g. stomaching. Serial dilution and plating on agar.	Not set
EN 13697	– *E. hirae* ATCC 10541 – *E.coli* ATCC 10536 – *P. aeruginosa* ATCC 15442 – *S. aureus* ATCC 6538	– 1.5-5×10^8 CFU/mL – 50 µl on Ø20 mm samples – Presence of interfering substance simulating clean and dirty conditions	Air-dried inoculum (18-25±1)°C	Using 10 mL neutralizer (e.g. SCDLP broth) and shaking with glass beads. Serial dilution and plating on agar.	≥4-log reduction from water control after 5 min ± 10 s contact
EPA Test Method for Efficacy of Copper Alloy Surfaces as a Sanitizer EPA Test Method for the Continuous Reduction of Bacterial Contamination on Copper Alloy Surfaces	– *S. aureus* ATCC 6538 – *E. aerogens* ATCC 13048 – *P. aeruginosa* ATCC 15442 – *S. aureus* MRSA ATCC 33592 – *E.coli* O157:H7 ATCC 35150	– Approx. 10^8 CFU /mL – 20 µl on 25.4×25.4 mm samples – Presence of interfering substance simulating dirty conditions	Air-dried inoculum (25±1)°C	Using 20 mL neutralizer (e.g. Letheen broth) and sonicating. Serial dilution and plating on agar.	≥3-log reduction (99.9% reduction in numbers) from control surfaces after 2 h ≥1-log reduction (90% reduction in numbers) at all recovering 2 h staggered-times over 24 h
EPA Test Method for Residual Self-Sanitizing Activity of Copper Alloy Surfaces		– Approx. 10^8 CFU /mL – 10 µl on 25.4×25.4 mm samples – Dry/wet wear cycles and re-inoculation			≥3-log reduction (99.9% reduction in numbers) from control surfaces after 2 h (and 12 wear cycles)
Immersive inoculation method					
ASTM E2149 − 13a	*E. coli* ATCC 25922	– 1.5-3.0×10^5 CFU/mL – 25.8 cm^2 sample size – Shaking at max. stroke	50 ±0.5 mL bacterial KH_2PO_4 buffer solution	After 1 h serial dilution and plating on agar	Not set

4.1.1 Methods based on direct inoculation

The JIS Z-2801/ISO 22196 test method evaluates the antimicrobial activity and efficacy against bacteria on the surface of antimicrobial products (plastics, ceramics and metals) [57,58] (Table VI). Bacterial cells (initial concentration of approx. 10^5 CFU/mL) are exposed and recovered from the test surfaces by detachment in soybean casein lecithin polysorbate broth using a stomacher, prior to serial diluting and plating [57,58]. The test conditions have the drawback of high humidity that, in the light of dry applications, does not correspond to typical indoor environments. In addition, there is no specific threshold level of antimicrobial efficacy (i.e. log reduction after a set time). Hence, this test can be used for an indication of antibacterial activity within an arbitrary chosen exposure time. In EN 13697, a higher concentration of inoculum (approx. 10^8 CFU/mL) is applied on the surface, and a reduction of at least 4 log (as compared to a water control) after 5 minutes contact is set for the test success [59] (Table VI). However, this test is designed for surface liquid detergents and it should be modified to accommodate antibacterial surfaces. The procedure establishes that the bacterial inoculum is left to air dry on the surface, prior to the (eventual) addition of detergent. Here, bacteria are recovered from the surfaces by immersion in SCDLP and shaking with glass beads. Bacteria can remain attached to glass beads and the vial count may be biased, especially if compared to a water control and not an inert surface. Air-drying the inoculum is relevant for the intended application of a copper alloy surface, but additional longer contact times would be required.

In 2015, the U.S. EPA released tailored protocols for testing and evaluating the antibacterial efficacy of copper alloys with the intention of providing harmonized test conditions closely resembling real-life applications of such surfaces, e.g. environmental indoor items in healthcare facilities [60–62] (Table VI). The first two protocols evaluate the sanitizing efficacy of copper alloys on test organisms after 2 hours exposure and over a 24-hour interval in presence of a continuous bacterial contamination [60,61]. The 20 µl inoculum consists of a bacterial suspension and an organic soil load (fetal bovine serum and Triton X-100) that simulates dirty conditions, and it is let to air dry for 20-40 minutes before the beginning of the exposure period [60,61]. Bacteria are recovered from the test surfaces using a Letheen broth and sonicating, followed by serial dilution and plating on agar. An air-dried inoculum is consistent with the application of copper alloy surface as environmental touch-items, and the reinoculation procedure well simulates the overtime build-up of bacterial contamination. According to the

acceptance criteria of these protocols, cold sprayed copper coatings demonstrated sanitizing efficacy against *S. aureus* MRSA, and the sanitizing and continuous antibacterial activity against *S. aureus* MSSA and MRSA, *E. aerogenes* and *P. aeruginosa* of the electroplated copper-silver alloy coating was also demonstrated in this study [63], [Paper 3]. However, 2 hours exposure may be too long, in view of real-life applications and the efficacy of copper alloy surfaces, therefore additional shorter exposures would be appropriate. Also, presence of nutrient broth in the bacterial inoculum may have a protective action, decreasing the efficacy of the copper alloy. The third U.S. EPA protocol aims at assessing the sanitizing efficacy of copper alloys (according to the procedure outlined in the first protocol) after exposure to wet and dry wear cycles using an aluminum oxide pad as abrasive material, simulating surface abrasion conditions. Surface wear is very relevant for the real-life application, especially in the case of copper alloy coatings, and the surface wear resistance greatly depends on the hardness of the material and the counter-material in the test conditions. Therefore, the choice of the counter-material should be more specific that a general-purpose abrasive pad, and reflect, as far as possible, the wear conditions in the real-life intended applications. In 2016, the U.S. EPA released a combined version of these three protocols with the addition of exposure to aggressive chemicals (sodium hypochlorite, hydrogen peroxide and EDTA), which can likely influence the efficacy (and durability) of copper alloys [62].

Besides the official standardized tests, other methods have been developed and used to evaluate the efficacy of copper alloy surfaces. Some of these methods are summarized in Table VII.

Table VII. Test methods from literature for assessing antibacterial efficacy of copper alloys are listed. *inoculated surfaces were covered with a sterile cover slip.

Ref.	Test organisms	Inoculum level and volume	Test conditions	Cell recovering
Kredl et al. [46]	*S. aureus* ATCC 6538	– 10^7 CFU/mL in 0.85% NaCl solution – 50 µl on 200 mm samples	Dry	Using 10 mL nutrient broth and shaking for 15 min.
Ozkan et al. [48]	*E. coli* ATCC 25922 *S. aureus* NCTC8325-4	– 10^6 CFU/mL in PBS – 100 µl on 10×10 mm samples	Wet*	Using PBS and vortexing for 20 s.
McLean et al. [64]	*S. epidermidis* (clinical isolate)	– 2.7×10^6 CFU/mL in DI – 200 µl on Ø7 mm samples	Dry	Using 1 mL PBS and vortexing for 30 s.
Wilks et al. [65]	*E. coli* O157:H7	– 10^7 CFU/mL in TSB – 20 µl on 25.4×25.4 mm coupons	Dry	Using 10 mL PBS and vortexing with glass beads for 1 min
Santo et al. [66]	*E. coli* K-12	– 1.4×10^9 CFU from cotton swab – 0.17 µl on 25.4×25.4 mm coupons	Dry	Using 10 mL PBS and vortexing with glass beads for 1 min.

The test procedures are similar, i.e. inoculation and spread on surfaces, followed by recovering using vortexing/shaking with or without glass beads in buffer or nutrient broth. In all cases serial dilution and plating on agar is performed. The exposure is usually dry (following the drying of inoculum at the surface) under ambient conditions, in order to simulate indoor environments. In the method used by Ozkan et al., the inoculated surfaces were covered with sterile cover slip, aiming to provide good contact between the bacteria and the surface, but preventing the inoculum to dry [48]. The only "true" dry method was developed by Santo et al. where 40 µl inoculum was applied to a sterile cotton swab and spread evenly across a metal test coupon, so to have surfaces in a dry regime since the beginning of exposure [66]. However, the overall amount of liquid applied on the surface was only 7% (corresponding to 1.4×10^9 cells) of the total 40 µl volume (2.8×10^{10} cells) applied to the swab, due to binding of cells to the cotton fibers [66].

Five groups of copper alloys and stainless steel were tested using the wet plating method developed by Wilks et al. [65]. 20 µl aliquot of *E. coli* O157 culture (approx.10^7 cells) were placed on the test material, incubated for 75-270 minutes, and recovered from the surface using glass beads in PBS, which was efficient at removing bacteria from the coupons. Among the

tested five groups of copper alloys and stainless steel, pure copper (99.9 wt% Cu) had the highest antibacterial activity, followed by brasses, bronzes and copper-nickels [65]. However, increasing copper content in the alloy correlated with higher inhibitory effect only in few cases.

4.1.2 Methods based on immersive inoculation

ASTM E2149-13a is the most widely used standardized method to test the activity of antimicrobial-treated samples in a bacterial suspension under dynamic conditions [67] (Table VI). The test sample is immersed in *E. coli* ATCC 25922 suspension and incubated with shaking for 1 hour. After that, serial dilution and plating are used to determine the bacterial reduction in the test suspension. There is no specific value of log reduction to be achieved, therefore the test can be used to provide an indication of antibacterial activity after 1 hour exposure in these conditions. ASTM E2149-13a is, however, intended for non-leaching antimicrobial-treated samples, and it does not specifically account for the presence of an antimicrobial agent in solution. Not only the elution of the antibacterial agents, but also adhesion of viable cells to the sample can markedly affect the number of surviving cells recovered from the suspension. Antibacterial surface, cell-surface adhesion, ratio between sample area and suspension volume, mass transport from the surface to the bulk can influence the elution of antibacterial agents and so the number of viable cells. The composition of the testing suspension, in terms of presence of active compounds or nutrients, is a crucial aspect to consider, especially in the case of copper alloys, where copper ions can be released and interact with the surroundings.

In the present study, survival in suspension and attachment of *S. aureus* to copper-silver alloy coated surfaces were quantified by another (not officially standardized) method. This was done in order to evaluate in a combined manner the anti-adhesive and antibacterial properties of the alloy in buffer or nutrient broth [68], [Paper 1]. Test surfaces were immersed in a bacterial suspension (10^6 or 10^8 CFU/ml in PBS or BHI) for 30 minutes, 4 hours and 24 hours under static conditions. Attached cells were recovered by sonicating (4 minutes) and vortexing (15 s) the samples in new sterile PBS. This procedure was efficient at removing bacteria from the test coupons [69]. Serial diluting and plating on agar was performed from these and the previous test suspensions. Attachment of *S. aureus* to copper-silver alloy coated surfaces was not affected when the inoculum level increased from 10^6 to 10^8 CFU/ml in PBS, but survival in suspension with alloy coated samples increased by 1 log unit after 24 hours [Paper 1]. The

presence of nutrients had, however, the most dramatic effect on bacterial survival after exposure to copper-silver alloy coated surfaces. When *S. aureus* 8325 at a level of 10^6 CFU/ml was suspended in BHI, the attachment to the alloy coated surfaces increased by 2 log units as compared to PBS, already after 30 minutes. Also, no difference in terms of survival in suspension was observed between copper-silver alloy and stainless steel [Paper 1]. Although there was an increased copper ion concentration in the nutrient broth as compared to the buffer, the amino acids of the meat extracts sequestrated and reduced the amount of free ions accessible, protecting the bacteria in proximity of the surface and in the bulk suspension [Paper 1] (see also section 2.1).

4.2 Microscopy and live/dead staining techniques

Microscopy combined with live/dead staining techniques can give indications regarding the general efficacy of an antibacterial surface, but higher loads of bacteria are usually necessary. Live/dead staining in combination with confocal or epi-fluorescent microscopy exploits luminescence in order to detect bacterial viability in terms of e.g. membrane permeability, and also to count bacterial cells, if flow cytometry is used [70]. Cell membrane damage is the primary effect of the contact killing by copper surfaces, and this can be easily assessed by using the live/dead staining technique, which allows to discriminate between bacterial cells with intact (SYTO 9 stain, green fluorescence) and damaged (propidium iodide, red fluorescence) membranes [15,18]. However, the stains can sometimes interact with the substrate, producing high fluorescent backgrounds, when they are used on immobilized surfaces [70]. Regular indicator dyes lose their fluorescence upon contact with copper surfaces [71]. Copper surfaces interfere with the emission signal of propidium iodide due to their characteristic light absorption, resulting in the decrease or elimination of the fluorescent signal [72], [Paper 3]. Therefore cells are routinely removed from copper surfaces prior to the staining procedure and inspection [71]. Hence, only a post-visualization and not an *in situ* monitoring of contact killing of bacteria on copper surfaces is possible. In the present study, this problem was overcome by using a modified live/dead staining technique in combination with confocal laser scanning microscopy (CLSM) to visualize inactivation of bacterial biofilms at the copper-silver alloy coated surface [Paper 3]. A dead stain characterized by an emission spectrum shifted to longer wavelengths was used, hence its visualization in contact with copper surfaces was possible for

an extended period. Contact killing of *S. aureus* 8325 and *P. aeruginosa* PAO1 by copper-silver alloy coated surfaces was followed *in situ* over a period of 60 minutes with only a negligible reduction of the fluorescent signal over time [Paper 3]. Dead cells were already present at the alloy coated surfaces after the first minutes of exposure (Figure 11 and Figure 12). There were more *S. aureus* dead cells than live cells after 10 minutes, while there was no appreciable reduction in live cells exposed to stainless steel surfaces (Figure 11). *P. aeruginosa* cells were killed more slowly on the alloy coated surfaces, and the number of dead cells in the biofilm was larger than the number of live cells after 60 minutes (Figure 12). Likewise, there was no reduction in number of *P. aeruginosa* live cells in contact with stainless steel surfaces (Figure 12).

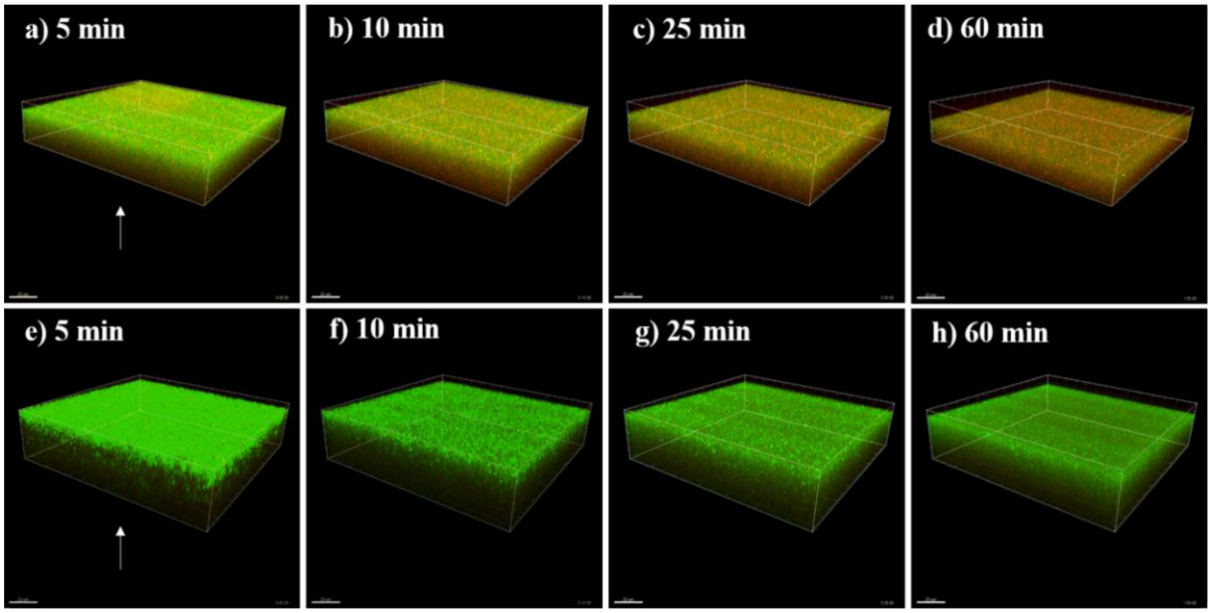

Figure 11. *Staphylococcus aureus* 8325 live and dead cells exposed to copper-silver alloy coated (a-d) and uncoated (e-h) AISI 316 surfaces monitored at the beginning of exposure (a, e), after 10 minutes (b, f), 25 minutes (c, g) and 60 minutes (d, h). The arrow indicates the position of the metallic surfaces. Cells are stained with a modified Live/Dead dye Stain mixture (0.2% of SYTO® 9 Green-Fluorescent Nucleic Acid and 0.2% of SYTOX® AADvanced™ Dead Cell Stain) and live cells appear green and dead cells stain red [Paper 3].

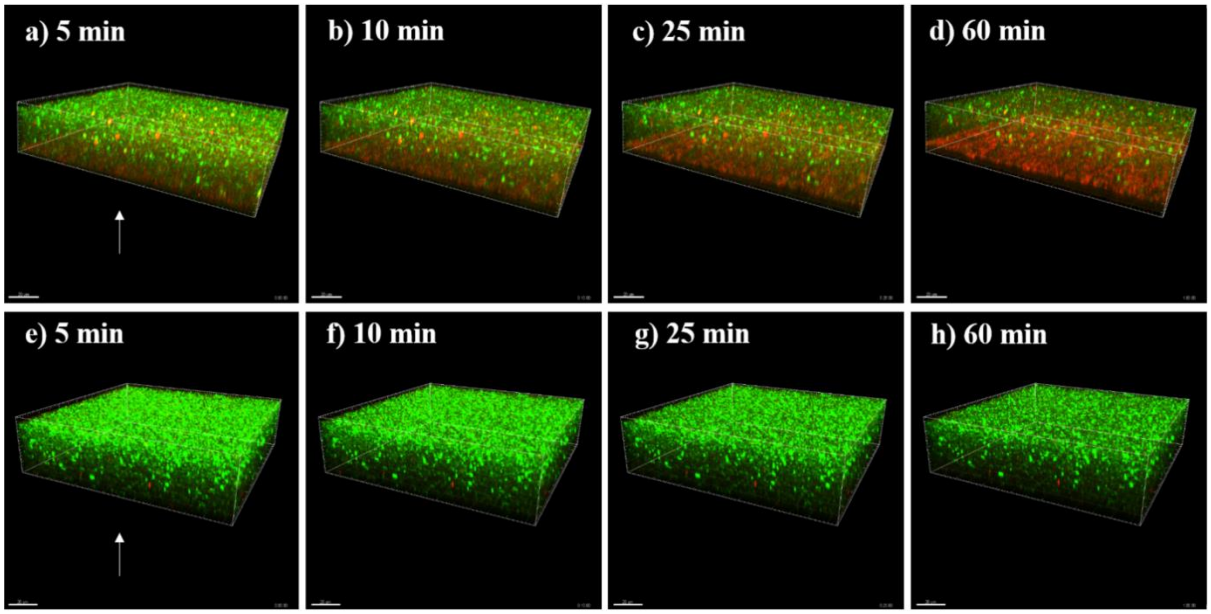

Figure 12. *Pseudomonas aeruginosa* PAO1 live and dead cells exposed to copper-silver alloy coated (a-d) and uncoated (e-h) AISI 316 surfaces monitored at the beginning of the exposure (a, e), after 10 minutes (b, f), 25 minutes (c, g) and 60 minutes (d, h). The arrow indicates the position of the metallic surfaces Cells are stained with a modified Live/Dead dye Stain mixture (0.2% of SYTO® 9 Green-Fluorescent Nucleic Acid and 0.2% of SYTOX® AADvanced™ Dead Cell Stain) and live cells appear green and dead cells stain red [Paper 3].

This experimental set up also allowed using the CLSM images to quantify the biomass at the surface (using Comstat2 software) as ratio of live and dead cells [73,74]. After 25 minutes, the remaining *S. aureus* live cells were less than 20% and the majority of cells were dead after 60 minutes on alloy coated surfaces, whereas the percentage of live cells on stainless steel surfaces was on average above 80% over the whole exposure period (Figure 13a and Figure 13b). In case of *P. aeruginosa* biofilm, Figure 13c clearly shows when the ratio of live and dead cells shifted in favor to the latter on the alloy coated surfaces, and Figure 13d presents the absence of killing on stainless steel surfaces.

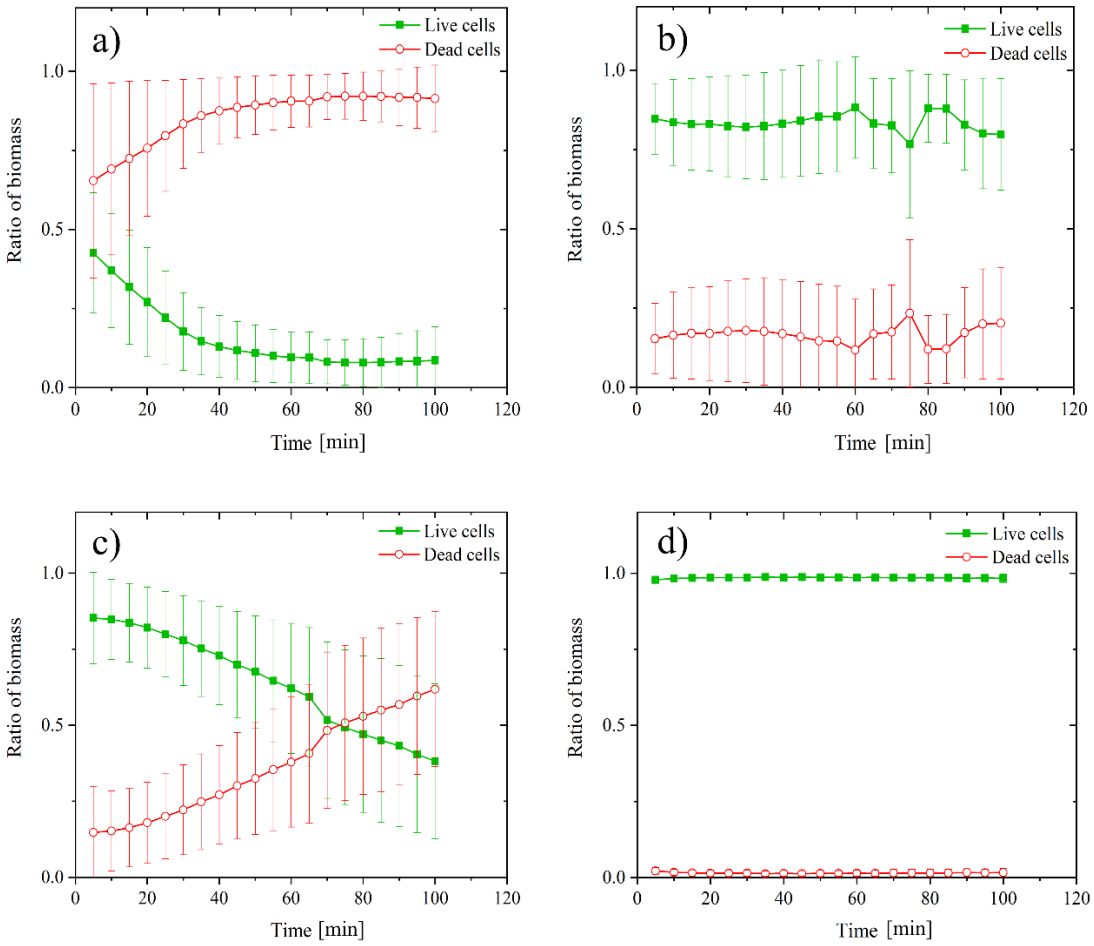

Figure 13. Ratio of *Staphylococcus aureus* 8325 live and dead cells exposed to copper-silver alloy coated (a) and uncoated (b) AISI 316 surfaces and *Pseudomonas aeruginosa* PAO1 live and dead cells exposed to copper-silver alloy coated (c) and uncoated (d) AISI 316 surfaces [Paper 3].

4.3 Conclusions on the methods for determining the antibacterial activity of copper alloy and copper-based surfaces

Among the enumeration-based test methods that use direct inoculation, the U.S. EPA test methods are the most suitable for copper alloys, and in particular for the copper-silver alloy coating, since they account for the real-life conditions of environmental surfaces. The test surfaces are exposed to a bacterial inoculum under dry conditions and to an overtime build-up of organic material, which can affect the antibacterial activity of the copper alloy surfaces. The U.S. EPA combined protocol takes also into account the effect of chemical exposure and

abrasion on the antibacterial activity, again relevant in real-life environments. However, the wear conditions in the test should be more specific for real-life abrasion and adapted to best suit copper-based surfaces (e.g. polymer substrates and coatings). For example, polyurethane as counter-material can simulate the contact with skin for touch-surface applications.
Enumeration-based test methods that use immersive inoculation have the purpose to evaluate the antibacterial activity of the test material in liquid conditions. In the present study, the adhesion/survival test method was used to assess how bacterial adhesion and survival in suspension are affected by a copper ions-releasing surface (i.e. the copper-silver alloy coating). This is relevant for copper alloys in applications that foresee more frequent contact with liquids and whether information about the levels of released ions is required.

Microscopy combined with live/dead staining techniques is a powerful tool to evaluate bacterial cell membrane damage, and it can be used to monitor contact killing of a dense bacterial layer at the surface of copper alloys, without the presence of interfering substances (e.g. chlorides and nutrients from buffer and/or broth). In the present study, the use of a modified live/dead staining technique in combination with CLSM allowed to visualize *in situ* the inactivation of bacterial biofilm at the copper-silver alloy coated surfaces, and revealed a faster killing in case of *S. aureus* as compared to *P. aeruginosa.*

5. Influence of environmental conditions on the antibacterial efficacy of copper alloys

Dry or humid atmosphere induces oxidation of redox-active surfaces, such as metallic copper, in ambient conditions: cuprous or cupric oxide may be simultaneously present at the surface depending on the specific environmental conditions [16] (see section 2.3.2). Besides this naturally occurring oxidation, other indoor environmental factors can react with copper and its alloying elements and modify the surface chemistry of the copper alloy. Chemical detergents and disinfectants, build-up of dirt and filth (organic soiling), and abrasion of the surfaces are the factors, alone and in combination, affecting the antibacterial efficacy of copper alloys.

The influence of active compounds in disinfectants (hydrogen peroxide, ethyl alcohol, benzalkonium chloride and sodium hypochlorite) and chelating agents on the antibacterial efficacy of copper alloys is discussed in section 5.1. In particular, the effect of chlorides and phosphates, which are common in detergents, on the availability of copper ions is examined in the case of copper-silver alloy coated surfaces. The aim is to provide an answer to the following questions: can antibacterial copper alloy surfaces and chemical disinfectants interact? If this is the case, is the overall antibacterial efficacy compromised or enhanced, so that they can potentially be used synergistically? [75]

Section 5.2 addresses the impact of organic soiling and wear resistance on the antibacterial efficacy of copper alloys.

5.1 Influence of chemicals and complexing agents on the antibacterial efficacy of copper alloys

Chemical disinfectants can be divided in hydrogen peroxide solutions, alcohols, aldehydes, quaternary ammonium compounds and chlorine-releasing compounds [28]. This classification is based on their active compound, the main responsible for the chemical interaction with the surface.

Hydrogen peroxide induces oxidation of copper alloys surfaces. Cuprous oxide, cupric oxide and hydroxide formed a non-uniform film on copper surfaces already after 5 minutes exposure to 1 wt% hydrogen peroxide solution [76]. In the non-uniform film, cuprous oxide was mostly located at the interface between the metal and a layer consisting of cupric oxide and hydroxide

precipitates [76]. Cupric oxide has a reduced antibacterial efficacy, as compared to pure copper and cuprous oxide, and therefore reduced antibacterial efficacy may be expected [33]. Ethyl alcohol is used to remove copper oxides at high temperatures (130-200 °C), and copper complexes (e.g. CuCl phenethylamine) catalyze the oxidation of alcohols to aldehydes and ketones [77,78]. However, there was no appreciable interaction between an alcohol-based disinfectant (isopropyl and ethyl alcohol) and copper alloys surfaces, when the disinfectant was applied at the surface and its biocidal efficacy assessed under ambient conditions [75]. The same biocidal efficacy against *P. aeruginosa* and *S. aureus* was observed after 60 minutes exposure on copper alloys, stainless steel and surgical tiles, as compared to the water controls [75]. Therefore, interaction between copper alloys and alcohol-based disinfectants (commonly used for routine disinfection of open hard surfaces) is not expected, nor their use affects significantly the antibacterial efficacy of copper alloys.

In contrast, disinfectants based on quaternary ammonium compounds and aldehydes interact with copper alloys resulting in a synergistic effect [75]. The prEN 14885 standard requirements for disinfectants were met using a sub-effective concentration of a biocidal formulation based on benzalkonium chloride and glutaraldehyde on copper alloy (CuZn23AlCo) surfaces [75]. A 5-log reduction of *P. aeruginosa* level was achieved on copper alloys surfaces exposed to the benzalkonium chloride and glutaraldehyde-based disinfectant [75].

Also, sodium hypochlorite reacts with copper alloyed stainless steel surfaces and can restore their antibacterial activity (when stopped by the presence of organic soiling) more efficiently than ethyl alcohol [79,80]. Thus, there is indication that chlorine (in forms of hypochlorite ions or chloride ions) can enhance the antibacterial efficacy of copper. Other chlorine-releasing compounds (e.g. calcium hypochlorite, chlorine dioxide, chloramines) and sodium chloride are present in various detergents and cleaning products, not to mention hand soap and human sweat [81]. In chloride media, Cu(I) ions are released from copper surfaces and are stabilized by the presence of chloride ions [82,83]. In this way, copper ions in solution do not precipitate and can exert their antibacterial action.

In the present study, attachment of *S. aureus* 8325 to copper-silver alloy and pure copper coated surfaces was the lowest, as compared to attachment to pure silver coated and uncoated stainless steel surfaces, in chloride-containing media, i.e. PBS and 0.15 M NaCl solution [Paper 2] (Figure 14).

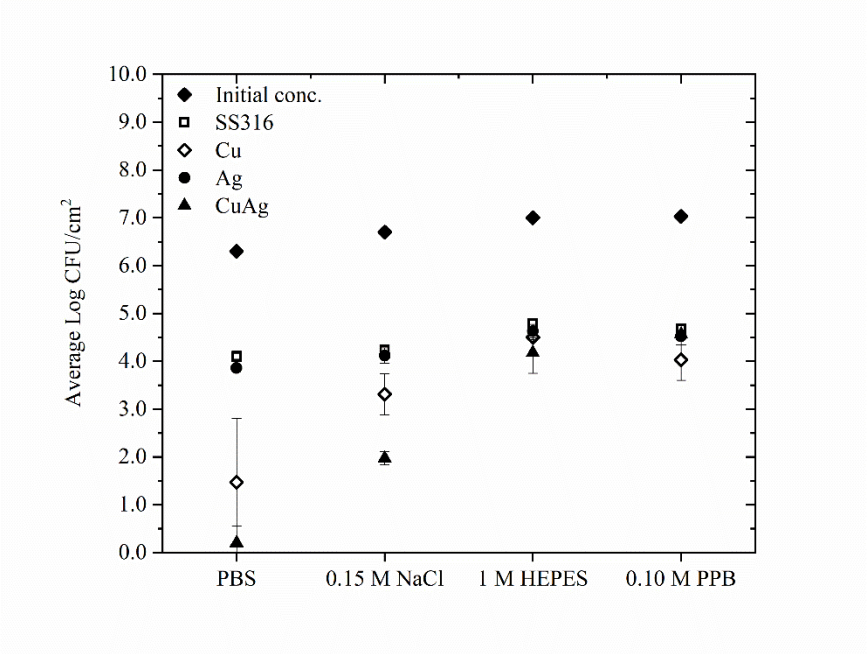

Figure 14. Attachment of *S. aureus* 8325 to AISI 316 stainless steel, pure silver, pure copper, and copper-silver alloy coated surfaces after 30 minutes in suspension with various diluents. Numbers are mean values ± standard deviation of three biological replicates [Paper 2].

In contrast, no difference in bacterial attachment among all tested surfaces was observed in absence of chlorides, i.e. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and potassium phosphate buffer [Paper 2]. Copper surfaces had also lower contact killing efficacy against *E. coli* suspended in HEPES buffer, as compared to PBS or Tris buffer solutions, under dry test conditions [84].

The HEPES buffer solution neither offered conditions for Cu(I) ions dissolution from metallic copper at neutral pH, nor had a strong complexing action on copper ions [Paper 2]. This explained the reduced contact killing efficacy in dry conditions, and lack of antibacterial activity in the suspension test [84], [Paper 2] (Figure 14).

Orthophosphates and polyphosphates can reduce the solubility of copper leading to formation of cupric phosphate scale, as observed in e.g. water pipes [85,86]. Therefore, it is possible that the formation of a cupric phosphate complex prevented further dissolution of cupric ions, so that bacterial attachment was not inhibited in presence of potassium phosphate buffer [Paper 2] (Figure 14). Interestingly, phosphates did not seem to reduce copper dissolution in PBS, and copper-silver alloy coated surfaces were, in fact, more efficient against bacterial adhesion than in 0.15 M NaCl solution [Paper 2] (Figure 14). A similar low attachment (approx. 4-log reduction as compared to stainless steel) of live bacteria to the copper-silver alloy coating was

also observed in artificial sweat, indicating that chloride ions from sweat can enhance the antibacterial activity of copper [16], [Paper 2].

Cu(I) and Cu(II) ions chelating agents, i.e. bathocuproine disulfonic acid (BCS) and EDTA respectively, extended the survival of *E. faecalis* on copper alloy surfaces [87]. The number of viable cells was reduced with 2-log when Cu(II) was chelated, and only 1-log reduced in absence of Cu(I) ions. Enterococcal clinical isolates were better protected against the antibacterial activity of copper when BCS neutralized Cu(I) ions, and this is in line with evidence of the higher toxicity of cuprous ions [33,87].

5.2 Influence of organic soiling and wear resistance on the antibacterial efficacy of copper alloys

The build-up of organic deposits on copper alloy surfaces during use is expected, and it is necessary to evaluate how organic contamination can affect the efficacy of copper alloy surfaces. In experiments simulating conditions with and without organic contamination, the antibacterial efficacy of nine commercial copper alloys was assessed using a modified version of JIS Z-2801/ ISO 22196 standard [88] (see subsection 4.1.1). *E.coli* or *S. aureus* inoculum suspended in saline solution and tryptic soy broth represented a clean and contaminated condition, respectively. Antibacterial efficacy against *E. coli* was reduced and delayed in presence of organic contamination (4-log reduction between 120 and 240 minutes), as compared to absence of organic contamination (7-log reduction between 15 and 120 minutes) [88]. *S. aureus* was reduced with 5-log between 45 and 210 minutes in absence of organic contamination, and between 120 and 300 minutes in presence of organic contamination [88]. Correlation between antibacterial efficacy and copper content in the alloy was only possible for *E. coli* in absence of organic contamination. However, in all cases, presence of simulated organic contamination reduced the antibacterial efficacy of copper alloys against *E. coli* and, to a lesser extent, *S. aureus*, due to the reaction between the amino acids and copper.

In another study, poultry carcass rinse water simulated the organic contamination on copper-tin alloy surfaces for applications in a poultry processing plan [89]. Monocultures of *S. enterica* and *L. monocytogenes*, and mixtures of each pathogens with *E. cloacae*, were reduced with

approx. 4-log between 20 and 30 minutes on dirty copper surfaces, whereas only 5 minutes were sufficient to eradicate the pathogens on clean copper alloy surfaces [89] (Figure 15).

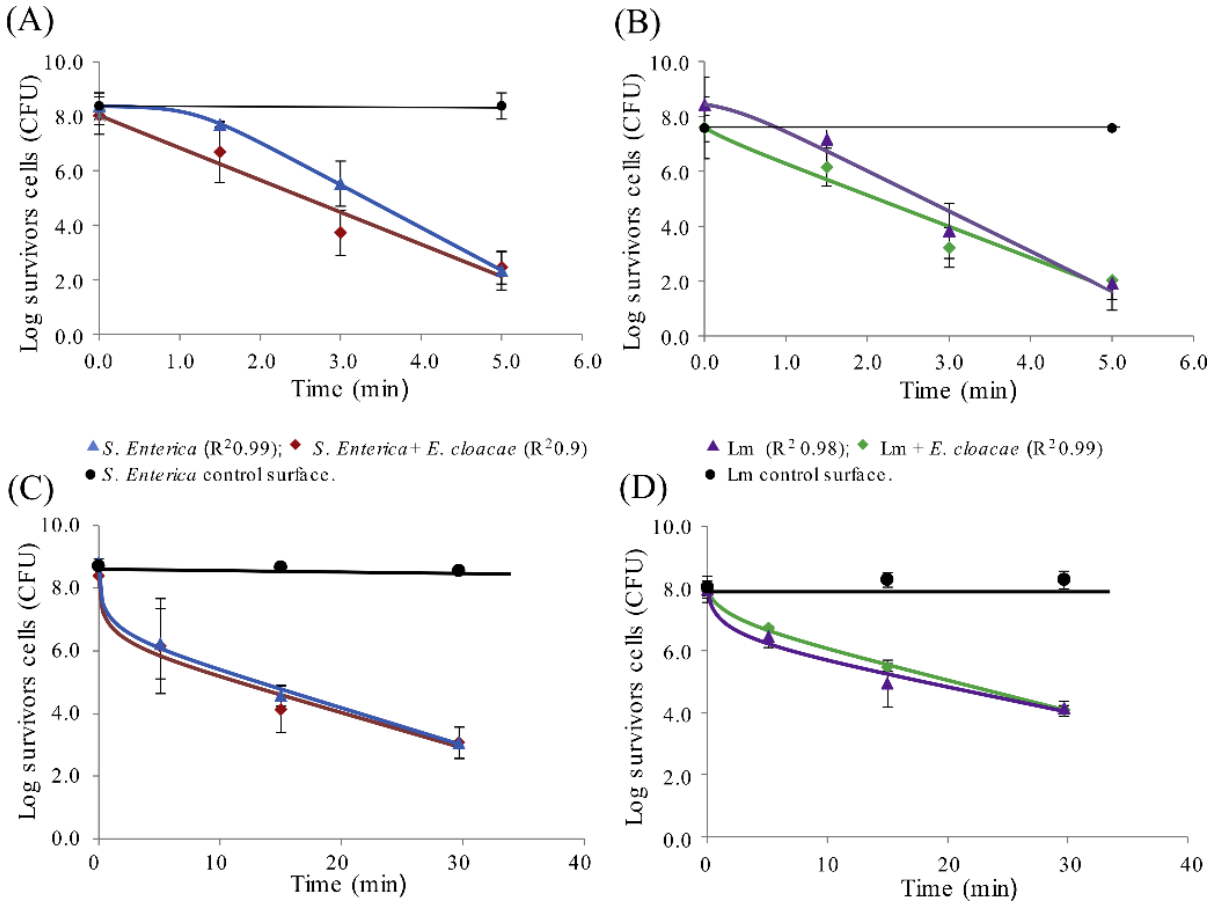

Figure 15. Inactivation kinetics curves for 40 µL inoculum (1×10^{10} CFU/mL) of *S. enterica* (A, C) and *L. monocytogenes* (B, D) on polished copper surfaces (A, B) and treated copper surfaces with poultry carcass rinse water (C, D). Pathogens were exposed individually and in a mixture with *E. cloacae*. The average of 3 repetitions and standard deviation are shown. Control surfaces were stainless steel coupons [89].

Although the antibacterial activity of copper-tin alloy surfaces was not affected by a mixed microbiota (*S. enterica* + *E. cloacae*, or *L. monocytogenes* + *E. cloacae*), it was clear that organic material can prolong the survival of bacteria.

Another factor that can influence the long-term antibacterial efficacy of copper surfaces is their wear resistance, which is even more relevant in case of coatings or copper nanoparticle/polymer composites. Their durability, i.e. the lifetime during which the coating is capable of maintaining its properties, is highly dependent on the exposure conditions. Besides the specific hardness of the material, the environment can influence the type of wear: the simple

abrasive action of a counter material can be intensified by the simultaneous presence of liquids or variably aggressive chemicals, resulting in tribocorrosion phenomena.

Cupron Enhanced EOS Surfaces (a blend of copper oxide, acrylic and polyester resins) had a residual sanitizing effect after abrasion and bacterial inoculation cycles, according to the U.S. EPA test method (see section 3.1, Table V and section 4.1, Table VI). The U.S. EPA unified test protocol combines cyclic exposure to abrasion and aggressive chemicals, in order to assess the sanitizing efficacy of copper alloys after this treatment [62]. However, such protocol is designed for bulk copper alloys, and surface wear is performed using a generic counter-material, as already discussed in chapter 4. Testing copper-based coatings with appropriate modification of the U.S. EPA protocol can give an indication of the expected durability, in relation to antibacterial performances, in applications that foresee occurrence of wear.

5.3 Conclusions on the influence of environmental conditions on the antibacterial efficacy of copper alloys

Among surface disinfectants, hydrogen peroxide can reduce the antibacterial activity of copper alloys by inducing the formation of cupric oxide, whereas ethyl or isopropyl alcohol does not affect significantly the surface chemistry of copper alloys under ambient conditions.

Chlorides can be found in disinfectants (benzalkonium chloride), detergents and sweat, and enhance the antibacterial activity of copper alloys, in particular the copper-silver alloy coating, by stabilizing and thus preventing the precipitation of copper ions released from the surface [75]. Organic contamination reduces the antibacterial activity of copper alloy surfaces by protecting bacteria and prolonging their survival. The build-up of organic material on a copper alloy surface prevents the direct metal-bacteria contact and reduces the amount of available copper ions [28]. Therefore, regular cleaning of copper alloy surfaces is necessary to remove layers of dirt and filth, especially in touch-surfaces applications. A compatible cleaning procedure (e.g. based on chlorides) can prolong and even optimize the antibacterial efficacy of copper alloy surfaces [75].

Antibacterial copper alloys need to have adequate wear properties, and abrasion resistance is closely related to the specific case and application, especially in the case of copper and copper alloy-based coatings.

Hence, field-testing is probably the most straightforward choice in order to evaluate and select the best-fit antibacterial coating solution, considering the number of interconnected environmental parameters that can affect its antibacterial properties.

6. Field-testing of copper alloy surfaces in clinical settings

The extensive laboratory evidence demonstrating the antibacterial properties of copper alloys has led to a number of clinical trials aimed at providing a real-world proof of the concept [90]. Two main questions are raised: will the items made of copper alloys carry less microorganisms if introduced into the clinical environment, as compared to the parallel items in standard materials? If there is such a difference, will a corresponding reduction in HCAIs be observed? [90]

In the case of coatings, such as the copper-silver alloy, indications of the chemical modification at the surface due to the environmental exposure can also be obtained performing a surface analysis prior and after field-testing [Paper 4]. The evaluation of microbial load on clinical items made of copper alloys in comparison to reference items, and infections reduction in hospital rooms where the items were installed is discussed in section 6.1. Also, difference in microbial load and species among various alloys is addressed. Microbial load on copper-silver alloy coated and uncoated door handles, isolates identification and surface modification after field-testing is examined in 6.2.

6.1 Evaluation of microbial load on clinical items made of copper alloys and HCAIs reduction in treated hospital rooms

In the U.S., a multihospital clinical trial of the six EPA registered copper alloys has aimed to answering these questions (Table IV). Six copper alloys objects (bed rail, call button, chair arms, IV pole, tray table and data input device) were installed and sampled weekly, together with the parallel non-copper objects, for 21 months [40,90]. The average microbial load on copper alloy surfaces was 465 CFU/100 cm^2, whereas conventional surfaces (plastics, coated carbon steel, aluminum, and stainless steel) carried 2,674 CFU/100 cm^2 [40]. In both cases, levels were above 250 CFU/100 cm^2, which is the proposed standard for microbial load on a surface immediately after terminal cleaning [91]. Above this threshold, the likelihood of microbial transmission from surfaces increases [91]. However, these are indicative figures: conventional surfaces carried a six-times higher microbial level, as compared to copper alloy surfaces, and this translates in an average reduction of 83% [90]. Therefore, copper alloys

reduced the microbial load as compared to the parallel items in standard materials in a real-life healthcare setting.

Moreover, the infection reduction, calculated as the difference between the percentage of infected patients in “non-copper” rooms (8.1%) and in “copper” rooms (3.4%), normalized against the percentage in “non-copper” rooms, was 58% (*p*-value= 0.013). And this answers positively also to the second question [90].

Another study compared the microbial load on “copper” and “non-copper” items in different facilities in Finland: a hospital, a kindergarten, a retirement home and an office building were included in the field-testing [92]. Floor drain lids, toilet flush buttons, door handles, light switches, closet touch surfaces, corridor handrails, front door handles and toilet support rails were made of copper or brass alloys by Abloy Oy, Aurubis, Vemta, PA Wheels and Cupori O, and were installed at the facilities [92]. The sampling intervals, cleaning practices and surface usage varied from facility to facility [92]. However, floor drain lids (32 total items) had the highest microbial levels, followed by 90 small area touch surfaces (toilet flush buttons, door handles, light switches, closet touch surfaces) and 92 large area touch surfaces (corridor handrail, front door handle, toilet support rail) [92]. Door handles previously demonstrated to harbor the highest levels of bacterial contamination in a clinical environment [11]. Here, door handles made of pure copper (99.8 wt% Cu) outperformed brass (60.5 wt% Cu, 36.5 wt% Zn) door handles that did not show on average significant differences as compared to the reference (chromed) material [92] (Table VIII).

Table VIII. Total aerobic plate count (± standard deviation) on pure copper (99.8 wt% Cu), brass and chromed (reference) door handles in three different facilities [92].

Location	Material	#items	Total aerobic plate count [CFU/cm^2]
Office	99.8 wt% Cu	4	3 ± 1
Office	Chromed (reference to Cu)	4	10 ± 4
Kindergarten	99.8 wt% Cu	3	9 ± 10
Kindergarten	Chromed (reference to Cu)	3	11 ± 13
Kindergarten	Brass (60.5 wt%Cu, 36.5 wt%Zn, max 3 wt% other)	7	47 ± 100
Kindergarten	Chromed (reference to brass)	10	12 ± 20
Retirement home	99.8 wt% Cu	9	6 ± 6
Retirement home	Chromed (reference to Cu)	9	140 ± 375
Retirement home	Brass (60.5 wt%Cu, 36.5 wt%Zn, max 3 wt% other)	6	3 ± 3
Retirement home	Chromed (reference to brass)	6	10 ± 12

There was no difference in the occurrence of enterococci, Gram-negatives and *Staphylococcus aureus* between brass and reference surfaces. Enterococci were equally present on all copper alloy and reference surfaces (15%), whereas copper alloy surfaces had lower levels of Gram-negatives and *Staphylococcus aureus* (21% and 2.6%, respectively) as compared to the reference surfaces (34% and 14%, respectively). Hence, a higher antibacterial efficacy of copper alloy surfaces against Gram-negatives and *S. aureus* was suggested [92].

6.2 Evaluation of microbial load and identification of isolates from copper-silver alloy coated door handles in clinical settings

During this PhD project, field-testing of copper-silver alloy coated door handles was carried out at a private clinic in Denmark and at a wound care center in Texas, U.S. [Paper 4]. In Denmark, copper-silver alloy coated door handles were installed at the doors of two doctors and two nurses' exam rooms, and stainless steel door handles of other parallel four offices were sampled as reference material. Weekly samplings were done for 6 weeks. The microbial load (in terms of total aerobic plate count) on copper-silver alloy coated and uncoated stainless steel door handles was 1.3 ± 0.4 and 2.4 ± 0.4 Log CFU/cm^2, respectively (p-value =0.0008),

indicating a superior antibacterial ability of copper-silver alloy with respect to stainless steel surfaces [Paper 4]. In Texas, copper-silver alloy coated door handles were installed at the doors of seven exam rooms, one public restroom and two laboratory rooms. Original satin brass door handles of other six exam rooms, three public restrooms and one laboratory room were sampled as reference material, and weekly samplings were performed for 6 weeks. The microbial load (in terms of total aerobic plate count) was evaluated for 4 weeks. Here, the copper-silver alloy coated and satin brass door handles had a microbial load of 0.8 ± 0.3 and 1.7 ± 0.4 Log CFU/cm^2, respectively (p-value = 0.0068) [Paper 4].

Both reference stainless steel door handles and satin brass door handles had a microbial load approx. twice as high as the copper-silver alloy coated door handles in the field tests [Paper 4]. Not surprisingly, the microbial load on the satin brass reference door handles was lower than the stainless steel reference door handles, due to the antibacterial activity of brass [Paper 4]. All surfaces in the field tests, except for stainless steel, had microbial levels below 2.4 Log CFU/cm^2 (the standard immediately after terminal cleaning).

There was no marked difference among the surfaces in terms of surviving bacterial species in the Danish clinic [Paper 4]. Most abundant were *Micrococcus luteus* and staphylococci (*S. hominis*, *epidermidis* and *capitis*) on both copper-silver alloy coated and uncoated door handles. Interestingly, *Staphylococcus aureus* was recovered from stainless steel but not copper-silver alloy coated door handles [Paper 4] (Figure 16).

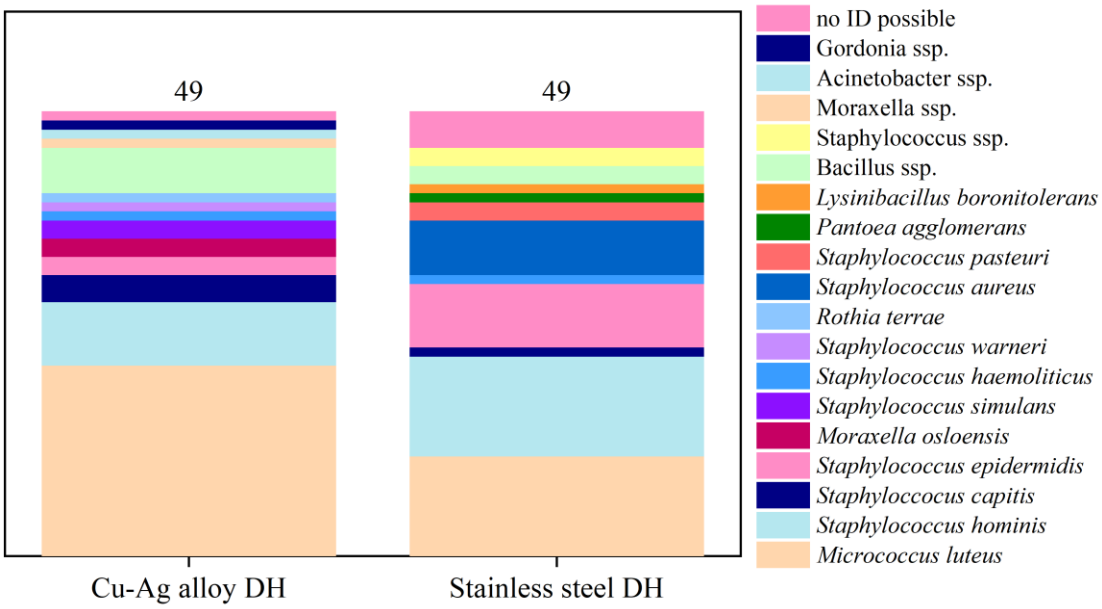

Figure 16. Species abundance of 98 isolates recovered from copper-silver alloy coated and uncoated stainless steel door handles at FamilieLægerne Espergærde and analyzed by MALDI/TOF. The species are ordered according to their abundance in the column stacks and correspondingly in the legend. Identification to the species level was not possible for Bacillus ssp. and score values below 2.00, thus the genus is reported. It was not possible to recover and identify one isolate from stainless steel door handle due to lack of growth.

Staphylococcus aureus was, however, detected on copper-silver alloy coated (on three out of the 16 test samples) and reference satin brass (on three out of the 24 control samples) door handles in the field test in Texas [Paper 4]. Seven out of the 16 test samples (44%) and 22 out of the 24 control samples (92%) were also positive for the *mecA* gene, and the *vanA* gene was detected in one sample from the control group. The greater occurrence of *mecA* gene in the control group could be simply due to the larger microbial load recovered from the satin brass door handles [Paper 4].

After the field tests, surface analysis identified presence of carbon on the copper-silver alloy coated door handles, and a reduction of the copper content in the alloy (approx. 5 wt%) was detected after the field test in Denmark [Paper 4]. Therefore, the lifetime of the coating was safely assumed to be 1 year; after that, the item should be re-coated to ensure the same

antibacterial activity. During installation, a complementary cleaning procedure would be advisable and necessary to remove dirt and filth from the surface [Paper 4].

6.3 Conclusions on field-testing of copper alloy surfaces in clinical settings

Items made of commercial copper alloys and installed in clinical settings carried reduced levels of microbial contamination, as compared to reference non-copper alloy items, and proved to reduce the infection rates in “copper-treated” hospital rooms.

In both field tests, copper-silver alloy coated door handles carried a lower bacterial load as compared to stainless steel or satin brass door handles, thus lowering the chances of transmission of antibiotic-susceptible and antibiotic-resistant bacteria. It follows that the copper-silver alloy coating has also potential to reduce microbial transmission and relates infections, in clinical settings or other environments sensitive to bacterial contamination. Selective higher antibacterial efficacy of copper alloy surfaces against Gram-negatives and *S. aureus*, as previously suggested, was not observed in the case of copper-silver alloy coating [92]. A general indiscriminate reduction of microbial levels and a lifetime of approx. 1 year between re-coating interventions are to be expected, should this coating be used in such real-life application.

7. The potential challenge of bacterial tolerance or resistance to copper and cross-resistance to other antimicrobials in clinical settings

A potential challenge when using copper ions-releasing materials is the risk of development of increased tolerant or resistant bacteria. This is especially of concern if clinically relevant bacteria continuously exposed to copper alloy surfaces would also develop cross-resistance to antibiotics. Intracellular copper levels in bacteria are strictly regulated by homeostatic control mechanisms, due to the inherent toxicity of copper at high levels. The three most common regulator mechanisms of copper homeostasis are:

i. export of copper in the extracellular environment with copper-ATPase pumps [17,18]
ii. sequestration of copper in the cytoplasm by copper-binding metal chaperones and by metallothionein in the periplasm or outside the cell [17,18]
iii. generation of the less toxic form Cu(II) by oxidation [17,18]

Copper ions are readily released from a copper surfaces, when bacteria are in contact [18] (see section 2). Although contact killing and killing by copper ions in solution are essentially different, resistance mechanisms against copper ions may induce an increased resistance or tolerance to dry copper alloy surfaces. Vice versa, there is the possibility that bacteria capable to survive on dry copper alloys may have a reduced sensitivity towards copper ions. This is especially valid when copper alloy surfaces are periodically exposed to dry and moist/wet regimes in clinical settings.

The possibility of bacterial tolerance or resistance to copper ions and copper alloy surfaces is discussed in section 7.1. Section 7.2 addresses the potential of cross-resistance to other antimicrobials induced by the prolonged exposure to copper surfaces.

7.1 Bacterial tolerance or resistance to copper ions and copper alloy surfaces

Both *E. coli* W3110 wild-type and *E. coli* W3110(pPA173) carrying the *pco* (plasmid-borne copper resistance) gene were completely killed after 1 minute exposure to dry pure copper (99.9 wt% Cu) surfaces [66]. Therefore, the Pco copper resistance system, which gives

increased resistance to copper ions, did not protect *E.coli* under exposure to dry copper surfaces (see subsection 4.1.1, Table VII for test procedure) [66]. However, the chromosomally encoded CopA, Cus, and CueO resistance systems contributed to extend *E. coli* survival on copper alloys (C75200 max 62% Cu, 18% Ni and 21% Zn and C28000 59-69% Cu, 38-41% Zn). *E. coli* wild-type was killed after 5 minutes contact, whereas the *copA cusCFBA cueO* deletion mutant strain was already killed after 1 minute [66].

Bacteria can be isolated from copper-rich environments, such as European copper alloy coins, and some were able to survive dry exposure to pure copper (99.9 wt% Cu) surfaces [66,93]. Nevertheless, numbers of survivors decreased dramatically overtime and none survived after 1 month of exposure to the copper alloy surfaces [93] (Figure 17).

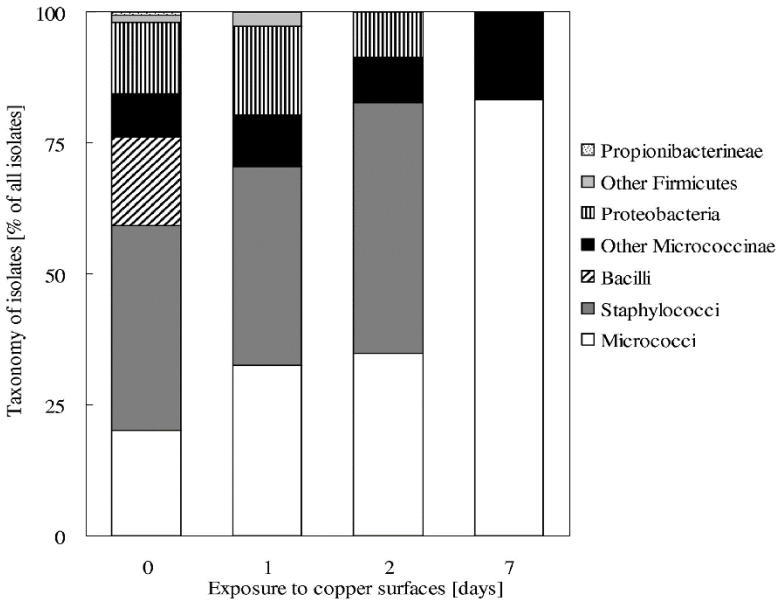

Figure 17. Relative species abundances of 294 bacteria isolated from European copper coins and exposed to dry pure copper (99.9 wt% Cu) surfaces after 1 day (71 survivors), 2 days (23 survivors), and 7 days (6 survivors) are shown [93].

The isolates able to survive 2 days on dry copper surfaces were equally or even more sensitive to moist copper surfaces (40 µl aliquot of approx. 10^9 cells in PBS as standing droplet), as compared to their type or control strain [93]. *Micrococcous luteus* DSM 20030, for example, survived eight times longer than the corresponding coin isolate. All the isolates from the copper alloy coins were also sensitive to solid media containing increasing $CuCl_2$ concentrations

(corresponding to a copper ions exposure) [93]. Hence, bacterial tolerance or resistance towards exposure to dry copper surfaces does not confer increased protection in moist conditions or against copper ions.

The surface attachment of an *E. faecium* isolate carrying the plasmid-localized *tcrB* copper resistance was compared to the corresponding *tcrB*-negative strain in suspension with copper-silver alloy coated surfaces in 1:16 BHI in PBS [94].

After 2 hours exposure, numbers of attached *tcrB*-negative strain were below 2 Log CFU/cm^2, whereas *tcrB*-positive *E. faecium* levels were still 3 Log CFU/cm^2. After 24 hours, neither *tcrB*-negative nor positive *E. faecium* was recovered from the copper-silver alloy coated surfaces [94]. The *tcrB* copper resistance gene and sequestering action of nutrients practically abolished the toxic action of copper ions, however, the adhesion of *E. faecium* to the copper-silver alloy coated surface was approx. 1-log reduced after 2 hours, and 5-log reduced after 24 hours, as compared to stainless steel [94]. This may indicate that the contact between bacteria and the copper-silver alloy coated surfaces, even in a liquid suspension, could have contributed to reduce the adhesion.

In the present study, an ad hoc adaptive laboratory evolution experimental setup was used to evaluate the possibility of tolerance or resistance to a copper-silver alloy coating. *S. aureus* JE2 was grown in a nutrient broth (BHI) and in contact with a copper-silver alloy coated coupons, with daily transfer to new substrate and coupon. After 5 re-inoculations, the transfer was made to a diluted substrate (1:2 BHI in PBS) with new coupons, and the BHI concentration was further diminished after 5 days. From day 10 to 30, *S. aureus* JE2 was exposed to copper-silver alloy coated coupons in 1:4 BHI in PBS. By reducing the amount of organic nutrients over time, an increased exposure to the alloy coated surface was obtained, thus potentially triggering resistance against the alloy. The substrate dilution was maintained at the same level after 15 days to ensure bacterial survival to similar levels of 6-8 Log CFU/mL over the remaining period. After 30 days (equivalent to 180-210 generations) of this adaptive laboratory evolution experiment, there was no difference in survival between *S. aureus* JE2 adapted lineages and wild-type exposed to both copper-silver alloy coated and uncoated surfaces [94]. In addition, a selection of these adapted and control strains showed similar mutational profiles and mutation rates.

7.2 Cross-resistance to antibiotics induced by copper alloys

The staphylococci isolated from coins did not exhibit increased resistance to 11 antibiotics, suggesting no co-selection of metallic copper resistance and resistance against antibiotics [93]. *S. aureus* JE2 adapted lineages to copper-silver alloy coating and wild-type had similar susceptibility to 11 tested antibiotics [94]. Furthermore, no co-selection of antibiotic resistance would be expected in *E. faecium i*solates. The correlation between acquired copper resistance (mediated by the plasmid-localized *tcrB* gene) and resistance towards glycopeptides and macrolides has been disproved after the ban of the same class-antibiotic growth promoters (avoparcin and tylosin) [95,96].

7.3 Conclusions on the potential challenge of bacterial tolerance or resistance to copper and cross-resistance to other antimicrobials in clinical settings

Systems conferring copper ions resistance do not necessarily protect against killing by dry copper surfaces, and tolerance to dry exposure does not increase survival to moist copper surfaces or copper ions. Copper-resistant *E. faecium* isolate could not attach to copper-silver alloy coated surfaces after 24 hours, but levels of attached copper-resistant strain were higher than the corresponding sensitive strain after 2 hours. This indicates the combined action of surface contact and copper ions release in the antibacterial mechanism of copper alloys. *S. aureus* JE2 did not develop increased tolerance or resistance to copper-silver alloy coated surfaces after 30 days exposure in an adaptive laboratory evolution experiment.

Development of cross-resistance to other antimicrobials in staphylococci should not be expected, since no co-selection for antibiotic resistance was observed in staphylococci isolated from coins and in *S. aureus* JE2 adapted lineages to copper-silver alloy coating.

On the basis of these results, resistance to copper alloys and developing of cross-resistance to antibiotics do not seem a very likely scenario, although longer exposure times should be considered in further experiments, and a constant monitoring should be performed in the case of a widespread use of such alloys [93].

8. Concluding remarks and future perspectives

The electroplated copper-silver alloy coating is a potential surface solution strategy to reduce bacterial load and subsequently infectious diseases in hospitals and healthcare settings. In addition, such coatings can reduce microbial contamination in biopharmaceutical industry and food production environments. Bacteria exposed to the alloy coated surface are rapidly killed both in dry and wet conditions. The high surface area to volume ratio and the galvanic coupling of copper and silver are responsible for the antibacterial activity of the copper-silver alloy coating. At the copper-silver alloy coated surfaces, reduction and oxidation occur, as shown by electrochemical measurement and pH monitoring, likely causing the observed killing [Paper 3]. pH fluorescent indicators and copper indicators measuring the free copper concentration at the interface could be used to further investigate the electrochemical reaction at the copper-silver alloy coated surface.

Whilst it is likely that most microorganisms will be affected by the copper-silver alloy coating, further experiments are required to address this. Spore-forming bacterial species and other bacteria common in e.g. food environment such as *Listeria monocytogenes* and *Salmonella enterica*, filamentous fungi and yeasts (e.g. *Candida albicans*), and human norovirus should be considered, also in the light of different applications.

The surface morphology and composition of the copper-silver alloy coating have been characterized using scanning electron microscopy, energy-dispersive X-ray spectroscopy, X-ray diffraction analysis, and the electrochemical properties in chloride-containing and chloride-free media have been evaluated with potentiodynamic polarization measurements [Paper 1, 2]. The relatively high surface roughness of the coating is due to the electroplating process. The coating consists of mechanical mixture of copper and silver, where silver atoms have diffused into the unit cell of copper, and this can probably explain the reduced over time oxidation of the surface as compared to an electroplated copper surface [Paper 1, 4]. Further investigation and use of other surface techniques such as X-ray photoelectron spectroscopy and transmission electron microscopy could help to evaluate the oxidation of the surface and residual stress in the electroplated layer, thus elucidating this phenomenon.

The industrial scalability and production of the copper-silver alloy coating is feasible, and new or already installed objects can be coated. A regenerative design approach, where the items can be re-coated once the copper-silver alloy coating would have reached the end of its lifetime, can also be applied wherever necessary.

Since chlorides enhance the antibacterial activity of copper alloys and build-up of organic material reduces their activity, a combined strategy using copper alloys and compatible cleaning procedures should be considered to maximize the overall antibacterial action. Evaluation of the antibacterial performance of the copper-silver alloy coating after exposure to common commercial disinfectants should be performed, even if compatible with the copper alloy surfaces. A procedure similar to that outlined in the U.S. EPA combined test protocol could be used. The U.S. EPA test methods are the most suitable for copper alloys, however, they should be in part adapted to suit also copper-based coatings. In the light of application as touch-surfaces, simulation of abrasion by hand contact (using e.g. polyurethane as counter-material) in dry and wet conditions can give indication of durability of the surface in daily usage conditions. Harsher wear condition, simulating scratching of the surface with a harder material, should also be taken into account and alternated to the (mild) daily abrasion test conditions. However, field-testing is a decisive test to evaluate the durability and antibacterial performance, since the combination of affecting parameters depends on the specific application. Copper-silver alloy coated door handles had lower microbial load than conventional surfaces during field-testing in clinical settings. Longer installation time and different applications would be required for a more precise estimation of the coating performances.

The understanding of the electrochemical reactivity of metals could be used to produce other combinations of redox-active metals, or an active system based on a galvanic couple, tailoring the choice of elements to the specific environment and application. Additionally, composition, state and surface structure could be varied to achieve the desired activity rate. It is however crucial that microbiology and materials science join forces to fulfill the needs and requirements in antimicrobial surface applications.

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Paper 1

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An electroplated copper–silver alloy as antibacterial coating on stainless steel

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ABSTRACT

Transfer and growth of pathogenic microorganisms must be prevented in many areas such as the clinical sector. One element of transfer is the adhesion of pathogens to different surfaces and the purpose of the present study was to develop and investigate the antibacterial efficacy of stainless steel electroplated with a copper-silver alloy with the aim of developing antibacterial surfaces for the medical and health care sector. The microstructural characterization showed a porous microstructure of electroplated copper-silver coating and a homogeneous alloy with presence of interstitial silver. The copper-silver alloy coating showed active corrosion behavior in chloride-containing environments. ICP-MS measurements revealed a selective and localized dissolution of copper ions in wet conditions due to its galvanic coupling with silver. No live bacteria adhered to the copper-silver surfaces when exposed to suspensions of *S. aureus* and *E. coli* at a level of 10^8 CFU/ml whereas 10^4 CFU/cm^2 adhered after 24 h on the stainless steel controls. In addition, the Cu-Ag alloy caused a significant reduction of bacteria in the suspensions. The coating was superior in its antibacterial activity as compared to pure copper and silver electroplated surfaces. Therefore, the results showed that the electroplated copper-silver coating represents an effective and potentially economically feasible way of limiting surface spreading of pathogens.

1. Introduction

Healthcare-associated infections (HCAIs) are one of the major causes of patient morbidity during hospitalization [1]. The European Centre for Disease Prevention and Control estimated that, on any given day in 2011–12, 81,089 patients were affected by HCAIs in European acute care hospitals, and the total annual number of patients with an HCAI was estimated at 3.2 million [2]. In 2014, 8% of the patients hospitalized for more than two days in an intensive care unit (ICU) in one of the 15 European countries reporting data, had at least one ICU-acquired healthcare-associated infection [3]. In 2015, the percentage of patients affected by ICU-HCAIs increased to 8.3% [4].

High frequency of HCAIs such as urinary tract infections, pneumonia, post-surgical complications is often associated with the use of invasive devices [1], but also a range of items including hospital furniture (bedrails, frames, door handles) can easily carry pathogenic microorganisms and be a vehicle of proliferation and transmission. Live bacteria adhere easily to different surfaces [5] and this can lead to the formation of structured and specialized bacterial communities (biofilms), which are often less sensitive to antimicrobial agents, such as disinfectants and surfactants. Thus, surfaces that minimize or even prevent bacterial adhesion could be a leading strategy in the control of HCAIs.

Surface treatments with antibacterial activity are receiving increasing attention and scientific interest since this could be a way of limiting transfer of bacteria and other infectious agents. Copper surfaces appear to be one of the best candidates due to their inherent biocidal properties [6–8], especially in environments where normal sanitization techniques are not sufficient to control the presence (or proliferation) of microorganisms or when the pathogenic agents have developed resistance against the compounds used [7,9]. When exposed to dry air, copper will be oxidized, however, this does not affect its biocidal properties, which makes it suitable for prolonged exposures under those conditions [10].

On the other hand, copper is also a fundamental trace element present in human body and it is necessary in a number of biological processes in most living organisms. More than 30 types of copper-containing proteins have been discovered as far [7].

The biocidal properties of copper are due to combination of several mechanisms that involve the redox couple Cu^+/Cu^{2+} [8,11]. Copper

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ions have the ability to cycle between Cu^{2+} and Cu^{+} at biologically relevant redox-potentials and Cu^{+} is considerably more toxic to bacteria than Cu^{2+} [6,12]. Cu^{+} ions are Fenton active, i.e. they can generate highly reactive oxygen species (ROS) when the further oxidization from Cu^{+} to Cu^{2+} occurs, and ROS can cause peroxidation and oxidation of proteins [13,14]. Free copper ions in high concentrations can also damage Fe-S clusters in metallo-proteins by occupying the metal site and therefore inactivating the protein [15]. In *Escherichia coli*, Fe-S clusters are specific target for copper toxicity, however, copper ions decrease oxidative DNA damage when *E. coli* cells were exposed to hydrogen peroxide [14–16]. Therefore, this suggests that in vivo copper ion toxicity in bacteria is not mediated by oxidative DNA damage and membrane proteins or membrane lipids are probably the major targets of copper toxicity [14,16].

Copper and copper alloys like bronze and brass are widely used in applications that foresee skin contact such as jewelry, electronics and hydraulic systems. Furthermore, copper or copper alloys items such as door knobs, bathroom fixtures, tables, armrests, etc., are already available on the market and had recently received more interest [6].

One concern of such surface alloys could be the development of bacterial Cu-resistance and cross-resistance to antibiotics. It had been demonstrated [17] that especially resistant strains, such as Gram-positive staphylococci and micrococci, *Kocuria palustris*, and *Brachybacterium conglomeratum* can survive on dry copper surfaces for 48 h or more. However, when these dry-surface-resistant strains were exposed to moist copper surfaces, resistance levels were close to those of control strains. This suggested that resistance mechanisms against dry metallic copper differ from those responsible for defense against wet surfaces or dissolved copper ions. Furthermore, the investigated staphylococci did not exhibit increased levels of resistance to antibiotics [17].

Copper surfaces obtained by deposition of copper through cold spray exhibit high killing efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) due to the copper microstructure that enhances the ionic diffusivity [18]. Both laboratory tests and clinical trials [19] have confirmed the superior effectiveness of copper alloys in killing bacteria when compared to components made of standard materials in hospital rooms (58% reduction in the infection rates). In vitro testing using the USA Environmental Protection Agency (EPA) approved testing protocols [20] have demonstrated the antibacterial efficacy of copper (I) oxide impregnated polymeric solid surfaces [10].

As for copper, the antimicrobial effect of silver has been known for centuries and silver is used as an antibacterial agent in different biomaterials such as urinary catheters, wound dressings and bone cement [21–23]. Also, silver has in vitro antimicrobial activity against MRSA [24].

Silver is inherently toxic to bacteria and it can inhibit bacterial growth by deactivation of membrane proteins, as Ag^{+} can bind to the thiol groups present in proteins [25]. However, silver and in particular silver nanoparticles due to their physicochemical properties, may also cause cytotoxicity and mitochondrial damage, although more targeted studies are still required to elucidate the role of mitochondrion in silver nanoparticles-induced toxicity [26].

The antibacterial effects of silver and copper have led to several studies combining these two antimicrobial components. Thus, copper and silver ions in combination can inactivate *Legionella pneumophila* in water distribution systems [27] and multi-layer silver-copper surface films sputter-coated on polymers for urinary catheters are efficient against *Pseudomonas aeruginosa* biofilm formation [28]. Silver-copper alloys, best known as sterling silver (92.5 wt% Ag and 7.5 wt% Cu), have been widely employed in jewelry and mint facilities, due to their superior strength conferred by the presence of copper [29]. However, copper-silver cast alloys with copper content between 50% and 94% [30] have only received limited attention, due to the limited solid solubility of the system (8.8 wt% Cu in the silver-rich phase and 8.0 wt% Ag in the copper-rich phase at the eutectic point), according to the copper-silver phase diagram [31].

However, one study has demonstrated that a copper-silver alloy with 10 wt% Ag obtained by intermixing of copper and silver on stainless steel through laser cladding process had a higher biocidal activity against *Escherichia coli* as compared to the pure elements [32].

Based on the above studies, we decided to investigate the antibacterial potential of an electroplated copper-silver alloy coating. Electroplating is one of the predominant surface technologies in Europe that aim at enhancing or providing various substrates with wear and corrosion protection, electrical conductivity and self-cleaning properties. Electroplating links and comprises a number of different sectors, thus it is one of the most significant manufacturing branches in the European economy [33]. Electroplating on different low-cost bulk materials characterized by various and complicated shape is easily feasible especially in a large-scale production. In fact, a number of key industries employ electroplating for reasons of economical and/or convenience factors, although other methods such evaporation and sputtering CVD (chemical vapor deposition) are an option [34]. Moreover, this process allows a regenerative design approach to recycle remaining coatings by stripping processes and further electroplate the items when the coating is worn off.

The purpose of the present study was to develop an antibacterial electroplated copper-silver alloy coating for stainless steel. The coating microstructure, chemical and electrochemical nature was characterized in detail by scanning electron microscopy, energy dispersive X-ray spectrometry, X-ray diffraction analysis and potentiodynamic polarization in different electrolyte solutions. The antibacterial properties of the copper-silver alloy coating and the ion release were investigated through bacterial adhesion tests and inductively coupled plasma mass spectroscopy (ICP-MS).

2. Materials and methods

2.1. Materials

The specimens were cut into 60 × 20 × 1 mm and 10 × 20 × 1 mm size coupons from AISI 316 and AISI 316L cold rolled sheet of steel having 2B surface finish, respectively. For the corrosion studies, cylindrical shape specimens were used according to ASTM G5-14 [35]. The chemical composition of AISI 316 and AISI 316L from supplier data sheet is presented in Table 1.

2.2. Surface preparation method

The AISI 316 and AISI 316L specimens were electroplated at a current of 4 A dm^{-2} for 1 min in a commercially modified copper-silver bath at Elplatek A/S Galvanord. To achieve the desired layer thickness i.e. 10 ± 0.8 μm, this process was repeated four times on each specimen.

AISI 316L specimens were electroplated at a current of 5 A dm^{-2} for 10 min in a commercial acidic copper bath and at a current of 1 A dm^{-2}

Table 1
The chemical composition of AISI 316 and AISI 316L from the supplier (LGM) data sheet.

EN10088-0	W.nr.	ASTM	C. % Max	Cr. %	Ni. %	Mo. %
X5CrNiMo17-12-2	1.4401	316	0.07	16.50–18.50	10.00–13.00	2.00–2.50
X2CrNiMo17-12-2	1.4404	316L	0.03	16.50–18.50	10.00–13.00	2.00–2.50

for 15 min in a commercial silver bath. Current density and time were chosen in order to achieve a coating thickness of 10 ± 0.5 μm.

Prior to the electroplating process, the specimens were cathodically degreased in a cyanide bath keeping the voltage at 3 ± 0.5 V for 2 min followed by rinsing with deionized water. After the rinsing, an activation step for the stainless steel substrate through a Wood's nickel strike was carried out at a current of 4.5 ± 0.5 A dm^{-2} for 2 min in order to ensure good adhesion between the electroplated coatings and the substrate. In the case of silver electroplating, a strike bath was performed at a current of 0.5 A dm^{-2} for 1 min to prevent immersion deposit and poor adhesion.

2.3. Microstructural characterization

2.3.1. Scanning electron microscopy and energy dispersive X-ray spectrometry

The microstructure and the chemical composition of the copper-silver alloy coated specimens were analyzed by scanning electron microscopy (SEM) (JEOL JSM 5900 Instrument operated at 13 kV) which was equipped with Oxford EDS detector and Oxford Inca software.

2.3.2. X-ray diffraction analysis

The crystalline structure of the deposited layer was determined by using X-ray diffraction. Chromium radiation (K_α = 2.29 Å) was chosen with a step time of 576 s and the scanning was performed from 32° to 78° values of 2θ angles with steps of 0.060° θ.

2.4. Potentiodynamic polarization

Potentiodynamic polarization scans were recorded according to ASTM G5-14 standard test method with an ACM (GillAC) potentiostate. A saturated calomel electrode and an iridium-oxide coated titanium electrode were used as reference and counter electrodes, respectively. The electrolytes used for polarization scans were EN 1811 artificial sweat media (NaCl 5.00 ± 0.01 g/l, CH_4N_2O 1.00 ± 0.01 g/l, $CH_3CHOHCOOH$ 940 ± 20 μl, DI H_2O 900 ml, pH 6.5 at 19 °C), 0.1 M Na_2CO_3 solution and phosphate-buffered saline buffer solution (PBS; Dulbecco A; Oxoid). Prior to the polarization scans, the OCP was monitored for 24 h. The measurements were performed on copper-silver alloy coated and AISI 316 specimens and conducted in replicas for consistency.

2.5. Antibacterial test

The bacterial adhesion to AISI 316L and copper-silver alloy coated coupons were tested using *Staphylococcus aureus* 8325 [36] and *Escherichia coli* MG1655 [37].

The bacterial adhesion to AISI 316L, pure copper, pure silver and copper-silver alloy electroplated coupons were tested using *Staphylococcus aureus* 8325 [36].

The bacteria were revived from −80 °C storage and grown on Brain Heart Infusion (BHI) agar plates (Oxoid, CM1135) at 25 °C overnight. The bacteria were inoculated in BHI broth (Oxoid, CM1135) and grown for two days at 25 °C. Ten-fold serial dilutions were made and bacterial cells transferred to phosphate-buffered saline solution (PBS; Dulbecco A; Oxoid) to an initial level of approx. 10^6 CFU/ml. In the test with the four different materials *S. aureus* at an initial level of approx. 10^7 CFU/ml was used.

The AISI 316L and copper-silver alloy coated coupons for testing with *S. aureus* and *E. coli* were cathodically degreased and sterilized by autoclaving. The AISI 316L, pure copper, pure silver and copper-silver alloy electroplated coupons (Fig. 1) live adherent bacteria for testing with *S. aureus* were sterilized by dry heat in order to avoid conditions for oxide formation on copper. Bacteria suspensions were added to sterile polystyrene tubes (Sterikin LDT; Bibby Sterin LDT; Stones; UK) containing the coupons and 2 ml of phosphate-buffered saline buffer solution (PBS; Dulbecco A; Oxoid). The tubes were incubated at 25 °C for ½, 4 and 24 h. The incubation time for the test with the four different materials against *S. aureus* was ½h only.

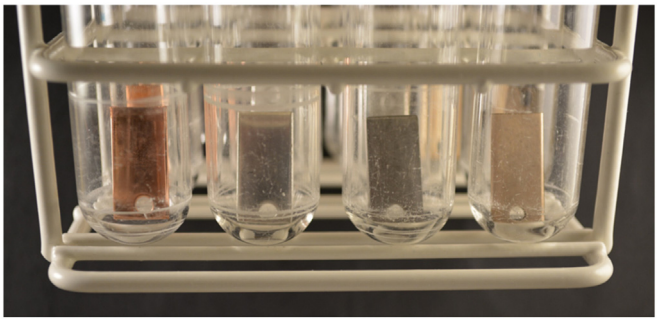

Fig. 1. From left to right: copper electroplated coupon, silver electroplated coupon, AISI 316L coupon and copper-silver alloy electroplated coupon after sterilization by dry heat.

Following the incubation time, the coupons were rinsed with 2 ml of sterile buffer solution and moved into new sterile tubes containing 2 ml of sterile buffer solution. These tubes were sonicated for 4 min at 25 °C (28-kHz, 2 × 150 W sonication bath, Delta 220, Deltasonic, Meaux, France) and vortexed at maximum speed for 15 s to further facilitate the detachment of bacteria from the surfaces [38]. The number of live bacteria attached on surfaces and the cell concentration in the suspension was determined by serial dilution and plating on BHI agar (Oxoid, CM1135) [39]. In order to enumerate the total number of live bacteria per unit of surface (CFU/cm^2), CFU/ml^{-1} values were recalculated taking into account the area (4 cm^2) and the volume of solution (2 ml).

For *S. aureus*, the antimicrobial test above was also performed with an initial diluted culture of 10^8 CFU/ml in PBS and an initial diluted culture of 10^6 CFU/ml in growth medium, BHI.

Experiments with AISI 316L and copper-silver coated coupons against *S. aureus* and *E. coli* included technical triplicates and all experiments were conducted in three biological replicates.

2.6. Ion release analysis and pH measurement

The instrument used to perform the ICP-MS analysis of copper and silver ion release was an ICAPq ICPMS (Thermo, Fisher Scientific GmbH, Bremen, Germany). The analysis was performed using the isotopes ^{63}Cu and ^{107}Ag, respectively, and was done using KED mode with helium as cell gas. External calibration with matrix matched calibrants and internal standardization (^{103}Rh) was done for the quantification and a ×100 dilution with milli-q water was carried out prior to the analysis. The bacterial (*S. aureus*) suspensions of PBS and BHI media where copper-silver alloy coated coupons were tested ½, 4 and 24 h, respectively, were 0,2 μm filtered and stored at −20 °C prior the analysis. PBS and BHI sterile solutions were analyzed as controls. The pH measurement of the test suspensions were carried out with a Radiometer PHM 95 pH/Ion-Meter calibrated before each set of measurement.

2.7. Statistical analysis

Bacterial cell numbers were log transformed and the average values among the triplicates of copper-silver coated and stainless steel coupons were calculated for each testing time. Statistical significance ($P < 0.05$) of the difference between the two surfaces was tested using the *t*-test. Numbers of live bacteria attached on surfaces and suspended bacteria in the testing solutions were compared by testing for the equality of the means assuming equal or unequal variance following the *F*-test.

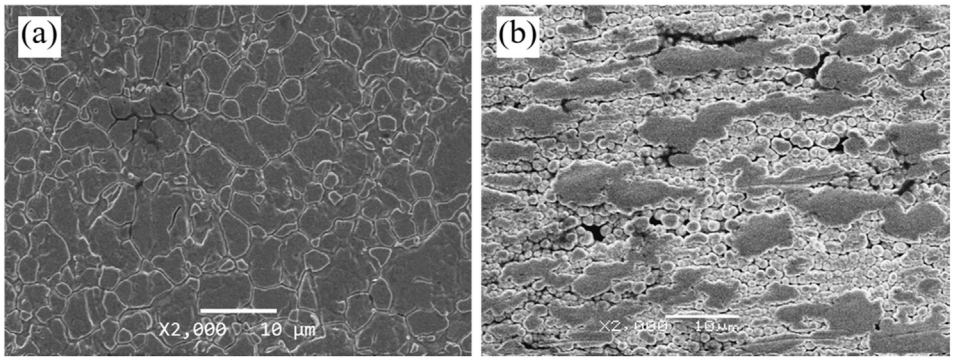

Fig. 2. Scanning electron microscopy of uncoated (a) and copper-silver coated (b) AISI 316 at 2000× magnification.

3. Results and discussion

3.1. Microstructural characterization

3.1.1. Surface morphology, composition and cross-sectional analysis of copper-silver surfaces

The surface morphology of the copper-silver alloy coating showed a rather uniform globular microstructure characterized by a marked distributed porosity in comparison with the stainless steel AISI 316 substrate (Fig. 2), which displayed a grain morphology typical of a 2B finished steel surface [40].

The deposition of the copper-silver alloy took place on grains and grain boundaries and the coating growth showed columnar morphology while the intrinsic porosities were present in between the columns (Fig. 3).

The presence of grooves and flat areas (marked in Fig. 3a and b) represents an artifact generated during the mechanical polishing of the coated surface. The cross-sectional analysis of the coating (Fig. 3c) showed that the porosities present at the coating surface did not penetrated down to steel substrate and the coating was well adherent with the steel substrate at microscopic scale. The composition of the copper-silver coating was 59 ± 2 wt% of copper and 39 ± 2 wt% of silver, as displayed by the surface EDS analysis (Fig. 3d).

Since the exposed area of the copper-silver alloy coating was larger with respect to a non-porous flat surface, bacteria that meet the surface would face a higher net contact with copper and silver. In fact, the surface roughness also influenced the contact killing [6] and it was demonstrated [15] that rough electroplated copper surfaces were more antibacterial than polished or rolled copper, since the release of copper ions per time was higher.

In addition, an aqueous layer could be more easily retained at the surface due to capillary forces [41] and in such wet conditions, a galvanic cell would be established between copper and silver in the electroplated deposit. Consequently, the morphological features of the copper-silver alloy coating could play a key role in its antiadhesive and antibacterial properties.

3.1.2. Phase analysis of copper-silver surfaces

The X-ray diffractogram of the copper-silver alloy coating detected Ag (111) and Ag (200) peaks at near 38° and 45° angle (blue dotted lines) (Fig. 4).

There were two characteristic peaks at 42° and 49° (marked by the red solid line) corresponding to the crystallographic directions of Cu (111) and Cu (200), respectively. These peaks were shifted towards

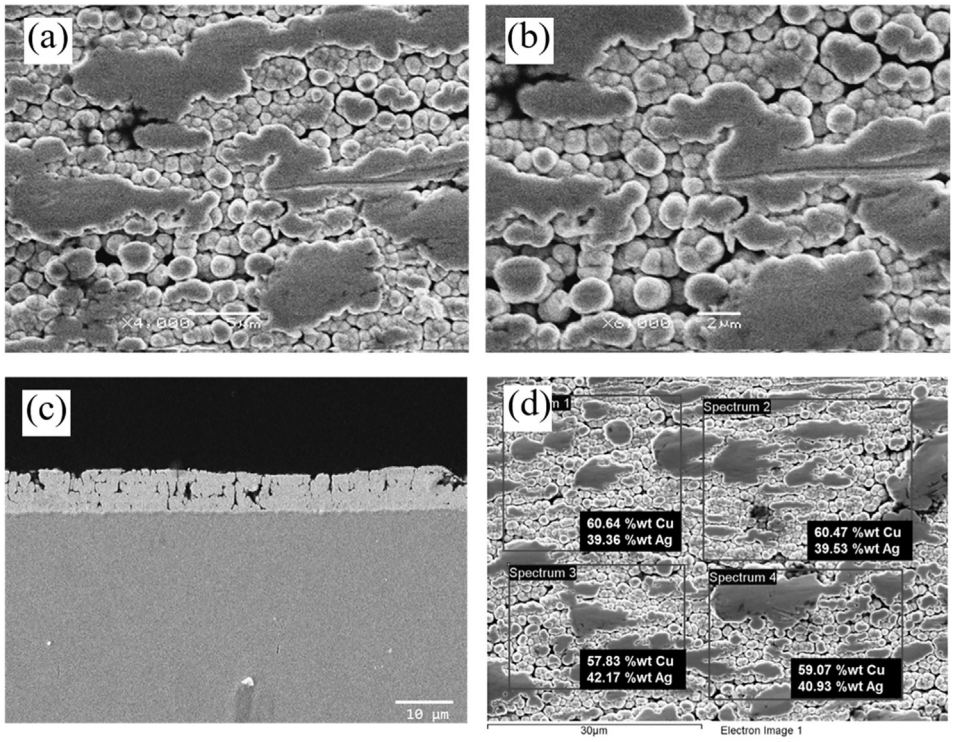

Fig. 3. Scanning electron microscopy of copper-silver coating. The same area was captured at different magnifications: 4000× (a) and 6000× (b). Cross-section of the copper-silver coating electroplated on AISI 316 substrate (1000× magnification) (c). Scanning electron microscopy and energy dispersive x-ray spectrometry on the inspected copper-silver coated plate (d).

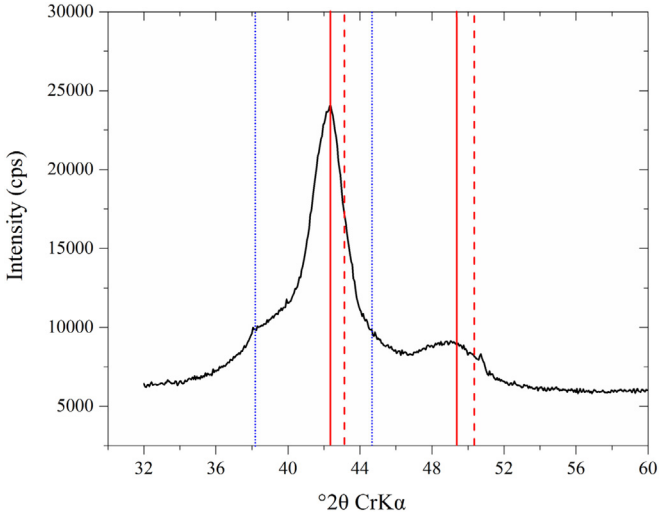

Fig. 4. XRD diffractogram of copper-silver coating. Cu (111) and (200) peaks were marked by a red solid line, instead the Cu (111) and (200) peaks representative of pure Cu were marked with a dashed red line. Ag (111) and (200) peaks were marked with a blue dotted line [42]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower angles in comparison with the position of the characteristic peaks of pure copper (marked by the red dashed line) suggesting that a certain amount of silver atoms had entered the copper crystal lattice and therefore had warped the unit cell, causing the observed shift of the diffraction peaks. The variation of the lattice parameters is estimated to approx. 2% according to the multiplying factor used by DIFFRACT.EVA software to simulate an isotropic dilatation of the crystal lattice [42].

The two elements were not expected to be soluble at room temperature according to the copper-silver phase diagram [31], however it has been reported [43] that the atomic solubility of silver atoms in bulk copper resulted of about 0.08 atom% at room temperature. Therefore, on the base of the present X-ray diffraction analysis, the copper-silver alloy coating can be defined as a homogenous mixture (alloy) of the two metals where a small amount of Ag atoms had diffused into the copper crystal structure.

3.2. Individual thermodynamic behavior of copper and silver

Potential-pH diagrams (Pourbaix diagrams) allowed a thermodynamic evaluation of the behavior of metals in aqueous environments at different electrochemical conditions. Here, the diagrams were calculated by means of HSC Chemistry software [44] for pure copper and pure silver in three different environments which resembled the solutions of interest: EN 1811 artificial sweat, phosphate-buffered saline buffer solution (PBS; Dulbecco A; Oxoid) (Fig. 5) and 0.1 M Na_2CO_3 (Fig. 6).

The purpose was to provide the experimental polarization measurements with a thermodynamic basis, which gave information about regions of metal stability, oxide formation and metal dissolution at potential and pH of interest. The calculated diagrams showed that around neutral pH conditions 7 ± 0.5 a protective AgCl layer was likely formed on silver in a chloride-containing environment above 250 mV (Fig. 5a and b) and Cu was dissolved in the form $CuCl_2^-$ between 100 and 400 mV (Fig. 5c and d). However, in presence of a solution containing 0.1 M Na_2CO_3, the pH raised up to 11. Under these strong alkaline pH conditions at 100 mV, silver (Fig. 6a) remained in its stability region, whereas Cu_2O and CuO formed on the copper surface (Fig. 6b). In dry environmental conditions the formation of Cu_2O was favored, while in humid conditions during long aging periods CuO was formed [6,10]. In addition, it was reported [45] that the presence of chloride ions shifted the formation of Cu_2O to more alkaline pH. Cuprous oxide showed the same antibacterial efficacy as pure copper, while cupric oxide exhibited much slower antibacterial killing [6,10] and this was correlated to the higher solubility of Cu_2O, i.e. higher ionic release, as compared to CuO. Therefore, this suggested that the long exposure of copper to humid atmosphere would reduce its antibacterial activity due to the formation of CuO.

Under oxidizing conditions or at high pH above 500 mV silver oxides would form and it has been suggested [6] that AgO was probably main responsible of observed antimicrobial effect of silver, since it has high solubility, even greater than Cu_2O.

3.3. Polarization behavior of copper-silver surfaces

The anodic behavior of AISI 316 and copper-silver alloy coating was studied in different electrolytes i.e. EN 1811 artificial sweat media, 0.1 M Na_2CO_3 solution and phosphate-buffered saline buffer solution (PBS), respectively (Fig. 7).

In all the electrolytes, AISI 316 displayed its typical passive nature. However, the change in the type of electrolyte shifted the corrosion potential values, i.e. +20 mV (vs. SHE) for artificial sweat, −100 mV (vs. SHE) for 0.1 M Na_2CO_3 and −75 mV (vs. SHE) for PBS. A similar phenomenon was also observed for the copper-silver alloy coating where the corrosion potential was +80 mV (vs. SHE) for artificial sweat, +100 mV (vs. SHE) for 0.1 M Na_2CO_3 and + 50 mV (vs. SHE) for PBS. Overall, the corrosion potential values for copper-silver alloy coating exhibited higher shift towards the noble side when compared to stainless steel, regardless of electrolyte type. The copper-silver alloy coating showed active behavior by exhibiting higher values of anodic current density when compared to stainless steel surface. In contrast, the copper-silver alloy coating showed passive nature in 0.1 M Na_2CO_3 solution and lower anodic current density values when compared the chloride-containing environments (artificial sweat and PBS). The higher anodic current density of the copper-silver alloy coating in comparison with stainless steel was probably due to the presence of copper, which speeded up the corrosion rate. On the other hand, the copper-silver alloy coating displayed a higher corrosion potential than stainless steel and this was likely because silver possessed higher cathodic potential than stainless steel. Moreover, the natural formation of the protective chromium oxide on the stainless steel surface conferred it the typical passive nature [46].

In accordance with previous studies [47,48] and consistent with the calculated Pourbaix diagrams (Fig. 5c and d), potentiodynamic polarization tests confirmed that copper dissolved from the surface of the coating in presence of a chloride-containing environments as the more toxic Cu^+ [12] in the form of soluble cuprous chloride ion complex $CuCl_2^-$.

Therefore, since the formation of copper oxides responsible for a passivation state was prevented in presence of chlorides at pH near neutrality, the corrosion rates increased in comparison with the case of 0.1 M Na_2CO_3 solution. In the latter case, a passivation state was likely reached due to the formation of copper oxide at potentials > 100 mV (Fig. 6b).

3.4. Bacterial adhesion and survival to copper-silver alloy coating and AISI 316L

3.4.1. Antibacterial effect against S. aureus

S. aureus, at a level of 10^6 CFU/ml buffer, adhered to the AISI 316L coupons at a level of 10^4 CFU/cm^2 whereas the number on the copper-silver alloy coated coupons was lower than 10 CFU/cm^2 (Table 2).

The differences were, at all time points, statistically significant ($P = 0.0001$). The copper-silver alloy surfaces maintained its efficacy in repelling the attachment of live bacteria when the initial concentration of *S. aureus* culture was approx. 10^8 CFU/ml (Table 2). The difference in

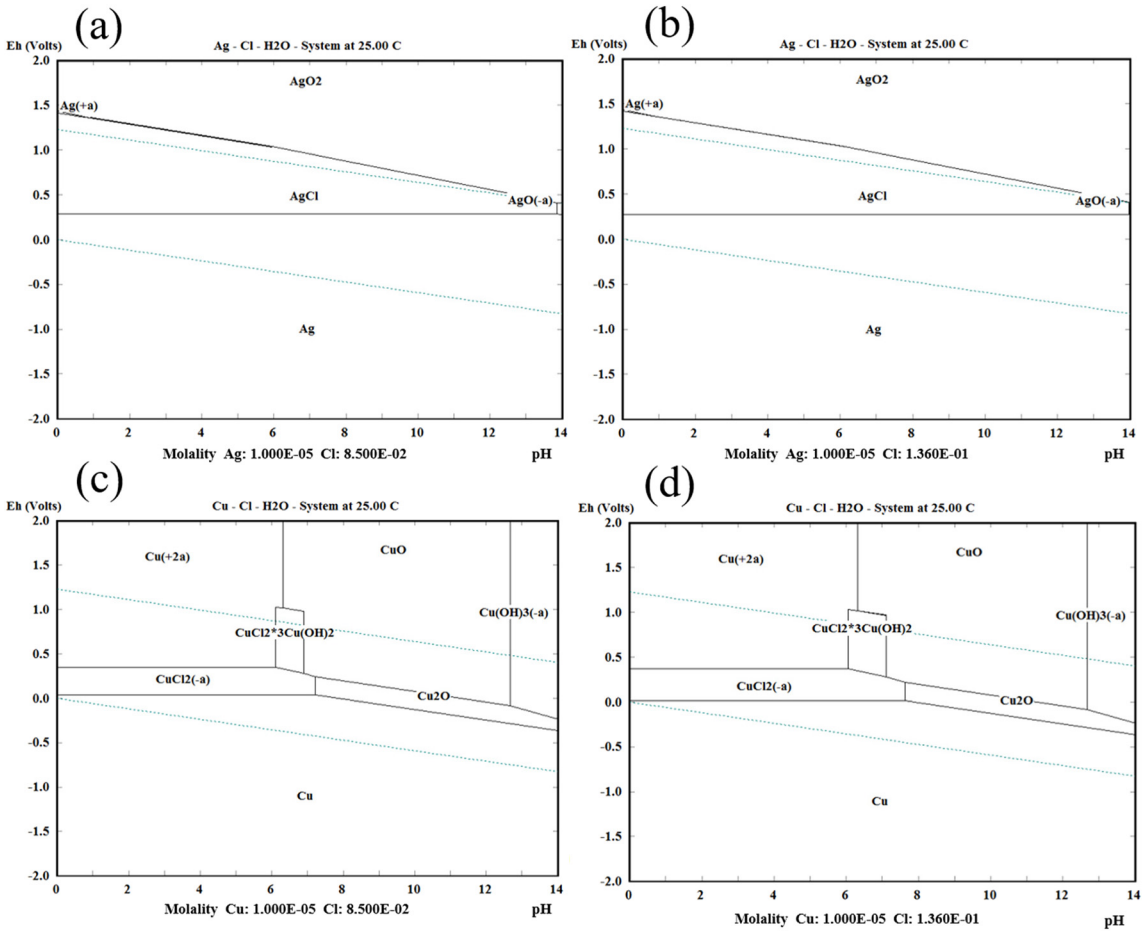

Fig. 5. Pourbaix diagrams (E-pH) of (a), (b) pure silver and (c), (d) pure copper (Cl–H_2O system at 25 °C) in the presence of 5 g/l of NaCl correspondent to the artificial sweat solution (a), (c) and 8 g/l of NaCl correspondent to phosphate-buffered saline buffer solution (b), (d).

numbers of bacteria was, again, clearly statistically significant ($P = 0.0004$).

The antibacterial effect of the copper-silver alloy coating was less pronounced when *S. aureus* was allowed to grow in BHI broth during attachment. The number of live adherent bacteria on the copper-silver alloy surfaces and the AISI 316L was initially approx. 10^2 and 10^3 CFU/cm^2 respectively. The number of live adherent bacteria increased from approx. 10^2 CFU/cm^2 to 10^5 CFU/cm^2 on the copper-silver alloy surfaces over 24 h and the number on the stainless steel controls was constantly 1–2 log units above (Table 2). The difference in numbers of live adherent bacteria on the two types of surfaces was not statistically significant ($P = 0.522$).

The numbers of *S. aureus* decreased from 10^6 CFU/ml to 10^2 CFU/ml in the PBS suspension in the presence of copper-silver alloy coupons while remaining constant where the AISI 316L coupons were immersed ($P = 0.042$) (Table 3).

There was a slight decrease in *S. aureus* numbers in BHI broth in the presence of copper-silver alloy coated coupons as compared to the AISI 316L (Table 3) but this was not statistically significant ($P = 0.963$).

S. aureus, at an initial level of 10^7 CFU/ml buffer, adhered to the

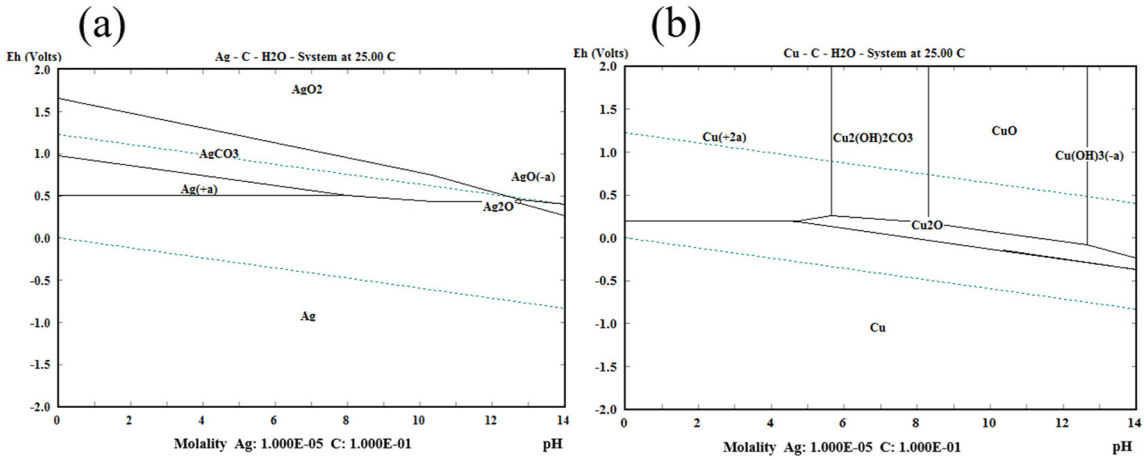

Fig. 6. Pourbaix diagrams (E-pH) of (a) pure silver and (b) pure copper (Cl–H_2O system at 25 °C) in the presence of 0.1 M Na_2CO_3 solution.

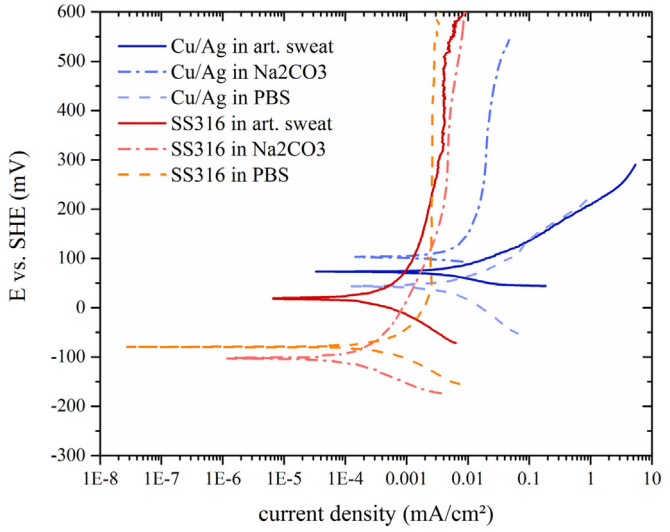

Fig. 7. Polarization curves of copper-silver coated AISI 316 and AISI 316 specimens tested in EN 1811 artificial sweat, and 0.1 M Na_2CO_3 solution and phosphate-buffered saline buffer solution. Potential (mV) values in ordinate are recalculated against the standard hydrogen electrode (SHE).

Table 2
Attachment of *S. aureus* to copper-silver alloy coated and uncoated stainless steel AISI 316L surfaces. Numbers are mean values ± standard deviations of six total biological replicates performed in two technical replicates. LOD = limit of detection (1 CFU).

Dilution media	Average initial cell concentration Log (CFU/ml)	Time (h)	Attachment (Log (CFU cm^{-2})) of *S. aureus*	
			Copper-silver	AISI 316L
PBS	6.3 ± 0.5	0.5	0.7 ± 1.0	3.9 ± 0.1
		4	< LOD	4.4 ± 0.1
		24	< LOD	4.1 ± 0.0
PBS	8.1 ± 0.3	0.5	0.7 ± 0.5	4.9 ± 0.2
		4	0.3 ± 0.4	5.8 ± 0.1
		24	0.1 ± 0.2	6.5 ± 0.2
BHI broth	6.6 ± 0.1	0.5	2.5 ± 0.4	3.2 ± 0.2
		4	2.6 ± 1.1	4.1 ± 0.0
		24	5.8 ± 0.3	6.8 ± 0.3

Table 3
Survival of *S. aureus* in suspension with copper-silver alloy coated and uncoated stainless steel AISI 316L surfaces. Numbers are mean values ± standard deviations of three biological replicates each performed in two technical replicates. *Only three replicates were considered. LOD = limit of detection (1 CFU).

Dilution media	Average initial cell concentration Log (CFU/ml)	Time (h)	Survival Log (CFU/ml) of *S. aureus* in suspension	
			Copper-silver	AISI 316L
PBS	6.3 ± 0.5	0.5	3.6 ± 2.9	6.4 ± 0.0
		4	0.8 ± 1.1	6.2 ± 0.0
		24	0.9 ± 1.3	5.8 ± 0.1
PBS	8.1 ± 0.3	0.5	6.7 ± 0.5	7.6 ± 0.1
		4	3.5 ± 1.0	7.3 ± 0.6
		24	2.0 ± 0.3	8.0 ± 0.1
BHI broth	6.6 ± 0.1	0.5	6.6 ± 0.1	6.5 ± 0.0
		4	7.2 ± 0.2*	6.7 ± 0.6
		24	8.6 ± 0.1	9.3 ± 0.1

AISI 316L and silver coupons at a level of approx. 10^5 CFU/cm^2 whereas the number on the copper coupons was approx. 10^3 CFU/cm^2 and on the copper-silver alloy coated coupons was lower than 10 CFU/cm^2 after 30 min (Table 4). These results confirmed that metallic silver is not antibacterial in test conditions where the silver ions release is not occurring, as it can be seen from the Pourbaix diagram (Fig. 5) and as stated previously [6]. On the other hand, copper had antibacterial activity with approx. two-log reduction in *S. aureus* attachment compared to stainless steel. The copper-silver alloy coating had the highest antibacterial efficacy in these test conditions, with approx. a four-log reduction after 30 min of exposure to *S. aureus*.

Table 4
Attachment of *S. aureus* to pure copper, pure silver, copper-silver alloy electroplated and uncoated stainless steel AISI 316L surfaces. Numbers are mean values ± standard deviations of three biological replicates.

Initial cell concentration Log (CFU/ml)	Time (h)	Attachment (Log (CFU cm^{-2})) of *S. aureus*			
		AISI 316L	Copper	Silver	Copper-silver
7.3	0.5	4.7 ± 0.1	2.5 ± 0.7	4.9 ± 0.1	0.1 ± 0.1

3.4.2. Antibacterial effect against E.coli

E.coli, at a level of 10^6 CFU/ml buffer, adhered to the AISI 316L surfaces at a level of approx. 10^2 CFU/cm^2 whereas the number on the copper-silver alloy coated coupons was lower than 10 CFU/cm^2 (Table 5).

A similar investigation [32] reported in literature has demonstrated the high antibacterial activity of a CuAg clad alloy (with 10 wt% Ag) against *E. coli* with six logs reduction in 180 min through wet plating testing [49], which was followed by pure copper (4 log reduction), silver and stainless steel that did not exhibit significant antimicrobial effect [32].

Even though the initial cell concentration was not indicated in the study [32], our results confirmed the reported findings within a six-time lower exposure time, as we observed a six logs reduction within 30 min. These results suggest that the killing activity of the copper-silver alloy electroplated coating may be faster than the CuAg clad alloy.

Besides, the study [32] addressed that the increased antimicrobial properties of the CuAg clad alloy may be primarily due to the higher release of copper ions, which in turn is governed by the surface electrochemistry. This also explained the faster efficacy observed in the copper-silver alloy electroplated coating, since the higher amount of silver in the coating (approx. 40 wt%) increased the cathodic and anodic areas ratio, which controlled the speed of the anodic reaction. Moreover, the electroplated copper-silver surface had a higher roughness (Figs. 2 and 3), which increased the bacteria-metal contact area and the release of copper ions per time [6,15,50].

The numbers of *E.coli* decreased from 10^6 CFU/ml to approx. 10^3 CFU/ml in the PBS suspension in the presence of copper-silver alloy coupons while remaining constant where the AISI 316L coupons were immersed, but the difference in numbers was not statistically significant ($P = 0.221$) (Table 5).

Table 5
Attachment of *E. coli* to copper-silver alloy coated and uncoated stainless steel AISI 316L surfaces and survival in suspension. The average initial cell concentration was 5.8 Log (CFU/ml). Numbers are mean values ± standard deviations of three biological replicates. LOD = limit of detection (1 CFU).

Time (h)	Attachment and survival of *E. coli* on surfaces and in suspension			
	Copper-silver		AISI 316L	
	Adhesion Log (CFU cm^{-2})	Suspension Log (CFU/ml)	Adhesion Log (CFU cm^{-2})	Suspension Log (CFU/ml)
0.5	< LOD	5.6 ± 0.1	2.5 ± 0.5	5.6 ± 0.0
4	< LOD	4.6 ± 0.2	2.7 ± 0.2	5.7 ± 0.0
24	< LOD	3.4 ± 0.7	2.5 ± 0.4	5.0 ± 0.3

Table 6
Copper and silver ion concentrations released in PBS and BHI by the copper-silver alloy electroplated surfaces and the stainless steel controls at the different times measured by ICP-MS. Sterile (PBS and BHI) and filtered media that had been in contact only with bacteria were also tested as further controls.

Material	Average initial cell concentration Log (CFU/ml)	Metal ions concentration [$\mu g \cdot l^{-1}$]					
		After ½ h		After 4 h		After 24 h	
		Cu	Ag	Cu	Ag	Cu	Ag
Copper-silver	6.3 ± 0.5	3041	9	3108	15	3539	11
AISI 316L		1749	< 5	2168	< 5	3236	13
PBS filtered						220	< 5
PBS sterile						169	< 5
Copper-silver	8.1 ± 0.3	80,511	29	87,954	70	87,789	31
AISI 316L		2264	< 5	3567	< 5	3931	< 5
PBS filtered						387	< 5
PBS sterile						174	< 5
Copper-silver	6.6 ± 0.1	13,994	7	41,201	13	48,648	26
AISI 316L		< 10	0	139	< 5	< 10	< 5
BHI filtered						< 10	< 5
BHI sterile						< 10	< 5

3.5. Ion release and pH variation during the bacterial adhesion tests

Copper ion concentration in the PBS suspensions where the copper-silver alloy coated coupons were immersed with an initial concentration of *S. aureus* of approx. 10^6 CFU/ml increased over time and reached 3500 µg/l after 24 h (Table 6).

When the initial concentration of *S. aureus* culture was 10^8 CFU/ml, the copper ion concentration was significantly increased and reached a level of almost 88,000 µg/l after 24 h. This can be attributed to the higher bacterial load but also to the media carry-over from the initial diluted bacterial suspensions.

Copper ions in aqueous solutions easily form coordination compounds with organic species such as proteins and carbohydrates, therefore medium composition and pH influences greatly the sensitivity of bacteria towards copper ions [51].

Under the same conditions, the release of silver ions was negligible compared to copper (Table 6) in accordance with previous results [32]. From the stainless steel controls, the release of copper ions was lower but in the same order of magnitude in comparison with the copper-silver alloy coated surfaces immersed in suspension with the initial concentration of *S. aureus* of approx. 10^6 CFU/ml (Table 6). We did not expect copper ion release from the stainless steel, however copper is often added to stainless steel during the metallurgical process in order to enhance its resistance to corrosion (increase the pitting potential) [52], even if not always indicated by the supplier, as in the present case.

Copper ion concentration in the BHI suspensions with an initial concentration of *S. aureus* of approx. 10^6 CFU/ml increased over time with values in between the concentrations measured in the PBS suspensions. In this case, the ion release increased to almost 49,000 µg/l after 24 h and the release of metal ions from the stainless steel controls was negligible (Table 6). The presence of complex medium speeded up the release of copper ions compared to the buffer solution with the same initial cell concentration (Table 6).

The pH increased linearly from 7.3 ± 0.1 to 7.7 ± 0.1 over the 24 h where the copper-silver alloy coated coupons were immersed in buffer with *S. aureus*. In contrast, pH remained stable at about 7.3 ± 0.1 in buffer with stainless steel surfaces emerged (Table 7).

The pH of the BHI suspensions were lower and no significant difference was between the copper-silver surfaces and the stainless steel was observed after ½ h and 4 h (Table 7). After 24 h, the pH increased in both the stainless steel and the copper-silver suspensions, but more markedly in the latter.

The concentration of copper ions increased in all the suspensions, whereas in comparison silver was released only in negligible amounts.

Table 7
Values of pH measured in PBS and BHI where the copper-silver alloy electroplated surfaces and the stainless steel controls were tested at the different times. The pH was also measured in sterile (PBS and BHI) and filtered media that had been in contact only with bacteria.

Material	Average initial cell concentration Log (CFU/ml)	pH		
		After ½ h	After 4 h	After 24 h
Copper-silver	6.3 ± 0.5	7.3 ± 0.1	7.5 ± 0.1	7.7 ± 0.1
AISI 316L		7.3 ± 0.1	7.3 ± 0.1	7.3 ± 0.1
PBS filtered				7.2 ± 0.1
PBS sterile				7.2 ± 0.1
Copper-silver	8.1 ± 0.3	7.2 ± 0.1	7.3 ± 0.1	7.7 ± 0.1
AISI 316L		7.2 ± 0.1	7.2 ± 0.1	7.2 ± 0.1
PBS filtered				7.2 ± 0.1
PBS sterile				7.2 ± 0.1
Copper-silver	6.6 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	6.5 ± 0.1
AISI 316L		6.1 ± 0.1	6.1 ± 0.1	6.2 ± 0.1
BHI filtered				6.1 ± 0.1
BHI sterile				6.9 ± 0.1

This further indicates that in presence of a chloride-containing environment silver is protected by the selective corrosion, i.e. dissolution, of copper induced by the galvanic coupling. The increase of pH in the PBS suspensions was probably due to the progressive precipitation of $CuCl_2^-$ (Fig. 5c and d) following the overtime dissolution of copper ions. Therefore, the shift towards a more alkaline environment could add a further damaging effect to the biocidal action of the copper ions.

The charged amino acids of the meat extracts in BHI, acting as cation "sink," could have reduced the amount of free ions accessible to bacteria and the ions complexation could have also maintained the pH of the suspension more stable to lower values, protecting in this way the bacteria in their growing environment [53].

4. Conclusions

In this study, an electroplated copper-silver alloy coating was developed and characterized in its microstructure, chemical and electrochemical nature. The copper-silver alloy coating (59 ± 2 wt% Cu and 39 ± 2 wt% Ag) had a significant antibacterial effect against *S. aureus* and *E. coli* as compared to AISI 316L stainless steel. The coating was also superior in antibacterial activity against *S. aureus* when compared to pure copper electroplated surfaces.

The electrochemical mechanism, the copper and silver areas ratio and the porous microstructure were believed responsible. In presence of a chloride-containing environment, bacteria would be exposed to metallic copper at the interface, Cu^+ ions in the surroundings and metallic silver where the pH raises locally. Therefore, the galvanic coupling would allow the copper-silver alloy electroplated coating to maintain its antibacterial efficiency in the intended working conditions. The electroplated copper-silver alloy coating could therefore be an effective coating solution for reasons of economy and convenience in the burdensome struggle against the proliferation and transmission of pathogens in hospitals and intensive care units. However, further investigations are required to assess the behavior of the coating in dry environmental conditions and the kinetics of the bacterial inactivation.

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Paper 2

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Influence of chlorides and phosphates on the anti-adhesive, antibacterial and electrochemical properties of an electroplated copper-silver alloy.

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Influence of chlorides and phosphates on the antiadhesive, antibacterial, and electrochemical properties of an electroplated copper-silver alloy

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Antimicrobial surfaces such as copper alloys can reduce the spread of pathogenic microorganisms, e.g., in healthcare settings; however, the surface chemistry and thus the antibacterial activity are influenced by environmental parameters such as cleaning and disinfection procedures. Therefore, the purpose of the present study was to assess how copper-complexing compounds (chlorides and phosphates), common to the clinical environment, can affect the surface chemistry and the antiadhesive and antibacterial properties of a newly developed antibacterial copper-silver alloy and the single alloying metals. The authors demonstrated that the antiadhesion efficacy against *S. aureus* 8325 was the highest when the copper-silver alloy and copper surfaces (four- and two-log bacterial reduction compared to stainless steel controls, respectively) were exposed to chloride-containing suspensions. This was explained by the electrochemical activity of copper that dissolved as Cu^{+}, highly toxic to the bacterial cells, in the presence of Cl^{-} and eventually formed a chlorine- and oxygen-rich layer with the incorporation of phosphorus, if also phosphates were present. If chlorides were omitted from the wet environment, there was no difference ($P > 0.05$) in bacterial counts on copper-silver alloy, copper, silver, and AISI 316 stainless steel control surfaces, due to the fact that no oxidizing conditions were established and therefore there was no dissolution of copper ions from copper-silver alloy and copper surfaces. However, under dry conditions, copper-silver alloy and pure copper surfaces were antibacterial also in the absence of chlorides, suggesting a marked difference between dry and wet conditions in terms of the interactions between surfaces and bacteria. The authors conclude that an attentive design of control policies integrating disinfection interventions and antimicrobial surfaces, such as the copper-silver alloy coating, can be a beneficial solution in fighting the spread of antibiotic resistant bacterial strains and potentially reducing the number of disease outbreaks. *Published by the AVS.* https://doi.org/10.1116/1.5088936

I. INTRODUCTION

Hand-washing and infection control policies are first-line strategies against healthcare-associated infections (HCAIs) but ensuring complete compliance with the protocols is challenging and can be difficult in practice, as in the case of hand washing before and after every patient contact.[1,2] Moreover, HCAIs and the rapid spread of antimicrobial resistance pose a major challenge to the normal control policies and disinfection practices in hospitals and healthcare institutions.[3,4] Hospital patients are particularly susceptible to infections because of their underlying diseases and medical interventions. On average, 5%–10% of inpatients acquire a nosocomial infection and the rates are higher in surgical and intensive care units.[1,3]

Since bacteria readily adhere to surfaces, these may serve as vectors for transmission of pathogens, and therefore reduction in pathogen spread and consequently HCAIs may be obtained by improving environmental disinfection.[5,6] A further improvement to the existing disinfection protocols may be obtained by integrating surfaces that prevent bacterial adhesion or exert a microbiocidal effect. Antimicrobial surfaces are most efficient when a direct contact between surfaces and microbes is ensured, and regular cleaning that removes organic deposits is essential.[7] However, the cleaning and disinfection products are likely to interact chemically with antibacterial surfaces potentially influencing their biocidal properties. Therefore, frequency of disinfection, combinations of detergents, disinfectants, and antimicrobial surfaces need to be assessed in a combined manner to achieve optimal impact.[5]

Over the past decade, several studies have been dedicated to developing new effective antimicrobial coatings.[8] Metallic copper and copper alloys have intrinsic antibacterial properties that make them especially suitable for applications where other metals lack effectiveness.[9,10] The high efficacy of metallic copper and copper alloys is attributed to the so-called "contact killing" mechanism,[9,11] while other metals such as silver and zinc exert their antimicrobial activity in the form of oxides and/or nanoparticles.[7] Many studies have demonstrated *in vitro* antimicrobial efficacy of metallic copper surfaces.[12] Interestingly, dry-copper surface-resistant isolates *K. palustris*,

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B. conglomeratum, *S. panni*, and *P. oleovorans* were killed by exposure to wet copper surfaces,[13] suggesting different resistance mechanisms depending on the exposure conditions, wet or dry, and reduced chances to develop cross-resistance to antibiotics.

Importantly, these dry-copper surface-resistant isolates did not exhibit increased resistance to antibiotics,[13] which would have implied risk of cross-resistance.

These promising results have led several authors[14,15] to assess the antimicrobial efficacy of copper alloy surfaces in clinical trials at different hospital and facilities. Nevertheless, a recent review[16] emphasized that the effect of copper surfaces on HCAIs incidence is still unclear due to limited published data and the lack of robust designs. The lack of a common protocol, with arbitrary choices in the experimental design, may have affected the evaluation of copper antimicrobial activity both in *in vitro* and *in situ* studies.

From this perspective, more studies that relate surface properties of copper alloy and impact of environmental factors on their antimicrobial activity are required to predict the performance of copper alloys in real-life settings. More comparable data are also needed to understand how *in vitro* and *in situ* test conditions influence antimicrobial activities of these copper alloys. It has been demonstrated that contamination of the surfaces by organic compounds decreased the antibacterial efficacy of copper alloys,[17] while cleaning of the alloys with sodium hypochlorite restored their activity by removing the surface contaminants.[18,19] Also, a recent study[20] found synergism in antibacterial activity between copper alloy surfaces and a disinfectant based on benzalkonium chloride and glutaraldehyde. The authors concluded that this class of disinfectants and copper alloys can be combined in a dual strategy to reduce microbial contamination. However, due to the complexity of the killing mechanism of copper ions and copper alloys, there is a need for further elucidations on the interaction between copper alloys, microorganisms, and chemical disinfectants.[20] Knowledge about the surface chemistry of copper alloys and the changes that follow upon exposure to organic contamination and disinfectants could be crucial for the prediction of the antimicrobial performance.

Copper-complexing compounds such as benzalkonium chloride, chloroacetic acid, sodium chloride, and phosphates are present in many disinfectants and cleaning products. Such compounds can alter the surface chemistry of copper alloys and therefore affect the antimicrobial performances.[7] In hospital settings, surfaces are constantly handled and thus exposed to sweat and soaps that contain chlorine and phosphorous complexes. Under these conditions, copper chloride and copper phosphate compounds are readily formed[21,22] and may precipitate on copper alloys surfaces leading to a modification of their antimicrobial efficacy. Chlorides and phosphates are also found in common biological media and buffer, which are routinely used in *in vitro* studies evaluating antibacterial activity, such as saline solutions, potassium phosphate buffer (PPB), and phosphate saline buffer (PBS). It has, for instance, been shown that chlorine is incorporated into the Cu_2O layer when oxidation of copper was induced in PBS.[23]

In a previous study,[24] we demonstrated the high antibacterial activity of a newly developed copper-silver alloy coating, using standardized bacterial adhesion tests in PBS buffer. The purpose of the present study was to evaluate how chlorides and phosphates can influence the antiadhesion and antibacterial properties of the copper-silver alloy coating during standardized *in vitro* testing, and to relate these findings to the electrochemical properties of the alloy and the pure metals under the same conditions. Overall, we wanted to predict the behavior and performance of the copper-silver alloy coating in real-life settings with exposure to disinfectants, detergents, and sweat. We also aim to provide an understanding of the chemical modifications that can occur at the surface of copper and silver based materials in the presence of chlorides and phosphates, which can explain their antibacterial properties, both in previous and future *in vitro* and *in situ* studies.

II. EXPERIMENTAL SETUP AND METHODOLOGY

A. Preparation of electroplated surfaces

AISI 316 cold rolled sheet of steel with 2B surface finish (X5CrNiMo17-12-2)[24] was cut into $10 \times 20 \times 1$ mm size coupons. Cylindrical shaped specimens were used for potentiodynamic polarization studies according to ASTM G5-14.[25] Copper-silver alloy coated samples were prepared by electroplating AISI 316 specimens at a current density of 4 A dm^{-2} for 1 min in a commercially modified copper-silver bath at Elplatek A/S Galvanord. Pure copper and pure silver coated samples were obtained by electroplating AISI 316 specimens at a current density of 5 A dm^{-2} for 10 min in a commercial acidic copper bath and at a current density of 1 A dm^{-2} for 15 min in a commercial silver bath, respectively. Prior to the electroplating process, the AISI 316 surfaces were cathodically degreased at 3 ± 0.5 V for 2 min, rinsed with deionized water, and activated through a Wood's nickel strike at a current of 4.5 ± 0.5 A dm^{-2} for 2 min.[24] Prior to silver electroplating, a strike bath was performed at a current density of 0.5 A dm^{-2} for 1 min. The electroplated copper-silver alloy, pure copper, and pure silver AISI 316 specimens were used as test coupons, uncoated AISI 316 specimens as control coupons. Prior to antibacterial tests, specimens were sterilized by dry autoclaving in individual glass tubes.

B. Bacterial adhesion tests in suspension

Staphylococcus aureus 8325 (Ref. 26) was revived from −80 °C storage and grown on Brain Heart Infusion (BHI) agar plates (Oxoid, CM1135) at 25 °C overnight. Four single colonies were inoculated in BHI broth (Oxoid, CM1135) and grown at 25 °C overnight. Tenfold serial dilutions of the overnight bacterial culture to 10^7–10^8 CFU/ml were made in PBS solution (Dulbecco A; Oxoid), 0.1M PPB (Merck Millipore), 1M HEPES buffer solution (Sigma-Aldrich), 0.06, 0.09, 0.14, 0.15, 0.17M sodium chloride solution (Merck Millipore), or EN 1811 artificial sweat.[27] Concentrations of 0.14 and 0.09M corresponded to the sodium chloride concentrations in PBS and artificial sweat, respectively. Three milliliters of each

diluted bacterial suspension was added to sterile polystyrene tubes (Sterkin LDT; Bibby Sterin LDT; Stones; UK) containing the test coupons and incubated at 25 °C for 30 min. After 30 min, the coupons were rinsed with 2 ml of the sterile diluent and moved to sterile polystyrene tubes containing 2 ml of the sterile diluent. The strongly adhering bacteria were liberated by sonication of the tubes for 4 min at 25 °C (28 kHz; 2 × 150 W sonication bath, Delta 220, Deltasonic Meaux, France) and vortexing at maximum speed for 15 s.[28] The sonication was performed in order to detach the live attached bacteria and allow their collection in buffer. This procedure had been previously assessed[29] to be optimal for the recovering of adhered bacterial cells.

The density of live attached bacteria on surfaces and bacterial survival in suspension was determined by serial dilution of the 2 and 3 ml test suspensions and plating on BHI-agar. The total number of live attached bacteria per unit of surface (CFU/cm^2) was calculated from CFU/ml values considering the coupons area (4 cm^2) and the suspension volume (3 ml).[24] All experiments were conducted in three biological replicates and each included technical replicates.

C. Statistical analysis

Bacterial cell numbers were log transformed and a single factor analysis of variance among the groups of triplicates was performed to test the null hypothesis. If the null hypothesis was rejected, statistical significance ($P < 0.05$) of the difference between two groups was tested using the *t*-test after assessment of equal or unequal variance through *F*-test.

D. Live/Dead survival assay after dry exposure

Staphylococcus aureus 8325 was grown as described above and overnight bacterial cells were harvested by centrifugation at 5000*g* for 5 min and resuspended in 500 μl of PBS, 0.15M saline solution, 1M HEPES buffer solution or 0.10M PPB, respectively. Dry incubation was performed by applying 5 μl of bacterial suspensions on the coated and uncoated test coupons. The small amount of bacterial suspension was spread over the surface and allowed to air-dry completely and incubated at 25 °C for 30 min. Bacterial cells were liberated and recollected from the surfaces by a soft rinsing using 100 μl of sterile resuspension medium and stained with the Live/Dead dye mixture (L7007 LIVE/DEAD® BacLight Bacterial Viability Kits, 2004).[30] The suspensions were mixed thoroughly, incubated in dark at 25 °C for 15 min and immediately inspected at Olympus Model BX51 Fluorescence Microscope with a WIB excitation filter (460–490 nm) equipped with Olympus Power Supply for Mercury/Xenon Burner model U-RFL-T and with Olympus micro imaging software cellSens 1.5.

E. Potentiodynamic anodic polarization and Pourbaix diagrams

Potentiodynamic polarization scans were recorded with an ACM (GillAC) potentiostat with a sweep rate of 50 mV/min and a set limit current density of 300 mA/cm^2, according to the ASTM G5-14 standard test method.[25] The reference and the counter electrode were a saturated calomel electrode and an iridium-oxide coated titanium, respectively. PBS, 0.15M saline solution, 1M HEPES buffer solution, and 0.10M PPB were the electrolytes for the polarization scans. The open circuit potential was monitored for at least 2 h with a cell settle time of 10 s prior to the polarization measurements. The measurements were performed on pure copper, pure silver, copper-silver alloy coated and uncoated AISI 316 specimens, and conducted in replicas for consistency. All the potential values were recalculated against the standard hydrogen electrode (SHE).

Potential-*p*H predominance diagrams (Pourbaix diagrams) of copper and silver were calculated using MEDUSA/HYDRA (Ref. 31) for a temperature of 25 °C with a cationic concentration of 10^{-5}M. The calculated Pourbaix diagrams allow a thermodynamic prediction of the chemical species that can form in the metal-medium system at different *p*H 0–14 (horizontal axis) and potentials between −250 and 1250 mV (vertical axis). For the calculations, concentrations of 0.14M Cl^- and 0.01M PO_4^{-3} were considered for PBS, 0.15M of Cl^- for the saline solution, and 0.10M PO_4^{-3} for 0.10M PPB. 1M HEPES buffer solution was simplified to an aqueous solution with 0.67M CO_3^{-2}, since carbon and oxygen account for approx. 67% mass percent of the substance,[32] in order to allow the calculation with the program. The more powerful PREDOM2 tailored for complex chemical systems was used and 200 calculation steps in each axis were chosen. Soluble and solid complexes were selected considering the free energy of formation and no correction for activity coefficients was used.

F. Energy dispersive x-ray analysis

The chemical composition of the pure copper, pure silver, copper-silver alloy coated specimens was analyzed by scanning electron microscopy (SEM) (JEOL JSM 5900 Instrument operated at 13 kV and Hitachi TM3030 Plus Tabletop Microscope operated at 15 kV tabletop), equipped with Oxford energy dispersive x-ray spectrometry (EDS) detector and Oxford Inca software and Bruker Quantax 70 EDS System, respectively. The chemical composition of tested specimens was checked prior to and after potentiodynamic polarization measurements. EDS analysis was performed on three different spots at the surface of each sample and results averaged. SEM images are presented as supplementary material.[41]

III. RESULTS AND DISCUSSION

A. Survival of *S. aureus* 8325 cells adhering to metal surfaces

Determination of the ability of copper-silver alloy coated surfaces to mediate the transfer of live *S. aureus* 8325 from a suspension of approx. 10^7 cells cm^{-2} to a sterile medium is important for the evaluation of the alloy's ability to prevent the spread of bacterial contamination in wet environments.

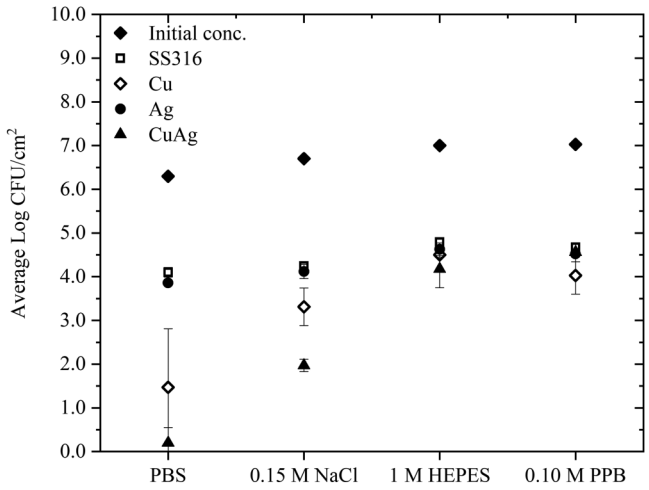

FIG. 1. Attachment of *S. aureus* 8325 by AISI 316, pure silver, pure copper, and copper-silver alloy coated surfaces after 30 min in suspension with various diluents. Numbers are mean values ± standard deviation of three biological replicates.

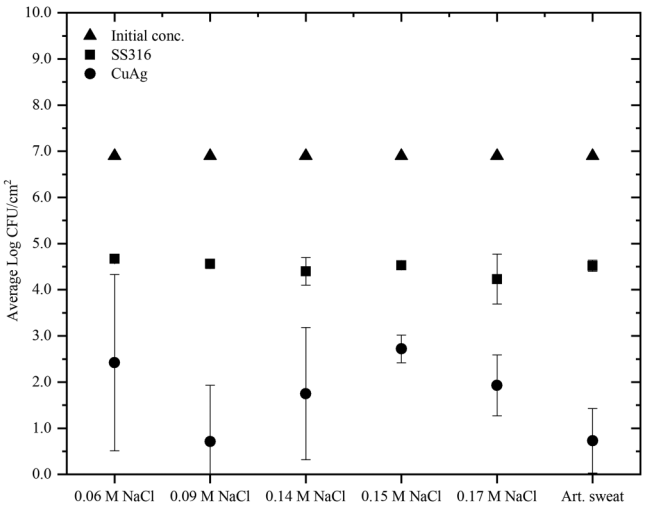

FIG. 2. Attachment of *S. aureus* 8325 by stainless steel AISI 316 and copper-silver alloy coated surfaces after 30 min in suspension with different NaCl concentrations. Numbers are mean values ± standard deviation of three biological replicates.

Staphylococcus aureus 8325 suspended in PBS adhered to AISI 316 and silver coated surfaces at a level of approx. 10^4 CFU/cm^2, but only at a level of level of approx. 10^2 CFU/cm^2 on copper surfaces after 30 min of exposure. On the copper-silver alloy, numbers were below 10 CFU/cm^2 (Fig. 1). These numbers were significantly different ($P < 0.05$), and the copper-silver alloy coated surfaces exhibited the highest antiadhesive effect, as shown previously.[24]

Similarly, the number adhering to copper-silver alloy coated surfaces was low (10^2 CFU/cm^2) when *S. aureus* was suspended in 0.15M saline solution. Levels on pure copper were 10^3 and 10^4 CFU/cm^2 on pure silver and AISI 316. The difference in numbers on surfaces was statistically significant ($P < 0.05$).

When suspended in 1M HEPES buffer and 0.10M PPB, *S. aureus* adhered to all the surfaces at a level between 10^4 and 10^5 CFU/cm^2, and numbers did not differ ($P \geq 0.05$). The *S. aureus* 8325 concentration in all the test suspensions with the metal surfaces was after 30 min equal to the initial concentration of approx. 10^7 CFU/ml. Therefore, the reduction in *S. aureus* attachment was not due to a general killing of the bacterial suspensions.

S. aureus 8325 suspended in sodium chloride solutions (0.06, 0.09, 0.14, 0.15, and 0.17M) and in artificial sweat adhered to a level of 10^4–10^5 CFU/cm^2 to AISI 316 surfaces (Fig. 2). On the copper-silver alloy coated surfaces, the number of attached bacteria was below 10 CFU/cm^2 in 0.09M NaCl solution and artificial sweat, approx. 10^2 CFU/cm^2 in 0.06, 0.14, and 0.17M NaCl solutions and approx. 10^3 CFU/cm^2 in 0.15M NaCl solution (Fig. 2).

From the data in Fig. 2, it clearly appears that both copper and chloride are needed to decrease the attachment of *S. aureus* 8325 and it possibly suggests a chloride concentration-dependent attachment of live bacteria. The difference in numbers between AISI 316 and copper-silver alloy coated surfaces was statistically significant ($P < 0.05$), and the concentration dependency appeared to reach a minimal attachment with approx. a four-log bacterial reduction in artificial sweat and 0.09M NaCl solution, followed by 0.14M NaCl solution with approx. a three-log bacterial reduction. This was mirrored in the attachment in phosphate-buffered saline solution, which could indicate a fortuitous optimal condition for prevention of contamination, but the high standard deviation in data for other concentrations prevented any firm conclusions.

B. Live/Dead *S. aureus* 8325 survival assay after dry exposure

While the analysis of attachment of live bacteria to metal surfaces under wet conditions proved fairly uncomplicated, the attachment of bacteria under dry conditions required a change in the assay procedure. When bacteria are dried on a surface, their envelope sticks and binds to the surface in a way that prevents quantification after mechanical release. Therefore, we took advantage of live-dead staining of bacteria to quantify the ratio between live and dead cells recovered from the dry surfaces but sacrificing the total quantification of the attached cells.

Copper induced a clumping phenotype in *S. aureus* 8385 that was not observed when the bacterial culture was dried on pure silver coated or AISI 316 coupons (Fig. 3).

Clumping is a phenotype that is closely related to the biofilm phenotype;[33] therefore, the induction of clumping by copper could, in theory, work against its antibacterial effect. It is also clear, however, that most cells appeared red after the live-dead staining, showing that the clumping phenotype did not prevent the copper-mediated killing. Obviously, drying *per se* kills many bacteria; therefore, only preliminary conclusions can be drawn from these experiments aside from the induction of clumping. Bacteria were able to survive drying on uncoated and pure silver coated AISI 316 surfaces, but the high proportion of dead cells when bacteria suspended in 1M HEPES were dried on silver (Fig. 3) was not

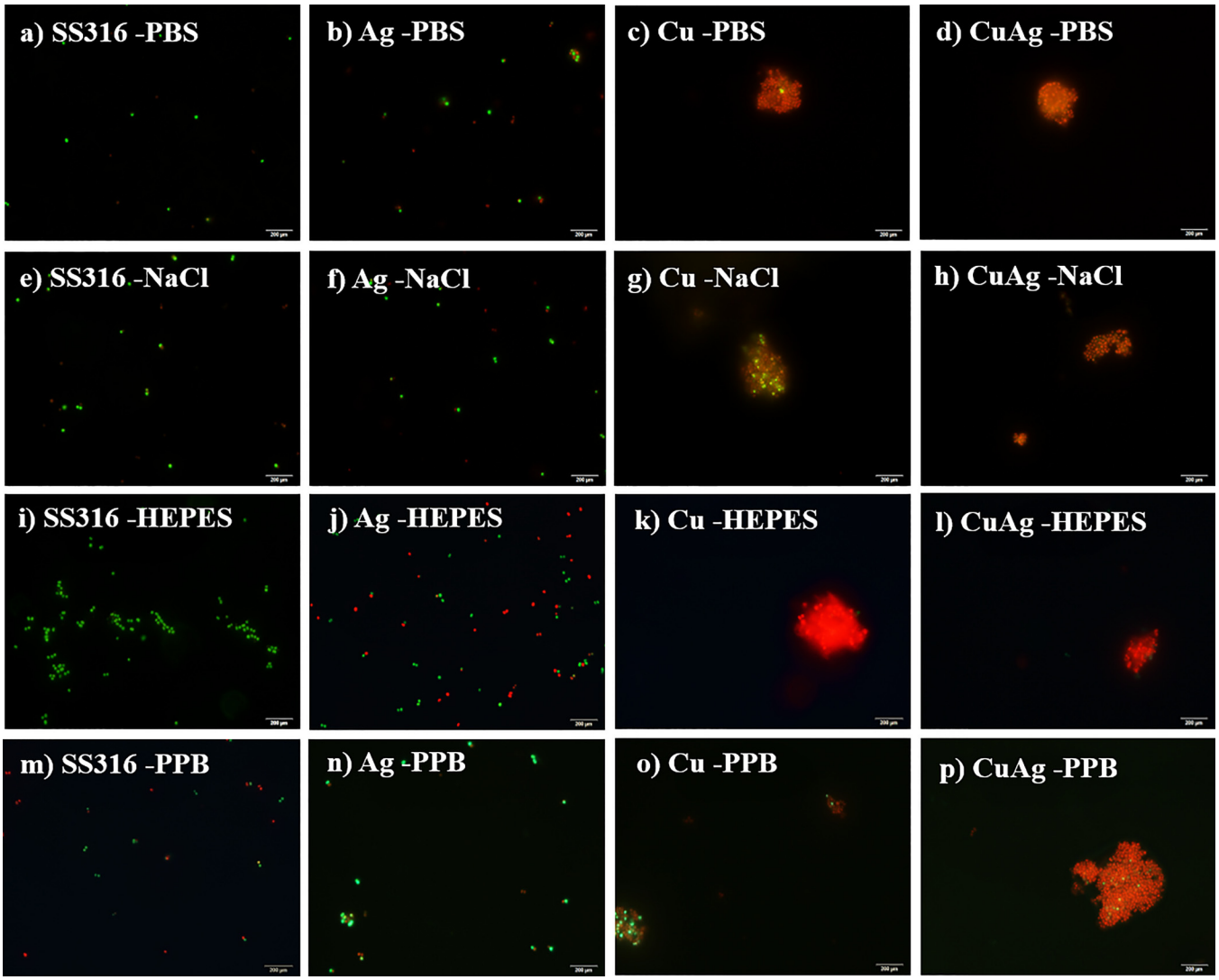

FIG. 3. Live/Dead survival assay after 30 min of dry exposure. *S. aureus* 8325 in suspension with PBS was exposed to stainless steel AISI 316 (a), pure silver (b), pure copper (c), and copper-silver alloy (d) coated specimens. *S. aureus* 8325 in suspension with 0.15M saline solution was exposed to stainless steel AISI 316 (e), pure silver (f), pure copper (g), and copper-silver alloy (h) coated specimens. *S. aureus* 8325 in suspension with 1M HEPES buffer solution was exposed to stainless steel AISI 316 (i), pure silver (j), pure copper (k), and copper-silver alloy (l) coated specimens. *S. aureus* 8325 in suspension with 0.10M PPB was exposed to stainless steel AISI 316 (m), pure silver (n), pure copper (o), and copper-silver alloy (p) coated specimens.

expected. We have not explored this any further in this study. Clumps of *S. aureus* 8385 recovered from pure copper coated surfaces had specks of live bacteria when bacteria were suspended in 0.15M NaCl solution or 0.10M PPB, while no live bacteria were detected when the cells were suspended in PBS or 1M HEPES. Clumps of *S. aureus* 8385 recovered from copper-silver alloy coated surfaces contained only few scattered, live cells when bacteria were suspended in 0.10M PPB; therefore, preliminary conclusions would be that the copper-silver coated surfaces can induce clumping during drying, but that bacteria in clumps are killed.

C. Electrochemical analysis

To explain the observed differences between the bacterial adhesion and antibacterial activity of electroplated copper, silver, and copper-silver alloy coated and uncoated AISI 316 in the tested media, we performed potentiodynamic anodic polarization measurements (Figs. 4 and 7). Information about corrosion mechanisms and susceptibility to corrosion in the specific medium (e.g., corrosion potential, oxidation peaks) are gained by monitoring the corrosion current density during anodic polarization.

The corrosion potential, also called the open circuit potential, is the characteristic value that the material displays when immersed in a particular medium and no current is applied. In other words, it is the virtually natural potential of the material when exposed to the corresponding real-life environment and the equilibrium is established. The corrosion potential can be used to evaluate whether the material will be more prone to corrode (low corrosion potential) or not (high corrosion potential) in the particular environment. The corrosion potential also determines the nobility of metals in the specific environment: the higher the corrosion potential, the nobler the material.

By sweeping the applied potential in the anodic (positive) direction from an arbitrary value below the corrosion potential, the test metals (working electrodes) are induced to corrode and liberate metals ions. These can form various soluble compounds or surface precipitates with the other surrounding chemicals in the media. As the output function of applied potential and corrosion current density, polarization curves display the corrosion potential and oxidation peaks corresponding to the formation of particular species. Polarization curve slopes indicate whether the material has an active (it corrodes) or a passive (it passivates) behavior in that solution and can also serve to evaluate corrosion rate.

The compounds that can form and predominate at certain potentials over the 0–14 *p*H range are instead predicted and shown by the Pourbaix diagrams calculated for each metal-medium system (Figs. 5, 6, 8, and 9). We could therefore in each case compare the anodic dissolution of the tested metals (as a function of applied potential and corrosion current density) to the prediction of the chemical species formed in solution or at the surface (as a function of the specific potential). In most cases, agreement with the prediction and the EDS analysis of the surface chemical composition was found.

1. Anodic polarization in PBS and 0.15M NaCl and EDS analysis

AISI 316 had no antibacterial activity against *S. aureus* 8325 and this can be explained, besides the intrinsic chemical composition, by its low electrochemical reactivity (passivity) due to the presence, at its surface, of a protective chromium oxide layer resistant to corrosion in most environments.[34] In the presence of chlorides, the corrosion rate increased sensibly only when the pitting potential (625 and 560 mV in PBS and 0.15M NaCl, respectively) was reached, which induced small delocalized corroding "pits," typical of stainless steels [Figs. 4(a) and 4(b)].

Likewise, silver does not readily oxidize under ambient condition due to its nobility (determined by the high corrosion potential); therefore, the silver surfaces were not effective in contact bacterial killing during short exposure times under these conditons.[9] At the near neutral *p*H, the Pourbaix diagrams show that metallic silver is predominant (silver is immune from corrosion) at potentials below +260 mV, while the formation of AgCl is predominant above +260 mV (Fig. 5).

The anodic behavior of electroplated silver in PBS and 0.15M NaCl revealed corrosion potentials of +230 and +120 mV, respectively [marked with a solid line in Figs. 5(a) and 5(b)], which were the highest corrosion potentials among the tested material. The corrosion potentials were located below +260 mV within silver immunity region, so it can be concluded that silver immersed in these media does not corrode.

By further sweeping the applied potential in the anodic direction, an anodic current density limitation (4 mA/cm^2) was reached at +375 and +330 mV in PBS and 0.15M NaCl, respectively [Figs. 4(a) and 4(b)]. This was due to the presence of AgCl that forms above +260 mV, according to the Pourbaix diagrams and acts as a protective layer stabilizing the corrosion current density to a limit value.

This was supported by previous findings[35,36] and the presence of AgCl was confirmed by EDS analysis that showed the presence of approx. 25 wt. % Cl (24.29 ± 0.45 and 24.81 ± 0.19 wt. % for the samples tested in PBS and 0.15M NaCl, respectively) at the surface.

Electroplated copper surfaces were effective in preventing the bacterial adhesion in the presence of chloride-containing suspensions, indicating an antiadhesion efficacy of copper and chloride in combination.

During anodic polarization measurements, electroplated copper displayed the expected active corroding behavior in PBS and 0.15M NaCl solution with low corrosion potentials of +10 and +15 mV, respectively (Fig. 4). These values were located within the corrosion region of copper, indicated by

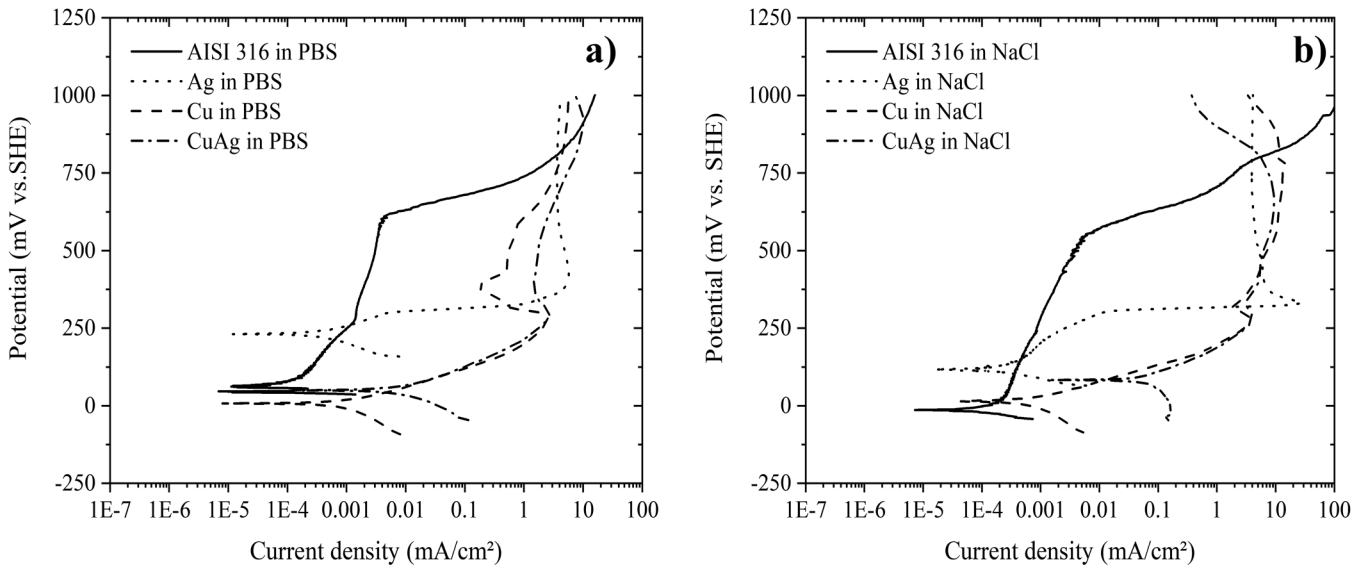

FIG. 4. Polarization curves of AISI 316, electroplated silver, copper, and copper-silver alloy in PBS (a), 0.15M NaCl solution (b). Potential (mV) values are recalculated against the SHE.

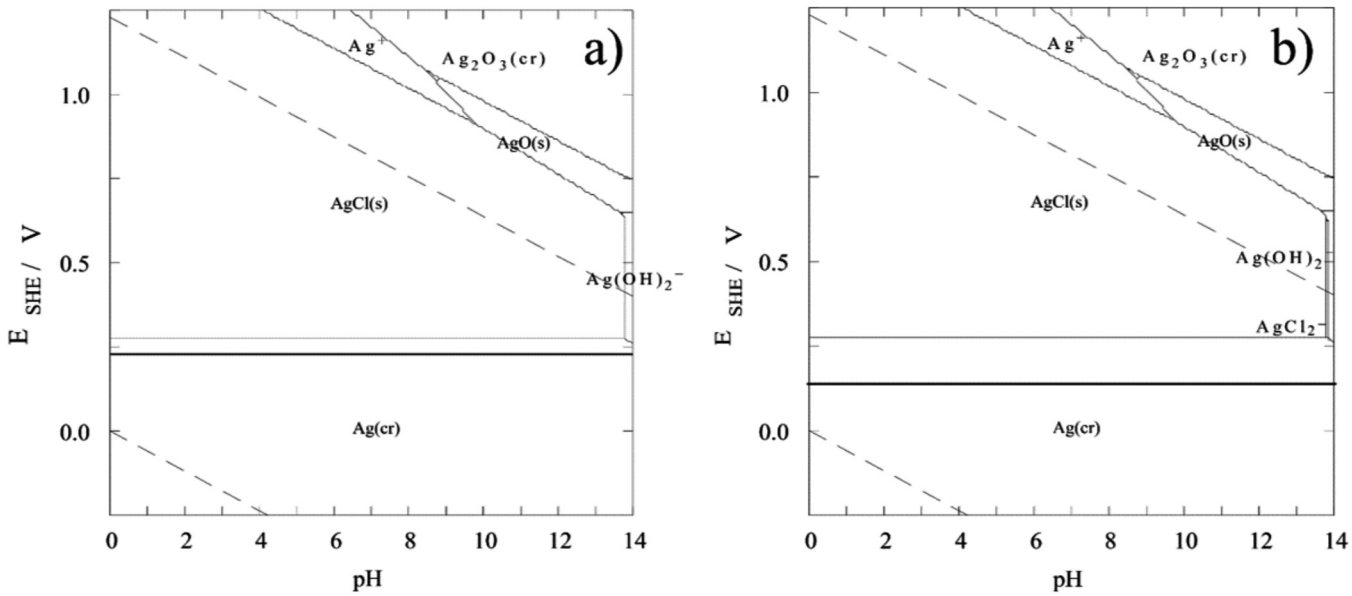

FIG. 5. Pourbaix diagrams (E-*p*H) of silver ($[Ag^+] = 10^{-5}$M) in (a) PBS ($[Cl^-] = 0.14$M, $[PO_4]^{3-} = 0.01$M), (b) 0.15M NaCl ($[Cl^-] = 0.15$M). Corrosion potential values are marked by the solid line.

the formation of $CuCl_2^-$ species[21] above 0 mV as suggested by the Pourbaix diagrams [Figs. 6(a) and 6(b)]. The correspondent release of copper as cuprous (Cu^+) ions,[37] highly toxic to the bacterial cells, can explain the observed antiadhesion behavior.

The EDS analysis on the samples exposed to PBS and 0.15M NaCl showed the presence of chlorine (6.36 ± 2.43 and 17.44 ± 0.43 wt. %, respectively) and oxygen (25.29 ± 6.03 and 18.16 ± 2.32 wt. %, respectively) at the surface. At neutral *p*H, the stable solid $Cu_2Cl(OH)_3$ (atacamite) is predicted to be formed above +250 mV [Fig. 6(b)] and a characteristic green color of the corrosion products further confirm it. In the presence of PBS, it was previously demonstrated[23] that when a sufficient amount of copper was dissolved in solution, Cu_2O oxide layer started to form and Cl^- was incorporated possibly by filling oxygen vacancies. When phosphates are present, $CuHPO_4$ can form above 250 mV at *p*H around neutrality according to the Pourbaix diagram [Fig. 6(a)] and accordingly, a small amount of phosphorous (5.50 ± 2.66 wt. %) was detected at the surfaces.

Similarly to electroplated copper, electroplated copper-silver alloy displayed an anodic behavior characterized by

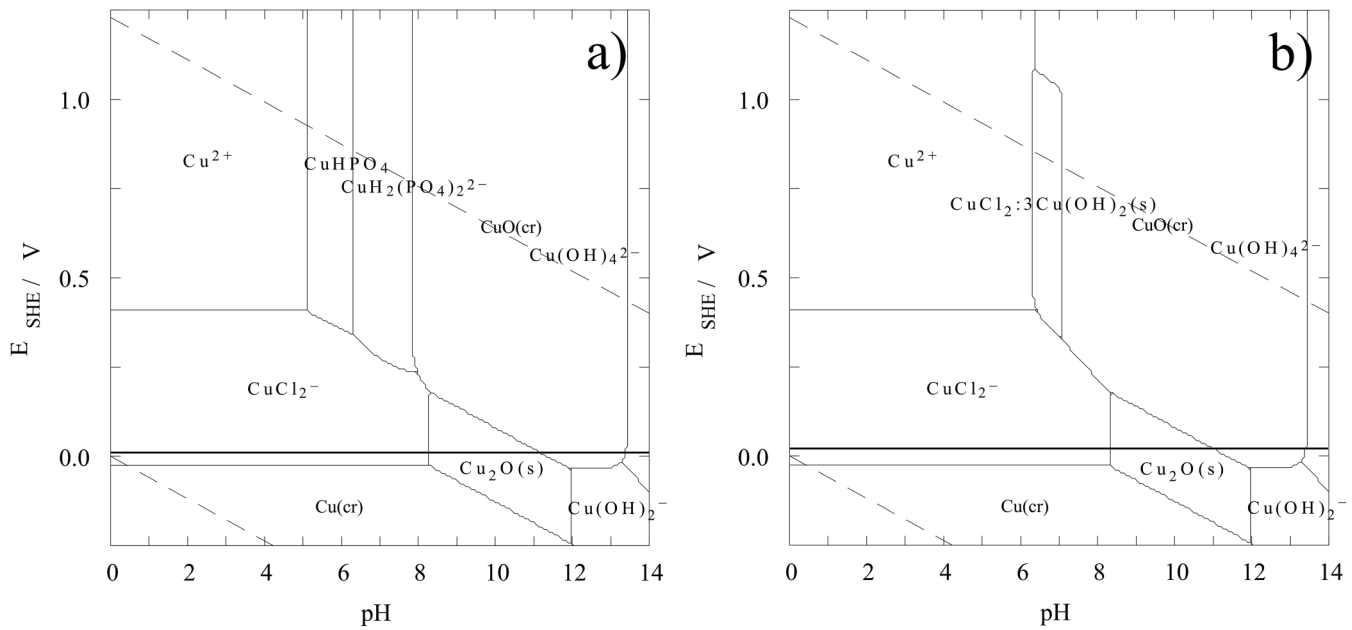

FIG. 6. Pourbaix diagrams (E-*p*H) of copper ($[Cu^+] = 10^{-5}$M) in (a) PBS ($[Cl^-] = 0.14$M, $[PO_4]^{3-} = 0.01$M), (b) 0.15M NaCl ($[Cl^-] = 0.15$M). Corrosion potential values are marked by the solid line.

corrosion current densities of 0.001 mA/cm^2, but higher corrosion potentials of +50 and +85 mV in PBS and 0.15M NaCl, respectively. This raise in corrosion potential was due to the presence of silver that increased the nobility of the alloy compared to pure copper (Fig. 4). Copper in the copper-silver alloy coating was oxidized to Cu^+ as indicated by the characteristic peaks at around 280 mV (Fig. 4).

The prevention of attachment of live bacteria was highest for the electroplated copper-silver alloy among the tested metals, and this can be linked to an increased and faster release of toxic cuprous ions due to the presence of silver in the alloy, which induced galvanic corrosion of copper.

The EDS analysis revealed the presence of chlorine (21.77 ± 4.54 wt. % Cl and 20.34 ± 4.96 wt. % in PBS and 0.15M NaCl, respectively) and oxygen (6.03 ± 1.53 and 13.55 ± 3.58 wt. % in PBS and 0.15M NaCl, respectively) at the surface, indicating that Cl and O were incorporated in the corrosion products.

2. Anodic polarization in 1M HEPES and 0.10 PPB and EDS analysis

Not surprisingly, AISI 316 displayed its passive nature also in 1M HEPES buffer and 0.10M PPB with similar corrosion potentials, but with no presence of pitting as chlorides were omitted from the environment (Fig. 7). This is again in line with its lack of antiadhesive efficacy observed in the bacterial adhesion tests.

Silver, at the top of the scale of nobility among the tested metals, showed the highest corrosion potential values (+160 and +240 mV in 1M HEPES buffer and 0.10M PPB) [Figs. 7(a) and 7(b)], amply comprised within the immunity region were metallic silver is stable [Figs. 8(a) and 8(b)].

It results that oxidation of silver was not reached in the bacterial adhesion tests conditions; therefore, silver did not display antiadhesive efficacy.

By further sweeping the applied potential in 1M HEPES buffer, an anodic current limitation was reached and the EDS analysis revealed a nonuniform surface composition, with areas where only traces (<5.00 wt. %) of oxygen and carbon were present and corrosion products made of silver, oxygen, carbon, and sulfur (24.42 ± 14.16, 32.66 ± 14.87, 31.23 ± 7.03, 5.17 ± 0.01 wt. %, respectively). According to the Pourbaix diagram [Fig. 8(a)], Ag_2CO_3 can form above 500 mV, which is a stable silver salt characterized by a white-yellow color,[38] similarly to the corrosion products that were present on the specimens. Considering the high affinity between silver and sulfur, it is likely that the latter was incorporated into the silver carbonate precipitates. In 0.10M PPB, the peak at 600 mV corresponding to Ag^+ formation was reached, also according to the calculated Pourbaix diagram [Fig. 8(b)]. The presence of oxygen and phosphorus (7.89 ± 2.36 and 6.00 ± 0.61 wt. %) was detected, possibly indicating the formation of the silver oxide at high potentials[38] [Fig. 8(b)] with the incorporation of phosphorus. Under oxidizing conditions and high *p*H, silver oxide (AgO) is formed and it has an antibacterial activity due to its solubility;[9] however, these conditions were not met in the bacterial adhesion tests.

Attachment of live bacteria in 1M HEPES and 0.10M PPB buffer was equal for all the tested metals, meaning that electroplated copper and copper-silver alloy did not show antiadhesive efficacy in these conditions.

This difference in antiadhesive behavior can be explained by the different electrochemical reactivity of these metals in the absence of chlorides, where no oxidizing conditions were established and therefore the metals had no antiadhesive efficacy. This implicates that wet copper surfaces have antiadhesive and antibacterial activity as long as there are conditions for the dissolution of copper ions.

In 1M HEPES buffer and 0.10M PPB, electroplated copper and copper-silver alloy displayed, in contrast to what observed in the presence of chlorides, corrosion potentials

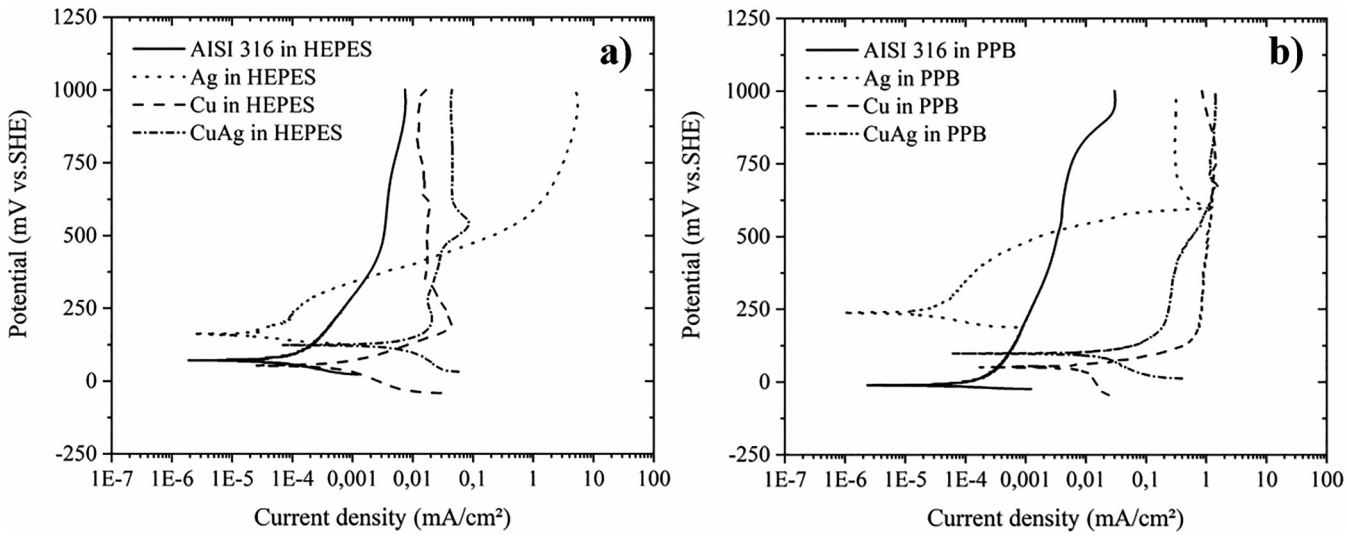

FIG. 7. Polarization curves of AISI 316, electroplated silver, copper, and copper-silver alloy in 1M HEPES (a) and 0.10M PPB (b). Potential (mV) values are recalculated against the SHE.

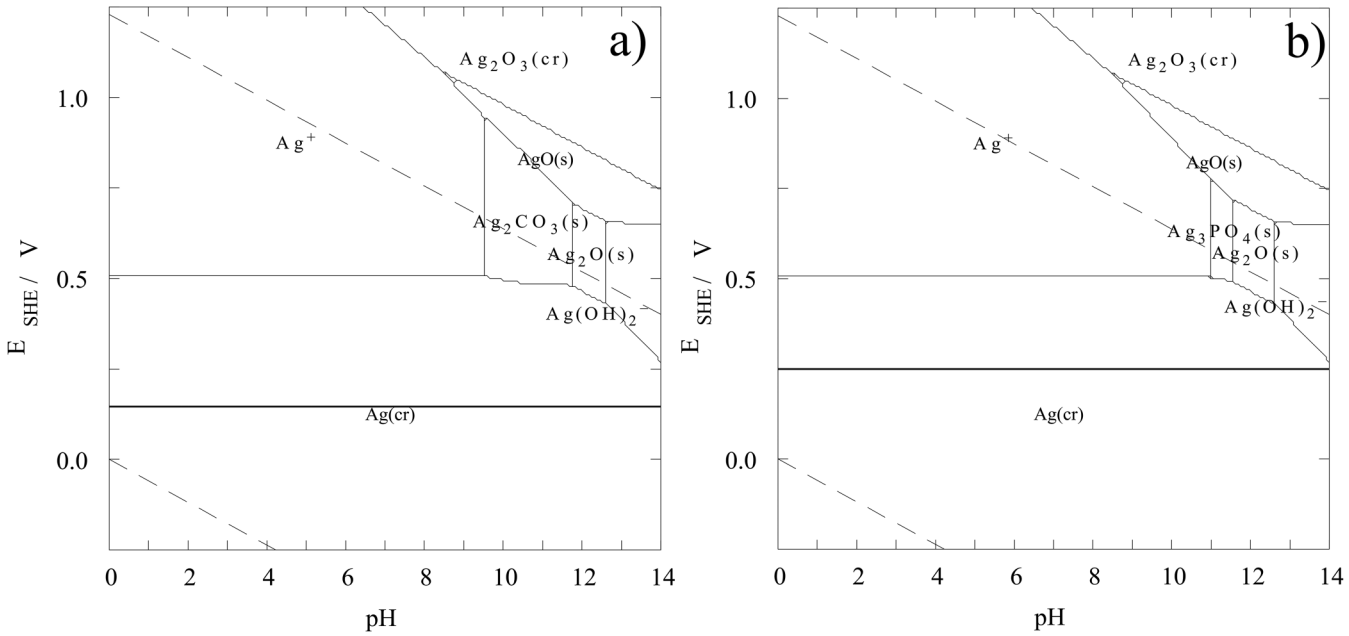

FIG. 8. Pourbaix diagrams (E-*p*H) of silver ([Ag^+] = 10^{-5}M) in (a) 1M HEPES ([CO3-2] = 0.67M) and (b) 0.10M PPB ([PO43-] = 0.1M). Corrosion potential values are marked by the solid line.

between +50 and +55 mV and an anodic current limitation at +180 mV [Figs. 7(a) and 7(b)]. These values fell well within the immunity region of copper, according to the calculated Pourbaix diagrams (Fig. 9); therefore, copper did not corrode in test conditions met in the bacterial adhesion tests.

This can be possibly explained by the interaction between HEPES and copper, since a strong copper binding property of the zwitterionic organic buffering agent and impurities present in the buffer itself was previously observed.[39] The EDS analysis on the copper and copper-silver alloy samples revealed, however, only traces (<5.00 wt. %) of oxygen and carbon, suggesting the weak character of the bond between HEPES and metallic copper.

In the presence of phosphates, it is likely that the formation of cupric phosphate complex acted as a protective layer, as it was reported[40] that orthophosphates can reduce the solubility of copper solids in equilibrium with water, presumably by formation of a cupric phosphate scale [$Cu_3(PO_4)_2$] and

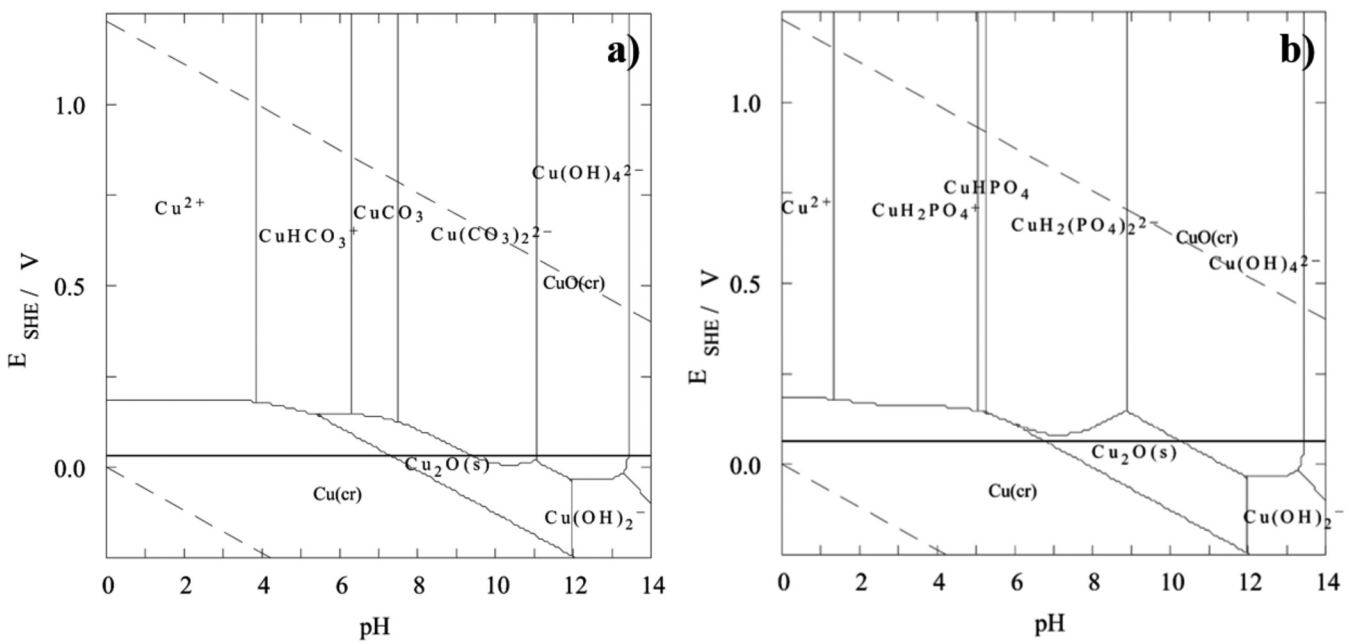

FIG. 9. Pourbaix diagrams (E-*p*H) of copper ([Cu^+] = 10^{-5}M) in (a) 1M HEPES ([CO3-2] = 0.67M) and (b) 0.10M PPB ([PO43-] = 0.1M). Corrosion potential values are marked by the solid line.

therefore acting as corrosion inhibitors. This was supported by the calculated Pourbaix diagrams [Fig. 9(b)] and by the EDS analysis, where the presence of oxygen (26.76 ± 0.45 and 22.75 ± 4.13 wt. % on copper and copper-silver alloy surfaces, respectively), phosphorous (11.86 ± 0.10 and 8.05 ± 2.41 wt. % on copper and copper-silver alloy surfaces, respectively), and traces (<5.00 wt. %) of potassium were detected at the surface.

IV. SUMMARY AND CONCLUSIONS

Here, we evaluated the antiadhesive, antibacterial, and electrochemical properties of a copper-silver alloy coating and the single alloying metals in the presence of biological solutions containing chlorides and phosphates, only chlorides or phosphates, or none of these. In the presence of chlorides, the copper-silver alloy coating had the highest antiadhesive activity against *S. aureus* 8325 followed by copper, silver, and AISI 316 surfaces. No statistically significant difference ($P > 0.05$) in antiadhesive effect was found between the tested surfaces in the absence of chlorides. The antiadhesive activity of the tested materials can be explained by their electrochemical reactivity in the different solutions: copper-silver alloy and copper were electrochemically active in the presence of chlorides, whereas they were immune in chlorides-free environments, and the presence or absence of phosphates had no influence on the antiadhesion activity.

In the presence of a chloride-containing environment, the galvanic coupling of the metals in the alloy would induce oxidation of copper, so release of Cu^+ ions, and local *p*H raise at silver due to the corresponding reduction reaction.[24] Therefore, copper-silver alloy coated surfaces are expected to work synergistically with chlorides-containing solutions against bacterial adhesion in wet environments. However, this depends on chlorides concentration and it affects the life time of the coating, due to the dissolution of copper.

In contrast, *S. aureus* 8325 cells recovered from copper-silver alloy and pure copper surfaces appeared to be predominantly dead following the dry exposure regime, irrespective of the presence of chlorides and phosphates in the diluent. It is expected that the presence of chlorides and phosphates would have no influence on the antibacterial activity of copper-silver alloy coated surfaces in dry environments.

This is important considering the intended application of such copper-silver alloy coating, i.e., a surface coating for environmental touch-surfaces. Similar surfaces will face periodical wet conditions during, e.g., cleaning procedures that may trigger oxidation, followed by a dry regime.

The analysis of the chemical modification occurring at these surfaces as a result of exposure to the environmental factors is important to assess their antibacterial effect. This will contribute to understand and predict the behavior in real-life conditions and guide the choice of copper alloys as antimicrobial surfaces in the particular working environment. Moreover, this can also provide an explanation tool for the previously observed variability in antimicrobial activity of other copper alloys in different *in vitro* and *in situ* conditions.

As an initial step, it is important to select environmental factors common to multiple conditions, e.g., a complexing or active substance, rather than narrowing to a specific case, e.g., detergent or disinfectant formulation, to allow a broader investigation and provide valuable information for the state-of-the-art of antimicrobial copper alloys. Next, environmental factors, such as active substances and wear, should also be evaluated in a combined manner prior to field tests, which are the necessary step in order to tailor the material choice to the specific environment. We conclude that a mindfully evaluated and designed strategy, which combines antimicrobial copper alloy surfaces and disinfection methods, can be a valuable tool in fighting healthcare-associated infections.

ACKNOWLEDGMENTS

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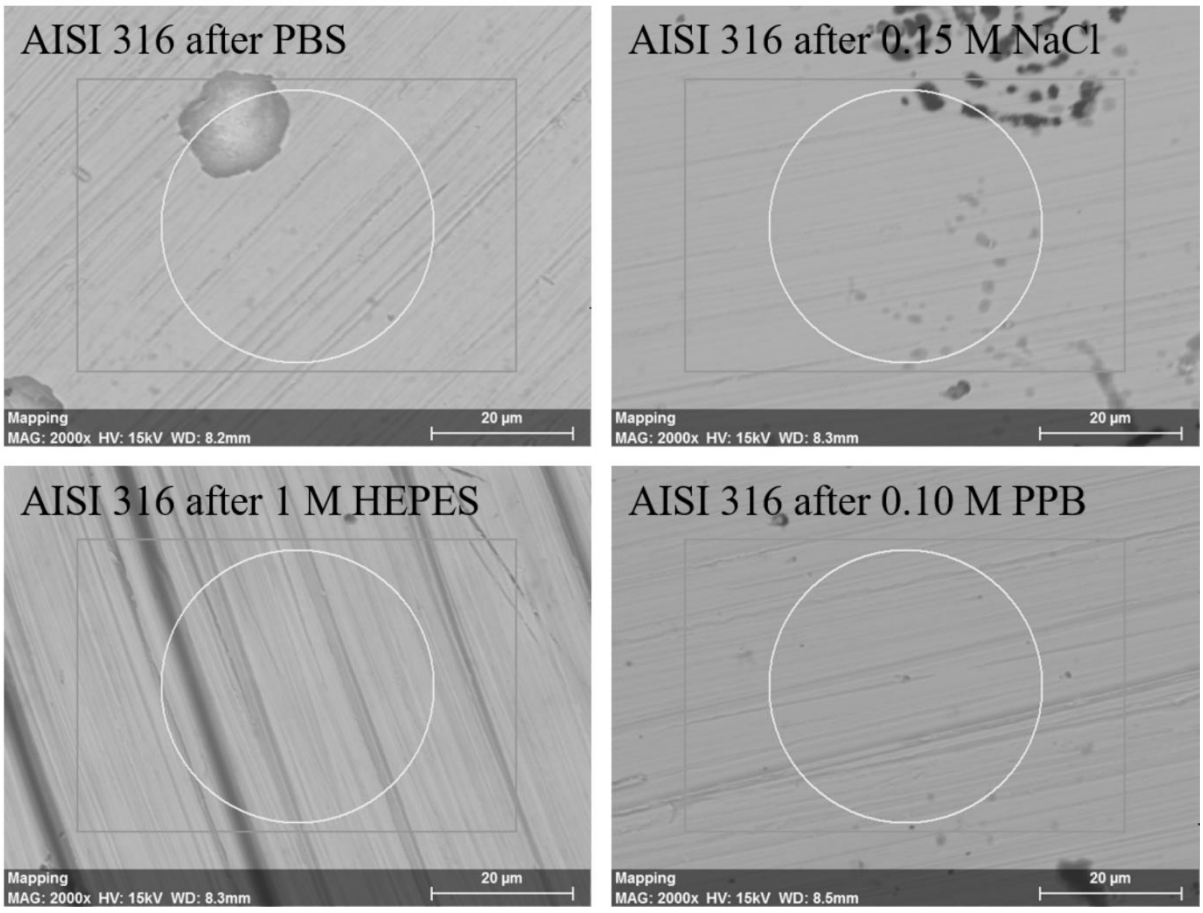
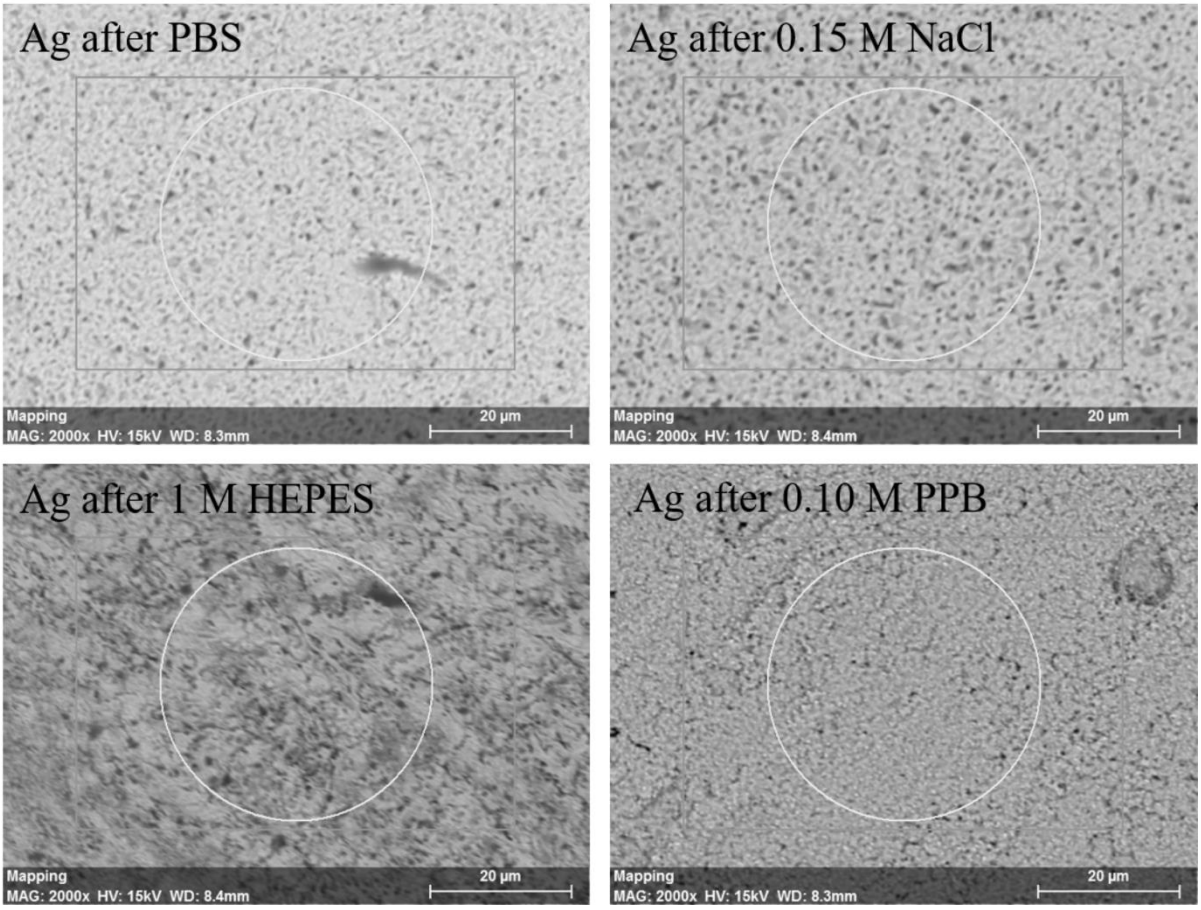
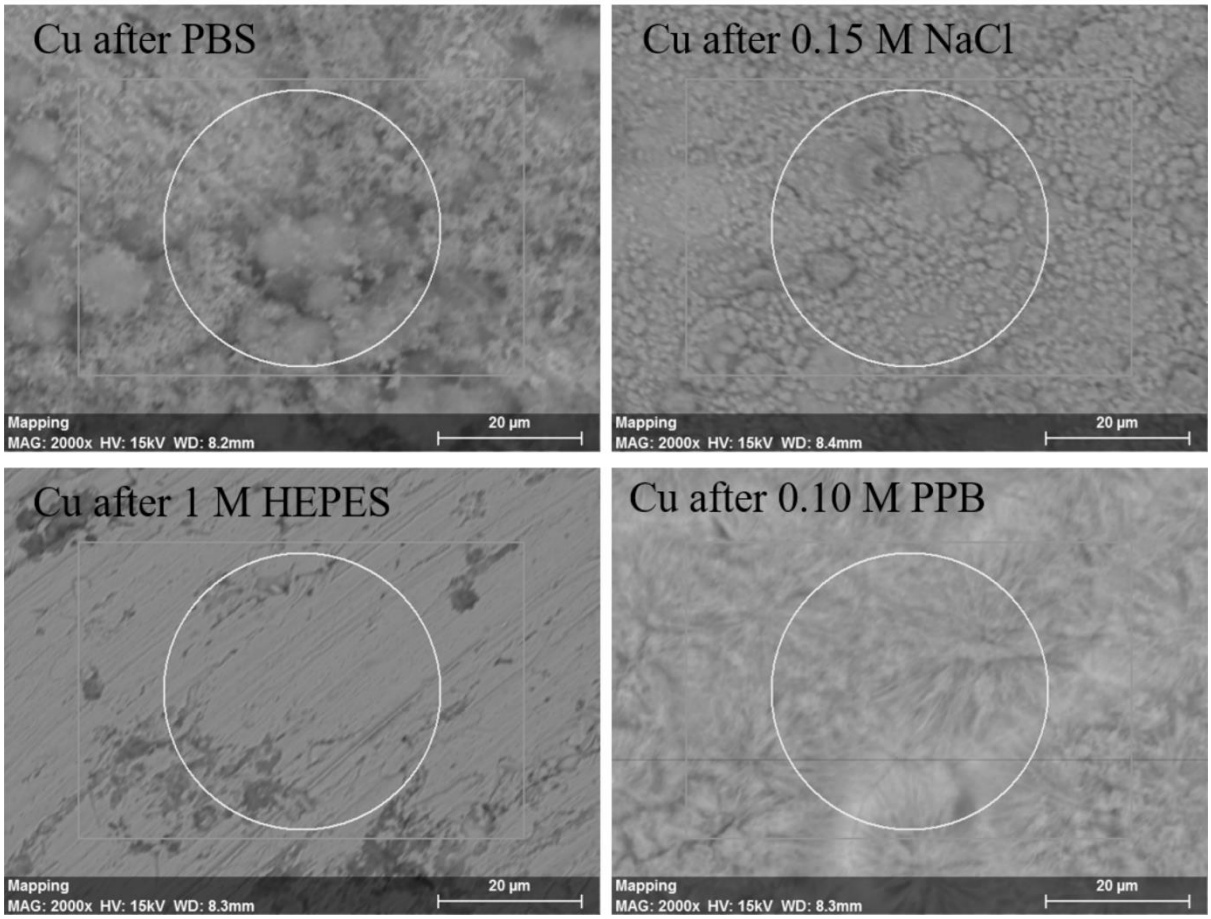
Supplemetary File



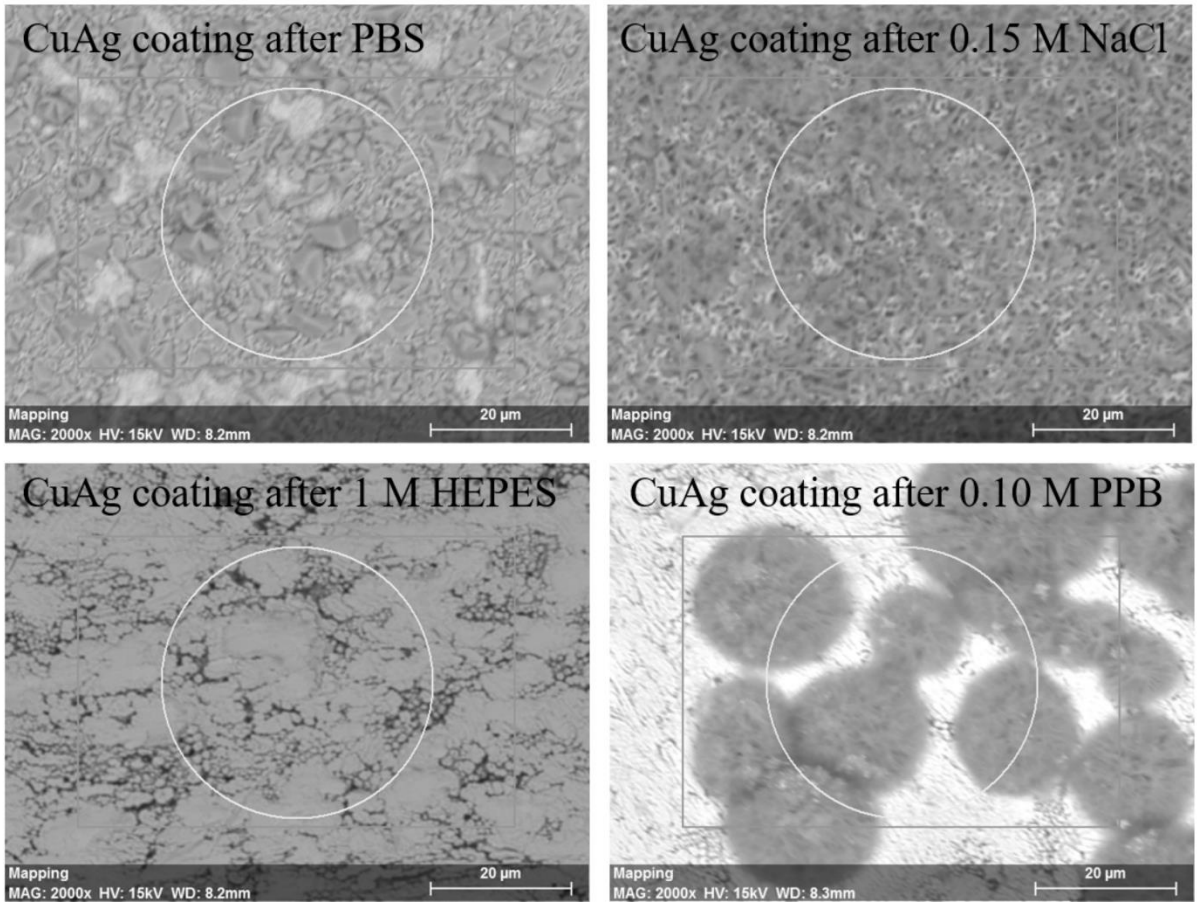
S1. SEM images of AISI 316 surfaces after potentiodynamic polarization testing.



S2. SEM images of electroplated silver surfaces after potentiodynamic polarization testing.



S3. SEM images of electroplated copper surfaces after potentiodynamic polarization testing.



S4. SEM images of electroplated copper-silver alloy surfaces after potentiodynamic polarization testing.

Paper 3

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In situ monitoring of the antibacterial activity of a copper-silver alloy using confocal laser scanning microscopy and pH microsensors

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In Situ Monitoring of the Antibacterial Activity of a Copper–Silver Alloy Using Confocal Laser Scanning Microscopy and pH Microsensors

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The antibacterial efficacy of a copper–silver alloy coating under conditions resembling build up of dry surface bacterial biofilms is successfully demonstrated according to US EPA test methods with a ≥99.9% reduction of test organisms over a 24 h period. A tailor-made confocal imaging protocol is designed to visualize in situ the killing of bacterial biofilms at the copper–silver alloy surface and monitor the kinetics for 100 min. The copper–silver alloy coating eradicates a biofilm of Gram-positive bacteria within 5 min while a biofilm of Gram-negative bacteria are killed more slowly. In situ pH monitoring indicates a 2-log units increase at the interface between the metallic surface and bacterial biofilm; however, the viability of the bacteria is not directly affected by this raise (pH 8.0–9.5) when tested in buffer. The OH^- production, as a result of the interaction between the electrochemically active surface and the bacterial biofilm under environmental conditions, is thus one aspect of the contact-mediated killing of the copper–silver alloy coating and not the direct cause of the observed antibacterial efficacy. The combination of oxidation of bacterial cells, release of copper ions, and local pH raise characterizes the antibacterial activity of the copper–silver alloy-coated dry surface.

1. Introduction

Microorganisms attach to both inert and biological surfaces and readily form biofilms.[1] This is especially problematic in healthcare settings, where dry surface biofilms can survive for extended periods on a multitude of surfaces.[1–3] Microbial communities assembled in a biofilm are less susceptible to biocides, antibiotics, and physical stress.[1] Therefore, dry surface biofilms can play a significant role in transmission of healthcare-associated infections, and dry environmental surfaces are a persistent source for the transfer of pathogens.[1,3]

Copper and copper alloy surfaces have been receiving increasing attention in the recent years, as a method for reducing such bacterial attachment and biofilms and subsequently the spread of pathogenic microorganisms in healthcare settings, thus potentially alleviating the occurrence of hospital mediated infections.[4–6] Evidence of their antibacterial properties from laboratory experiments has led to several field test studies in healthcare facilities in Europe and USA to validate their performances in real-life conditions.[5,7,8] In 2015, the United States Environmental Protection Agency (US EPA) released tailored protocols for testing and evaluating the antibacterial efficacy of copper and copper alloy surfaces with the intention of providing harmonized test conditions closely resembling real-life applications of such surfaces, e.g., environmental indoor items in healthcare facilities.[9–11] The first two protocols allow evaluation of the sanitizing efficacy of copper alloys on test organisms after 2 h exposure and after a prolonged exposure to a bacterial contamination accumulated over a 24 h interval.[9,10] Several copper-based surfaces have demonstrated antimicrobial effectiveness according to these protocols.[12,13]

A copper–silver (90–10 wt%) alloy laser-clad coating for stainless steel exhibited enhanced killing of *Escherichia coli*, as compared to the pure elements, and it was correlated with an 28-fold increased release of copper ions.[14] Similarly, a copper–silver (60–40 wt%) alloy electroplated coating has recently demonstrated strong antibacterial activity against *Staphylococcus aureus* and *E.coli* when tested in suspension.[15,16] In these test

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conditions, the copper–silver alloy-coated surfaces released copper ions in the bacterial suspension and the release was enhanced by a concentrated bacterial suspension or presence of nutrient broth.[15] Copper, the less noble alloying element, protected silver from dissolution by its preferential oxidation, according to the principle of galvanic corrosion.[15,16] This was confirmed by measurements of silver that was detected only as traces in the suspensions.[14,16] Therefore, the galvanic coupling of the two metals in the alloy coating induces oxidation of copper, resulting in release of copper ions, and reduction reaction on silver, leading to a local pH increase, under environmental conditions, e.g., in the presence of chlorides.[15,16] When bacteria are exposed to a copper–silver alloy-coated surface, a galvanic series is established, where silver holds the highest electrochemical potential followed by copper and bacteria.[16,17]

It is currently understood that bacteria are killed on dry copper surfaces through a contact-mediated killing process.[18] Copper dissolving from the surfaces and accumulating at the aqueous interface between the metallic substrate and bacterial cells causes severe membrane damage and overload of copper ions in the cytoplasm.[18,19] This scenario is quite different from killing of bacteria by copper ions in suspension or in culture, where the "free" copper ions concentration is lower by several orders of magnitude and bacteria are under growth conditions.[18]

The antibacterial efficacy of the newly developed copper–silver alloy against bacteria in suspension has been evaluated as mentioned, and this could resemble exposure to disinfectants, detergents, and hand sweat in the intended applications.[16] However, such surfaces will mostly face dry or humid conditions in a healthcare setting. It is possible that the antibacterial efficacy of this alloy would be enhanced in this dry scenario, also considering that the copper–silver alloy is an electrochemical active surface and is expected to have a different behavior than other copper alloy surfaces.[15] The surface contact is the well-established primary killing factor of copper alloys surfaces and the killing rate is crucial for any real-life application. Moreover, evidence suggested that the killing process initiates immediately after surface contact is established, and the exposed surface area and rate of release of copper ions can easily influence the overall rate of contact killing.[20,21] Therefore, the aim of this study is to determine the antibacterial activity of the copper–silver alloy coating under closer to real-life conditions, e.g., under dry conditions allowing a bacterial biofilm build-up.

2. Results and Discussion

2.1. Validation of Antibacterial Efficacy Through US EPA Test Methods

Test cultures, neutralizer solution and carriers successfully passed all the sterility, viability, quantitation and antimicrobial susceptibility controls carried out following the guidelines of the US EPA Test methods procedures.[9,10] The initial concentration of test organisms was $\approx 10^8$ CFU mL^{-1} (**Table 1**) in line with the US EPA Test methods for Efficacy as Sanitizer (Protocol 1) and Continuous Reduction of Bacterial Contamination (Protocol 2) of Copper Alloy Surfaces.[9,10] *Staphylococcus aureus* ATCC 6538 and *Staphylococcus aureus* MRSA ATCC 33592 were effectively inactivated by the copper–silver alloy coating with a 5-log reduction compared to the stainless steel control carriers after 2 h (Protocol 1) and at all time points over the 24 h time interval (Protocol 2), yielding a percent reduction greater than 99.9% (Table 1).[9] Copper–silver alloy-coated surfaces also reduced *Enterobacter aerogenes* ATCC 13048 levels with 5-logs compared to the stainless steel control carriers in Protocols 1 and 2 at all time points except after 2 h, where the level on stainless steel controls was $\approx 10^2$ CFU per carrier. However, the percent reduction of the copper–silver alloy–coated compared to uncoated stainless steel surfaces was greater than 99.9% both in Protocols 1 and 2 (Table 1).[9] In Protocol 1 *Pseudomonas aeruginosa* ATCC 15442 was able to survive on copper–silver alloy-coated surfaces to a geometric mean of 5.9 CFU per carrier, therefore the percent reduction was 99.9% compared to the stainless steel control surfaces, where the geometric mean of surviving *P. aeruginosa* was 1.1×10^4 CFU per carrier. However, the percent reduction was greater than 99.9% in Protocol 2 from 2-log reduction (after 2 h) to 4-log reduction (after 6, 12, 18, 24 h) (Table 1). Therefore, the copper–silver alloy-coated surfaces passed successfully the acceptance criteria of the test methods, i.e., a percentage reduction ≥99.9% after 2 h exposure and ≥99.0% at all-time points over the 24 h time interval, respectively.[9,10]

2.2. Confocal Laser Scanning Microscopy (CLSM) and Biomass Quantification

In order to visualize bacterial cells with a compromised membrane after exposure to copper surfaces, live/dead staining technique and fluorescence microscopy are the obvious choices that easily allow differentiation between bacterial cells with intact (green fluorescence) and compromised (red fluorescence) membranes. However, it was observed that regular fluorescence indicator dyes lose their fluorescence upon contact with metallic copper surfaces, due to the light absorption of copper.[18,20] Cells could be simply removed from surfaces prior to the staining procedure and then inspected, but this would only allow a post-visualizaton of the damaging effect caused by contact killing after set exposure times and not an in situ follow-up at the copper surfaces.[18]

Here, *S. aureus* 8325 (**Figure 1**) and *P. aeruginosa* PAO1 (**Figure 2**) cells were exposed and visualized directly at the surface of copper–silver alloy-coated and uncoated AISI 316 samples using a modified live/dead staining procedure and CLSM during a time interval of 100 min. Within the first 10 min of exposure to the copper–silver alloy-coated surfaces, the number of *S. aureus* 8325 dead cells (red) surpassed the number of live cells (green) (**Figures** 1 and **3**). After 25 min, the remaining live cells were less than 20% (Figure 3a) and the majority of cells appeared red after 60 min (Figure 1d). In contrast, *S. aureus* 8325 cells exposed to AISI 316 surfaces remained alive (Figure 1e–h) and their percentage was approximately above 80% over the whole exposure period (Figure 3b). The number of *P. aeruginosa* PAO1 live cells exposed to copper–silver alloy-coated surfaces decreased over time from the beginning of exposure up to 60 min (Figure 2a–d), when the ratio of live

Table 1. Results of US EPA test methods for efficacy as sanitizer (Protocol 1) and continuous reduction of bacterial contamination (Protocol 2) of copper–silver alloy-coated surfaces. Limit of detection (LOD) = 2.3 CFU per carrier.

Microorganism	Protocol	Inoculum CFU mL^{-1}	Recovered geometric mean numbers CFU per carrier		Percentage reduction
			Control	Test	
S. aureus ATCC 6538	1	1.7×10^8	2.6×10^5	<LOD	>99.9%
	2–2 h	2.7×10^8	9.9×10^4	<LOD	>99.9%
	2–6 h		4.0×10^4	<LOD	>99.9%
	2–12 h		9.3×10^4	<LOD	>99.9%
	2–18 h		1.1×10^5	<LOD	>99.9%
	2–24 h		9.0×10^4	<LOD	>99.9%
E. aerogenes ATCC 13048	1	8.7×10^8	8.0×10^5	2.4	>99.9%
	2–2 h	5.6×10^8	1.3×10^2	<LOD	>99.9%
	2–6 h		1.6×10^5	<LOD	>99.9%
	2–12 h		1.8×10^5	<LOD	>99.9%
	2–18 h		7.5×10^4	<LOD	>99.9%
	2–24 h		2.4×10^4	<LOD	>99.9%
P. aeruginosa ATCC 15442	1	7.0×10^8	1.1×10^4	5.9	99.9%
	2–2 h	9.0×10^8	2.4×10^2	<LOD	>99.9%
	2–6 h		2.3×10^4	<LOD	>99.9%
	2–12 h		1.2×10^4	<LOD	>99.9%
	2–18 h		3.8×10^4	<LOD	>99.9%
	2–24 h		2.3×10^4	<LOD	>99.9%
MRSA ATCC 33592	1	3.3×10^8	1.0×10^6	<LOD	>99.9%
	2–2 h	1.7×10^8	8.9×10^4	<LOD	>99.9%
	2–6 h		1.0×10^5	<LOD	>99.9%
	2–12 h		1.2×10^5	<LOD	>99.9%
	2–18 h		1.9×10^5	<LOD	>99.9%
	2–24 h		1.4×10^5	<LOD	>99.9%

and dead cells shifted in favor of the latter and the number of dead cells started to increase (Figure 3c). On AISI 316 surfaces, *P. aeruginosa* PAO1 cells remained alive (Figure 2e–h) and their percentage was close to 100% (Figure 3d) over the 100 min exposure period.

The direct visualization at the metal surface confirmed the antibacterial efficacy of copper–silver alloy-coated surfaces as compared to uncoated stainless steel controls, as also observed in the US EPA protocols testing. The copper–silver alloy-coated surfaces caused a more rapid killing of *S. aureus* than of *P. aeruginosa* and a lower percentage reduction in numbers of *P. aeruginosa* was also observed in the US EPA Test Method for Efficacy as Sanitizer (Protocol 1). Copper oxide impregnated non-porous solid surfaces were tested using the US EPA test protocols and did not reach a 99.9% reduction of *P. aeruginosa* in all tests.[13] Thus, these findings might suggest that *P. aeruginosa* can, to some extend, withstand exposure and contact to copper-based surfaces. During contact killing, when copper dissolves from the copper–silver alloy-coated surface as triggered by the presence of the bacterial film, copper ions accumulate in that limited space.[18] Membrane damage then occurs and copper ions enter the bacterial cytoplasm.[18,19] The presence of two cell membranes separated by a periplasmic space in Gram-negative bacteria and potentially the different mechanisms of copper homeostasis, active before contact killing inhibits the metabolic activities, can explain the observed delay in killing of *P. aeruginosa*. However, copper homeostasis mechanisms in Gram-positive and negative bacteria have not yet been fully unraveled and detecting the concentration of free copper ions at the interface poses serious practical issues.[18]

2.3. pH Monitoring at Copper–Silver Alloy-Coated and Uncoated Surfaces

Close to the interface between the layer of *S. aureus* 8325 suspension and the copper–silver alloy-coated surface, the pH increased with a rate of ≈0.14 pH units min^{-1} reaching a plateau at pH above 9.0 after 20 min (**Figure 4**). In contrast, pH at the interface between the layer of *S. aureus* 8325

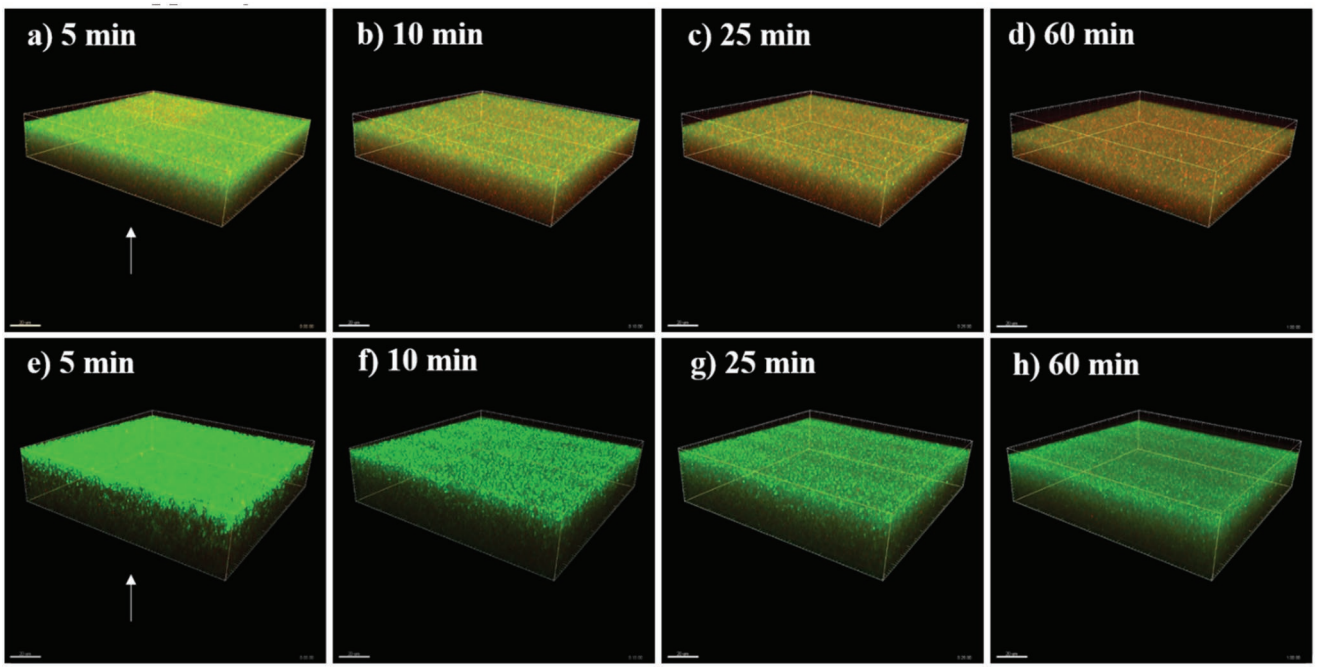

Figure 1. *Staphylococcus aureus 8325* live and dead cells exposed to a–d) copper–silver alloy-coated and e–h) uncoated AISI 316 surfaces monitored at the beginning of a,e) the exposure, and after b,f) 10 min, c,g) 25 min and d,h) 60 min. The arrow indicates the position of the metallic surfaces. Cells are stained with a modified live/dead dye stain mixture (0.2% of SYTO 9 green-fluorescent nucleic acid and 0.2% of SYTOX AADvanced dead cell stain) and live cells appear green and dead cells stain red.

suspension and the AISI 316 surface decreased after 10 min from values between 7.5 and 7.2 to values between 7.1 and 6.7 with a rate of ≈0.03 pH units min^{-1}. After 20 min, the pH reached plateau values between 7.0 and 6.5 (Figure 4). This clearly demonstrates the electrochemical activity of the copper–silver alloy-coated surface and the occurrence of the reduction

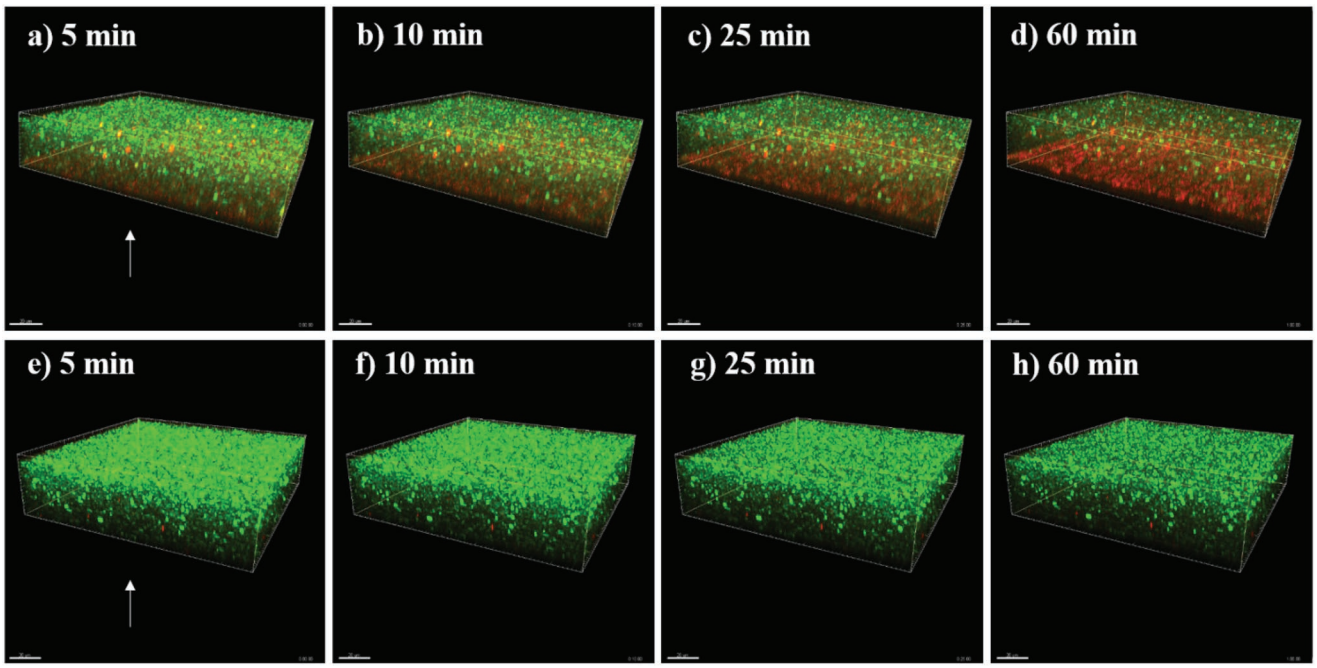

Figure 2. *Pseudomonas aeruginosa* PAO1 live and dead cells exposed to a–d) copper–silver alloy-coated and e–h) uncoated AISI 316 surfaces monitored at the beginning of a,e) the exposure, after b,f) 10 min, c,g) 25 min and d,h) 60 min. The arrow indicates the position of the metallic surfaces. Cells are stained with a modified live/dead dye stain mixture (0.2% of SYTO 9 green-fluorescent nucleic acid and 0.2% of SYTOX AADvanced dead cell stain) and live cells appear green and dead cells stain red.

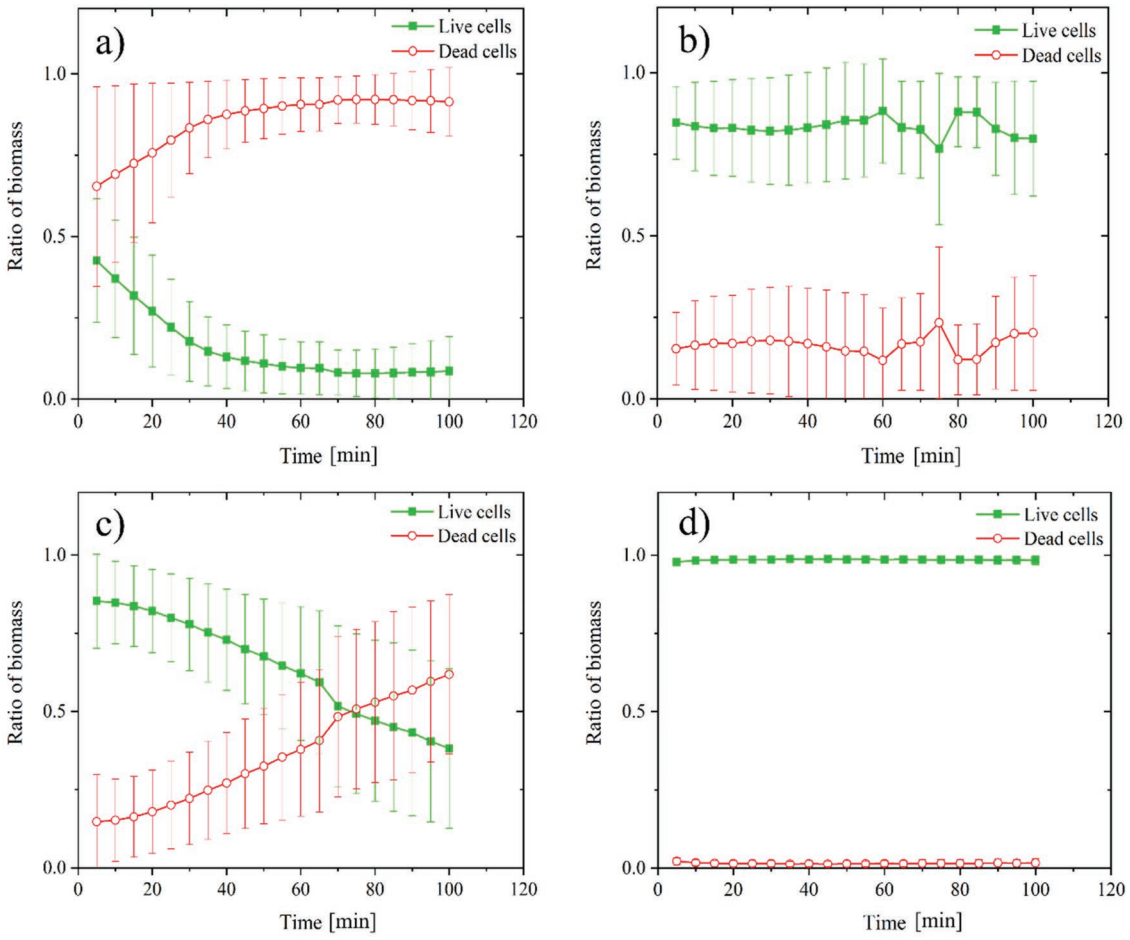

Figure 3. Ratio of *Staphylococcus aureus* 8325 live and dead cells exposed to a) copper–silver alloy-coated and b) uncoated AISI 316 surfaces and *Pseudomonas aeruginosa* PAO1 live and dead cells exposed to c) copper–silver alloy-coated and d) uncoated AISI 316 surfaces.

reaction ($O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$) at the aqueous interface with production of OH^- ions that raised locally the pH. If the copper–silver alloy is immersed in chloride-containing environments, galvanic corrosion conditions are established. In a 0.15 M saline solution, silver and copper exhibit corrosion potentials of 120 and 15 mV (vs standard hydrogen electrode), respectively.[16] Therefore, silver, the nobler metal in the galvanic couple, is protected at the expenses of copper and its dissolution rate increases with the increasing silver content in the alloy.[22] The membrane potential of *S. aureus* in the pH range from 5.0 to 7.0 is in the order of -100 mV (measured as distribution of [^{3}H]tetraphenylphosphonium TPP+).[17] Thus, in a three-element system consisting of the two alloyed metals and the *S. aureus* 8325 suspension in presence of the 0.15 M NaCl agarose matrix, a galvanic series is also established, where silver holds the highest electrochemical potential followed by copper and the bacterial material.[16,17] Consequently, the organic material readily oxidizes, since it possesses the lowest electrochemical potential, whereas the metallic alloy results the site of the reduction reaction. Copper is well known for its catalytic activity,[23–25] and this had potentially influenced the reaction rate and so the OH^- production rate. Moreover, the presence of the bacterial biofilm prevented the formation of copper oxide, maintaining the alloy-coated surface active, and provided enough material for the redox reaction to proceed at an equilibrium rate as indicated by the plateau after 20 min (Figure 4). In contrast, uncoated stainless steel was simply an inert substrate and the pH reduction was probably the result of an adjustment to optimal pH conditions from the unchallenged bacterial suspension in contact with the 0.15 M NaCl agarose matrix. If *S. aureus* 8325 suspension was not present at the interface between the agarose matrix and the copper–silver alloy-coated surfaces, pH increased with a rate of 0.69 pH units min^{-1} from values between 7.0 and 7.5 (**Figure 5**, replicas 2 and 3) to peak values between 9.5 and 10.0 after 4 min. In the case of replica 1 (Figure 5), the pH was already 9.0 at the beginning of the measurements and it reached its peak value of 9.4 after 1 min. Then, pH started to decrease with a slower rate of ≈0.12 pH units min^{-1} to reach values between 8.2 and 7.6 after 20 min. When the pH was monitored at the interface between the agarose matrix and the AISI 316 surfaces, it maintained approximately constant values between 6.4 and 6.7 for the whole duration of the measurement (Figure 5).

Once the contact between the 0.15 M NaCl agarose matrix and the copper–silver alloy-coated surface was established, the redox reaction readily initiated. In these conditions of bimetallic

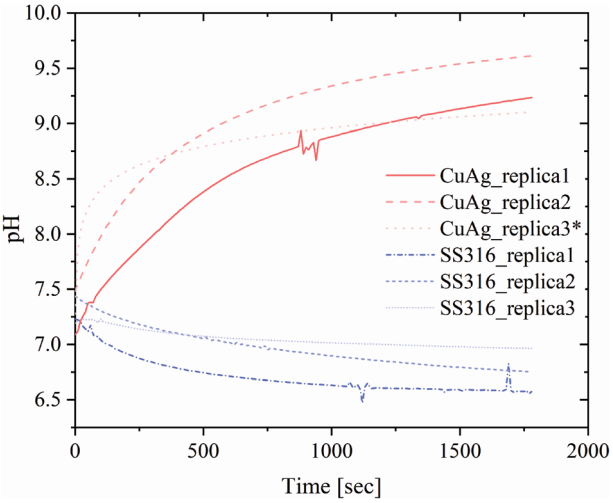

Figure 4. pH monitoring at the copper–silver alloy-coated and uncoated SS316 surfaces with 0.15 M NaCl 0.5% agarose matrix loaded with *Staphylococcus aureus* 8325 suspension. *the replicate was fitted with a model (indicated in the experimental section) that allowed extrapolation of its initial pH rise, due to a slower positioning of the sensor.

corrosion, the reduction reaction at silver sites produced OH^- that raised the pH and simultaneously copper dissolved from the alloy-coated surface. Copper ions subsequently reacted with the surrounding environment forming copper oxide Cu_2O.[26] The presence of Cu_2O led then to a pH decrease because of the establishment of new equilibrium conditions at the metal surface. Stainless steel surfaces were an electrochemically inactive substrate also in absence of a bacterial suspension layer, as clearly indicated by the constant pH value measured at the interface.

2.4. Exposure of *S. aureus* 8325 to 1 M Tris–HCl Buffer at pH 7.0 to 9.5

Due to the observed changes in pH at the copper–silver alloy-coated surface, we questioned whether this increase in pH

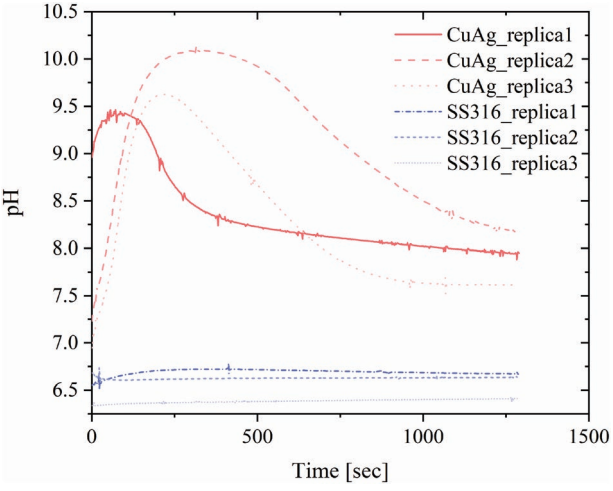

Figure 5. pH monitoring at the copper–silver alloy-coated and uncoated SS316 surfaces with unloaded 0.15 M NaCl 0.5% agarose matrix.

was the main cause of the rapid contact killing. Therefore, we exposed *S. aureus* 8325 at an initial concentration of ≈10^9 CFU mL^{-1} (corresponding to OD_{600} 2.0) to pH 8.0, 8.5, 9.0, and 9.5 in 1 M Tris–HCl buffer and incubated it at 25 °C for 1 and 24 h.

After 1 h exposure, *S. aureus* survived at levels between 10^8 and 10^9 CFU mL^{-1} and the cell level remained above 10^7 CFU mL^{-1} also after 24 h (**Table 2**). There was no statistically significant difference ($P > 0.01$) in survival after 1 and 24 h at the different pH. The 1-log reduction after 24 h exposure was caused by the buffering conditions, and it is not comparable to the 4 to 5-log reduction by the contact killing observed in the US EPA tests after 2 h. This indicates that *S. aureus* survival was not significantly influenced by the exposure to 1 M Tris–HCl buffer at 8.0–9.5 pH range. Therefore, it is not likely that the increase in pH at the copper–silver alloy-coated surface is the major cause of bacterial reduction, and it would rather have a secondary role in the contact-mediated killing by the copper–silver alloy-coated surface. Under environmental conditions and in presence of a bacterial biofilm at the interface, the galvanic coupling of copper and silver in the alloy would induce a redox reaction. Bacterial cells in contact with the alloy would oxidize, as they hold the lowest potential and a reduction reaction, resulting in OH^- production and local pH raise, would occur at the metal sites.

3. Conclusion

In this study, we demonstrated the antibacterial properties of a copper–silver alloy coating against bacterial contamination under dry and real-life like conditions. We used the US EPA test methods for efficacy as sanitizer and continuous reduction of bacterial contamination and a direct visualization by CLSM. The alloy passed successfully the EPA acceptance criteria of both test methods with a percentage reduction equal (*P. aeruginosa* ATCC 15442) or greater than 99.9% after 2 h exposure, and greater than 99.9% at all-time points over the 24 h time interval.

During the in situ monitoring of the contact killing at copper–silver alloy-coated and uncoated surfaces, we found a higher killing rate against bacterial biofilm of *S. aureus* 8325 than *P. aeruginosa* PAO1. Gram-positive alive cells were markedly reduced within the first minutes of exposure, whereas the ratio between alive and dead Gram-negative cells shifted toward the latter after 60 min of exposure. Membrane differences and different mechanisms of copper homeostasis may explain the slower killing rate in case of *P. aeruginosa* PAO1 bacterial biofilm.

pH measurement and monitoring at the copper–silver alloy-coated surfaces revealed a fast increase and reaching a plateau at pH 9.0 after 20 min, when *S. aureus* 8325 suspension was present at the interface between the surface and the agarose saline matrix. In absence of bacterial material, pH rapidly increased to ≈9.5 and dropped due to the formation of Cu_2O. No pH increase was detected at the uncoated control AISI 316 surface, due to the lack of electrochemical activity. When *S. aureus* 8325 was suspended in buffer solutions at different pH (range 8.0–9.5) no significant reduction in numbers was observed, indicating that pH could not be the sole responsible of the observed antibacterial properties. Therefore, OH^- production

Table 2. *Staphylococcus aureus* 8325 survival after 1 h and 24 h exposed to 1 M Tris–HCl buffer at pH 8.0, 8.5, 9.0, 9.5.

pH	Average Log CFU mL^{-1} after 1 h	Average Log CFU mL^{-1} after 24 h
8.0	8.3 ± 1.6	7.9 ± 1.3
8.5	8.9 ± 0.5	7.2 ± 0.6
9.0	9.2 ± 0.5	8.7 ± 0.5
9.5	9.4 ± 0.2	7.8 ± 0.5

is probably not the main reason for the contact-mediated killing phenomenon. Under environmental conditions and in presence of bacterial contamination, the galvanic coupling of copper and silver in the alloy would induce a redox reaction: oxidation of bacterial cells in contact with the alloy and reduction at the metal sites, resulting in local pH raise. In the same conditions, at surface areas not occupied by bacteria cells, the reduction reaction takes place at silver sites and oxidation reaction at copper, resulting in release of copper ions.

We conclude that the copper–silver alloy is an effective antibacterial against bacterial contamination under dry conditions. The redox reaction due to the galvanic coupling of the metals in the alloy likely induce oxidation of bacterial cells, release of copper ions and local pH raise under environmental conditions. The combination of these three factors is responsible for the observed antibacterial efficacy of this alloy coating and it would ensure its properties in the intended environmental applications in healthcare settings. The understanding of the electrochemical reactivity of metals can be used to produce other combination of redox active metals, or an active system based on a galvanic couple, tailoring the choice of elements to the specific environment and application.

4. Experimental Section

Materials and Surface Preparation Method: 2B surface finish AISI 316 cold rolled stainless steel sheet (X5CrNiMo17-12-2) was cut into 25.4 × 25.4 mm (1 × 1 in.) size carriers.[9,10,15] Carrier size of 25 × 75 mm was used for CLSM and pH monitoring measurements. The AISI 316 carriers were electroplated at a current of 4 A dm^{-2} for 1 min in a commercially modified copper–silver bath at Elplatek A/S Galvanord. Prior to the electroplating process, the specimens were cathodically degreased (3 ± 0.5 V for 2 min), rinsed in deionized water and surface activated in a Wood's nickel strike (4.5 ± 0.5 A dm^{-2} for 2 min). Copper–silver alloy-coated and uncoated AISI 316 carriers were used as test and control carriers, respectively.

Efficacy of Copper Alloys Surfaces as Sanitizer: The tests were performed according to the guidelines reported in the *Test method for Efficacy of Copper Alloy Surfaces as a Sanitizer* approved by the US EPA and using Good Laboratory Practice (GLP).[9] On the day prior to the test, five carriers per each material and organism were cleaned with 70% isopropyl alcohol, rinsed with deionized water, and allowed to air dry. After sterilization by dry heat, each carrier was placed in individual sterile plastic Petri dishes.[13] Six stainless steel and three copper–silver-coated carriers per organism were used for the carrier viability, carrier quantitation, neutralizer sterility, neutralizer confirmation, and carrier sterility controls according to the protocol guidelines.[9] The test controls were performed in parallel per each test. *Staphylococcus aureus* ATCC 6538, Methicillin Resistant *Staphylococcus aureus* (MRSA) ATCC 33592, *Enterobacter aerogenes* ATCC 13048, and *Pseudomonas aeruginosa* ATCC 15442 were revived from –80 °C stock cultures, streaked on Tryptone Soy Agar (TSA) (Oxoid CM0131) and incubated for 24 h at 36 ± 1 °C (27 ± 2 °C for *E. aerogenes*). Selected colonies were transferred to 1 mL Tryptone Soy Broth (TSB) (Oxoid CM0129) incubated for 24 ± 2 h at 36 ± 1 °C (27 ± 2 °C for *E. aerogenes*). Two 10 µL loopfuls of culture were transferred to 10 mL TSB and incubated for 24 ± 2 h at 36 ± 1 °C (27 ± 2 °C for *E. aerogenes*). This step was repeated three times. 4.7 mL of the bacteria suspension was transferred to a new tube and 0.25 mL heat-inactivated fetal bovine serum (FBS, Sigma F2442) and 0.05 mL Triton X-100 (Sigma-Aldrich) were added to yield 5% FSB and 0.01% Triton X-100 organic soil load. The carriers were spread with 0.02 mL of inoculum within 1/8 in. (≈3 mm) of the edges of the carriers and allowed to dry in a sterile bench for ≈20 min. A relative humidity of 25% and a laboratory temperature of 23 ± 2 °C were recorded during the experiments. After 120 min, the carriers were transferred to individual 50 mL falcon tubes containing 20 mL of neutralizer solution (Modified Letheen broth: Letheen broth + 0.07% Lecithin + 0.5% Tween 80). The tubes were sonicated for 5 min at 28 kHz (Delta 220; Deltasonic, Meaux, France) and rotated to collect bacteria. 10^{-1} to 10^{-4} serial dilutions in phosphate buffered saline (PBS) (Oxoid BR0014G) were made and 1 mL plated in duplicates on TSA plates. The plates were placed in a sterile bench with lids ajar in order to dry before the incubation for 48 h at 36 ± 1 °C (27 ± 2 °C for *E. aerogenes*). Plates with colony numbers in the range 5–300 were used in the evaluation. CFU per carrier were calculated as average number colonies per plate at respective dilution, multiplied by the dilution factor and the volume of the neutralized solution and divided by the volume plated. The geometric mean of the number of organisms surviving on control and test carriers was reported and used for the calculation of the percentage reduction.[9] Testing of the antimicrobial susceptibility of MRSA ATCC 33592 against oxacillin was also performed according to the EPA protocol guidelines. *Staphylococcus aureus* ATCC 25923 was used as control organism and the inhibition zone was interpreted according to the guidelines of Clinical and Laboratory Standards Institute.[27]

Continuous Reduction of Bacterial Contamination on Copper Alloy Surfaces: The tests were performed according to the guidelines reported in the Test method for the Continuous Reduction of Bacterial Contamination on Copper Alloy Surfaces approved by the US EPA and using Good Laboratory Practice (GLP).[10] The test procedure was followed as outlined in the previous section and five replicates per organism per time point were used.[13] The carriers (25 copper–silver coated test carriers, 15 stainless steel control carriers, 16 stainless steel carriers for quantitation and viability control per each organism) were inoculated with 5 µL of the inoculum at "time 0" and allowed to air dry in sterile conditions. At 2, 6, 12, 18, and 24 h after the initial inoculation, five copper–silver electroplated carriers, three stainless steel control carriers, and three stainless steel carriers for quantitation control were recovered. These carriers were inoculated one, two, four, six, and eight times, respectively. The remaining carriers were reinoculated with 5 µL of the inoculum after 3, 6, 9, 12, 15, 18, and 21 h. The recovered carriers were transferred to individual 50 mL falcon tubes containing 20 mL of neutralizer solution, sonicated for 5 min at 28 kH (Delta 220; Deltasonic, Meaux, France) and rotated to mix. Serial dilutions (10^{-1}–10^{-4}) were made in PBS and 1 mL plated in duplicates on TSA plates. After drying, the plates were incubated for 48 h at 36 ± 1 °C (27 ± 2 °C for *E. aerogenes*). Colony numbers in the range 5–300 were used in the calculations.

Modified Live/Dead Staining Assay and CLSM: A modified live/dead dye mixture containing 0.2% of SYTO 9 Green-Fluorescent Nucleic Acid Stain (Invitrogen, USA) and 0.2% of SYTOX AADvanced Dead Cell Stain (Invitrogen, USA) in MilliQ water was used to visualize and follow-up the killing process of bacterial films in contact with the copper–silver alloy-coated surface. SYTO 9 can penetrate both intact (live cells) and compromised (dead cells) membranes, while SYTOX AADvanced stains only compromised cells.[28] The modified dye mixture was designed to allow the direct inspection of bacterial cells on the copper–silver alloy-coated substrate. Copper surfaces were found to interfere and absorb the fluorescent signal of propidium iodide, which is the commonly used

dye for dead cell stain.[29] This effect is due to the characteristic light absorption of copper surfaces and results in decrease or elimination of the observed fluorescent signal.[30] SYTOX AADvanced was used instead since it is characterized by an emission spectrum shifted to longer wavelengths and therefore it is possible to visualize its signal in contact with copper surfaces. *Staphylococcus aureus* 8325 or *Pseudomonas aeruginosa* PAO1 were revived from –80 °C stock cultures, streaked on lysogeny broth (LB) agar plates (5 g L^{-1} yeast extract (Oxoid, Roskilde, Denmark), 10 g L^{-1} tryptone (Oxoid), 10 g L^{-1} NaCl (Merck, USA), pH 7.5) and incubated for 24 ± 2 h at 37 ± 1 °C. The modified live/dead dye mixture was applied on the inoculated plates that were incubated in dark for 5–10 min. Using a 5 µL inoculating loop, the stained bacteria were transferred from the plates to the copper–silver alloy-coated or uncoated AISI 316 25 × 75 mm carriers, mimicking a bacterial biofilm, and covered by a glass cover slide. The inoculated carriers were immediately inspected at a Zeiss LSM 880 inverted confocal laser scanning microscope using a Plan-Apochromat 63 × /1.40 oil differential interference contrast [DIC] objective (Zeiss, Germany). A 488 nm laser was used for excitation and a 561 nm filter for emission in order to capture both the signal from SYTO 9 (emission maxima 498 nm) and SYTOX AADvanced (emission maxima 647 nm). Bacteria at the metallic substrates were imaged as a 135 µm × 135 µm field with ≈0.5 µm increments in the *Z* direction. The stacks of images were captured every 5 min within 100 min time series.

Image Processing and Biomass Quantification: Image processing was done using the IMARIS software package (Bitplane AG, 451 Switzerland). Quantification of the biomass as ratio of live and dead cells was performed for three experimental repeats of each combination of test organism and material by using COMSTAT 2 (www.comstat.dk) using a threshold factor of 5 without connected volume filtering.[31,32]

pH Monitoring at the Metallic Surfaces: *Staphylococcus aureus* 8325 was from -80 °C stock culture, streaked on LB plates, and incubated for 24 ± 2 h at 36 ± 1 °C. A single colony was added to 5 mL LB broth and incubated for 24 ± 2 h at 36 ± 1 °C. Bacterial cells were harvested at 4000 g for 5 min, resuspended in 0.15 M NaCl solution, and adjusted to OD_{600} 2.0 by using a spectrophotometer (UV 1800, Shimadzu, Japan). 0.15 M NaCl solution 0.5% agarose was melted and 4 mL poured in a one-well glass slide (16 × 50 × 5 mm) with a removable well (Ibidi, Germany). The agarose was allowed to cool to room temperature and solidify for at least 10 min where after, the gel matrix was inverted in order to expose the smoother side. 250 µL of *S. aureus* 8325 bacterial suspension was spread on the surface and left to air dry for 5 min. pH measurements were done using pH microelectrodes (PH25, tip diameter ≈25 µm, Unisense A/S) with a linear range between pH 4–9, a 90% response time <10 s. The pH microelectrodes were used in combination with a reference microelectrode (REF-100, tip diameter of ≈100 µm; Unisense A/S) immersed in the agarose matrix to ensure electrical contact to the microelectrode. The pH microelectrode was calibrated from sensor readings in three pH buffers (pH 4.01, 7.00, and 10.01, at experimental temperature) and responded linearly to pH over the calibration range with a signal to pH ratio of ≈56 mV per pH unit. The pH electrodes were connected to a multimeter (Unisense A/S) and data acquisition was done in PC running software (SensorTrace Suite; Unisense A/S). During operation, the microsensors were mounted on a PC-interfaced motorized micromanipulator (MM33-2, MC-232; Unisense A/S) controlled by dedicated positioning software (SensorTrace Suite; Unisense A/S). The inoculated gel matrix was placed on copper–silver alloy-coated or uncoated AISI 316 substrate carrier (25 × 75 mm) and the electrodes were carefully positioned at a safe distance (<100 µm) from the metallic surface as rapidly as possible. However, in one replicate, the positioning of the sensor was slow and the initial rise in pH was not recorded. Therefore, this replicate was fitted with a model ($y = a(ln(x)) + b$) that allowed extrapolation of its initial pH rise. pH was also monitored at the surface of copper–silver alloy-coated or uncoated AISI 316 without bacterial inoculum. Here, the pH dynamics were faster than when bacteria were present so in order to capture the initial pH rise, the sensors were positioned close to the surface (<100 µm) and a drop of 0.5% low melting point agarose (Ultra Pure LMP Agarose, Invitrogen, USA) was deposited on the surface covering both the sensor and reference electrode tips. The drops (100 µL) were deposited at a temperature of 28 °C and the agarose solidified immediately upon contact with the alloy-coated surface.

Exposure of S. aureus 8325 *to 1* M *Tris-HCl Buffer at pH 8.0 to 9.5*: *Staphylococcus aureus* 8325 was revived from –80 °C stock culture, streaked on Brain Heart Infusion (BHI) agar plates (Oxoid, CM1135) and incubated for 24 ± 2 h at 36 ± 1 °C. Single colonies were added to 5 mL BHI broth and incubated for 24 ± 2 h at 36 ± 1 °C. 1 M Tris–HCl buffers (121.1 g Tris Base (Trizma, Sigma-Aldrich), 700 mL dH_2O) were prepared and the pH was adjusted to 8.0, 8.5, 9.0, 9.5 using concentrated HCl (Sigma-Aldrich). Bacterial suspensions were adjusted to OD_{600} 2.0 by using a spectrophotometer (Novaspec III Visible Spectrophotometer, Amersham Biosciences) and 1 mL was transferred in Eppendorf tubes (Eppendorf AG, Hamburg). Bacterial cells were harvested at 4000 g for 5 min and resuspended in 1 mL 1 M Tris–HCl buffers. Bacterial suspensions were sampled after 1 and 24 h exposure time. The density of bacterial survival in suspension (CFU mL^{-1}) was determined by serial dilution and plating on BHI-agar. All experiments were conducted in three biological replicates; average values and standard deviation among replicates are reported (Table 2).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

antibacterial activity, bacterial biofilms, confocal laser scanning microscopy, copper–silver alloy, electroplating

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Paper 4

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Copper-silver alloy coated door handles as a potential antibacterial strategy in real-life clinical settings

Manuscript in preparation

Copper-silver alloy coated door handles as a potential antibacterial strategy in real-life clinical settings

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Abstract (139 words)

A copper-silver alloy can be used as an alternative or complementary antibacterial strategy to other existing technologies and disinfection interventions in e.g. clinical settings. For this purpose, two field tests has been carried out. Copper-silver coated door handles had lower microbial load in terms of total aerobic plate count: 1.3 ± 0.4 Log CFU/cm^2 and 0.8 ± 0.3 Log CFU/cm^2 as compared to the reference uncoated material on-site: 2.4 ± 0.4 Log CFU/cm^2 on stainless steel and 1.7 ± 0.4 Log CFU/cm^2 on satin brass. No selective antibacterial activity in terms of reduction of specific bacterial species was observed. The durability (between a re-coating intervention) of the coating was estimated to approx. one year under the tested conditions of this study. Outcomes of this study confirm the possibility of a successful long-term implementation of this coating as antibacterial strategy.

1. Introduction

The extensive laboratory evidence demonstrating the antibacterial properties of copper alloys had led to a number of field tests aimed at providing a real-world proof of the concept especially in clinical settings [1–3]. Two main questions are at the basis of such field-testing: if introduced into the clinical environment: will the items made of copper alloys carry less microorganisms as compared to the parallel items in standard materials? If there is such a difference, will a corresponding reduction in healthcare-associated infections be observed? [1]

A multihospital clinical trial of six U.S. EPA registered antimicrobial copper alloys demonstrated that the microbial burden of copper alloy surfaces was six-times lower (465 CFU/100cm^2) as compared to conventional surfaces such as plastics, coated carbon steel, aluminum, and stainless steel (2,674 CFU/100cm^2) [4]. Levels were above 250 CFU/100cm^2 in both cases which is the proposed standard for microbial burden on a surface immediately after terminal cleaning [5]. However, there was a reduction in infections of 58% in "copper" rooms, compared to the "non-copper" rooms.

A study conducted in Finland installed different copper alloy and brass items in a hospital, in a kindergarten, a retirement home and an office building, and microbial levels were compared to conventional surfaces [3]. Door handles made of copper alloy (99.8 wt% Cu) outperformed brass (60.5 wt% Cu, 36.5 wt% Zn) door handles that, in terms of total aerobic plate count, did not show (on average) significant differences as compared to the reference (chromed) material [3]. Lower levels of both Gram-negative bacteria and *Staphylococcus aureus* were found on copper alloy (99.8 wt% Cu) surfaces as compared to brass and reference surfaces [3]. Door handles harbored the highest levels of bacterial contamination in a clinical environment [6]. Thus, we aim, in the present study, to investigate the antibacterial performances of copper-silver alloy coated door handles in the real-life environment of a private clinic (FamilieLægerne Espergærde) and a wound care center (Southwest Regional Wound Care Center), as compared to the corresponding reference on-site material. The identification of microbial isolates on different surfaces, and the evaluation of copper-silver alloy coating durability in these conditions complete this investigation in the light of a future use of this coating.

2. Material and methods

2.1 Door handles manufacturing and installation

Stainless steel door handles (Ruko Assa Abloy) were electroplated with a copper-silver alloy coating at Elplatek A/S. In the private clinic FamilieLægerne Espergærde (Egeskovvej 20, 3490 Kvistgård, Denmark), copper-silver alloy coated (test) door handles were installed at the doors of two doctors and two nurses's exam rooms. Stainless steel door handles of other parallel four offices were sampled as reference material, and weekly sampling was performed for a period of 6 weeks. At the Southwest Regional Wound Care Center (2002 Oxford Ave, Lubbock, TX 79410, USA), test door handles were installed at the doors of seven exam rooms, one public restroom and two laboratory rooms. Original satin brass door handles of other six exam rooms, three public restrooms and one laboratory room were sampled as reference material, and weekly sampling was performed for a total period of 6 weeks.

2.2 Microbiological sampling and MALDI-TOF analysis (Denmark)

Door handles surfaces (100 cm^2) were swabbed thoroughly with a flocked applicator sterile swab (BD™ ESwab Regular Collection Kit) including horizontal and vertical sweeps. The swab was inserted into the sampling tube containing 1 mL of Liquid Amies Medium, and the samples were transported to the laboratory at the Technical University of Denmark within 1 hour. The sampling tubes were sonicated for 2 min at 28 kHz (Delta 220; Deltasonic, Meaux, France) and vortexed for 15 s. 400 µl of the sampling suspensions were plated in duplicates on 5% blood agar (BA) plates (BD™, Franklin Lakes, USA). A 10 and 100 fold dilutions from the remaining 100 µl of the test and reference suspensions, respectively, was also made and plated. Additionally, all the sampling swabs were streaked on Brain Heart Infusion agar plates (BHI; Oxoid CM1135) to verify if any bacteria was left on the swabs. The plates were incubated at 37±1 °C, and total aerobic plate count was performed after 48 hours. Colony forming units (CFU) per plate corresponded to CFU per door handle surface (100 cm^2). Average values of CFU/100cm^2 for test and reference door handles at each sampling were log-transformed and presented as total average values and standard deviations. All the isolates from the test door handles at the third and last sampling, were re-streaked on BA plates and single colonies were stored for later identification at -80 °C in frozen medium (Tryptone Soy Broth 30 g/l, Glucose 5 g/l, Skim milk powder 20 g/l, Glycerol 40 g/l in distilled water). A corresponding number of single colonies was randomly selected from the reference door handles at these samplings and handled in the same way. Identification of species from isolates

was performed with Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) on a Microflex LT instrument (Bruker Daltonik GmbH, Germany). Protein profiles were acquired with the FlexControl 3.3 software (Bruker Daltonik GmbH) and analyzed with FlexAnalysis 3.3 (Bruker Daltonik GmbH). The database used to match spectra was Bruker Taxonomy (7311 MSPs). MALDI-TOF MS scores >2.0 were used to identify isolates to species level, while scores between 1.8 and 2.0 were used to identify to genus level. Scores below 1.8 were not considered in this study.

2.3 Microbiological sampling and analyses of bacterial load by direct sequence analysis (Texas)

The microbiological sampling and all analyses were performed on-site at the CAP accredited medical laboratory at the Southwest Regional Wound Care Center.

Door handles surfaces (100 cm^2) were swabbed with a sterile cotton swab including horizontal and vertical sweeps weekly. Cotton swabs were inserted into sterile screw caps micro tubes and 500 µl of phosphate-buffered saline solution (PBS; Dulbecco A; Oxoid) was added. Bacteria were detached from the cotton swabs by shaking at 20.0 Hz for 2 min using a Qiagen TissueLyser (Qiagen Inc., Valencia, CA, USA). At the first two samplings, the 500 µl were added to sterile screw caps micro tubes and genomic DNA was extracted using the Roche High Pure PCR Template Preparation kit (Roche Life Sciences, Indianapolis, IN, USA) according to manufacturer specifications. Sample lysates for DNA extraction were produced using the Qiagen TissueLyser and 0.5 mm zirconium oxide beads (Next Advance, Averill Park, NY, USA). Semi-quantitative determination of bacterial load using the universal 16S rRNA gene sequence was performed using TaqMan real-time PCR Assay with the LightCycler® 480 (Roche Life Sciences). Forward (5′-CCATGAAGTCGGAATCGCTAG-3′) and reverse (5′-GCTTGACGGGCGGTGT-3′) 16S rDNA primers (20 µM each) were used with a 16S rDNA probe (5′-TACAAGGCCCGGGAACGTATTCACCG-3′) in Quanta PerfeCTa® qPCR ToughMix (Quanta Biosciences, Beverly, MA, USA). The template DNA (2.5 µL) was added to the master mix containing primers and probe (10 µL each), and the reaction was run with the following thermal cycling profile: 50 °C for 2 min, 95 °C for 10 min, 35 cycles at 95 °C for 15 s, 60 °C for 1 min, and 40 °C for 30 sec. *E. coli* c600 (ATCC 23724, Manassas, VA, USA) genomic DNA was used as a positive 16S rDNA control and molecular grade water (Phenix Research Products, Chandler, NC, USA) was used as a no template control.

2.4 Microbiological sampling, and analyses of bacterial load by plating and sequence analysis (Texas)

For the last four weekly samplings (of a total of six samplings), the 500 µl from the swab collection tubes were plated on Tryptone Soy Agar (TSA) (Oxoid CM0131) plates. Plates were incubated at 37±1 ºC and the total aerobic plate count was performed after 48 hours. Average values of CFU/100cm^2 for test and reference door handles at each sampling were log-transformed and presented as total average values and standard deviations. Plates were then washed using 1 mL PBS and bacterial material was collected into sterile Eppendorf tubes. Genomic DNA was extracted from 500 µl of this suspension and the semi-quantitative determination of bacterial load (amplification of the 16S rRNA gene) was performed using the TaqMan real-time PCR Assay described above.

2.5 Microbiological sampling, and analyses of pathogen and resistance gene presence by plating and sequence analysis (Texas)

The possible presence of pathogenic bacteria and/or resistance genes was tested by using the TaqMan real-time PCR Assay for *Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Streptococcus pyrogenes, Streptococcus agalactiae,* and the *mecA* and *vanA* genes using primer sequences property of the CAP accredited medical laboratory. This analysis was done on all bacterial DNA extracted directly from swabs and on selected bacterial DNA extracted from the agar plates. At the third sampling, four DNA extracts from test door handles had Ct (cycle threshold)-values for the 16S rDNA gene below 30 (the cut-off value), and were selected for further screening to detect possible presence of pathogenic bacteria and/or resistance genes. Eight DNA extracts from the reference door handles (control) had Ct-values below 30, and here six samples were randomly chosen as representative of the control group and screened by PCR for presence of pathogens and resistance genes in the DNA extracts. The number of selected test (four) and control (six) DNA extracts were maintained at all the remaining samplings, but they were randomly chosen among the test and control DNA with Ct-values for the 16S rDNA gene below 30. The presence of *S. aureus*, *mecA* and *vanA* genes was indicated as positive or negative.

The remaining 500 µl of the 1 mL washing suspensions from the agar plates was spread on TSA plates and incubated at 37±1 ºC for 24 hours. Single colonies were restreaked on Mannitol salt agar and Cetrimide agar plates (selective for staphylococci and micrococcaceae, and *Pseudomonas aeruginosa*, respectively) and incubated at 37±1 ºC for 24 hours before visual

inspection. This was done to double-check results from TaqMan real-time PCR Assay for the presence or absence of these species.

2.4 EDS analysis on copper-silver alloy coated door handles prior and after field-testing

The chemical composition of selected copper-silver alloy coated door handles was checked prior to and after field-testing using Hitachi TM3030 Plus Tabletop Microscope operated at 15 kV equipped with Oxford Inca software and Bruker Quantax 70 energy dispersive x-ray spectrometry EDS System.. EDS analysis was performed on three different spots at the surface of each sample. The output values (normalized weight percentage) were averaged and re-calculated with respect to the total content of copper and silver in order to evaluate the difference before and after installation. Presence of other elements (C, O) was also reported in normalized weight percentage if greater than 5 wt%. This was done to evaluate possible changes in surface composition of the coating during usage.

2.5 Statistical analysis

Average values of CFU/100cm^2 for test and reference door handles at each sampling were log-transformed to obtain a normal or quasi-normal distribution. Values were tested for equal or unequal variance with the *F*-test and statistical significance of the difference between test and reference door handles was verified using the *t*-test.

3. Results and discussion

Both reference stainless steel door handles and satin brass door handles had a microbial load, in terms of average total aerobic plate count, approx. twice as high as the copper-silver coated door handles at FamilieLægerne Espergærde and Southwest Regional Wound Care Center (Table I). All surfaces in the field tests, except for stainless steel, had a microbial load below 2.4 Log CFU/cm^2 (the standard for acceptable microbial level on a surface immediately after terminal cleaning) [5]. Not surprisingly, the microbial load on the satin brass reference door handles was lower than on the stainless steel reference door handles, due to the antibacterial activity of brass.

Interestingly, there was no difference in the bacterial load as estimated by qPCR of the 16S rDNA gene between the coated and reference door handles (Ct-values of 24.5 and 24.6 respectively), when the DNA was directly extracted from the swabs. This can be due to the

fact that the recovered 16S rDNA was both from alive and dead bacterial cells on the surfaces, but it can also be caused by a selective killing targeting bacteria with fewer alleles of the 16S rDNA gene. The qPCR results when applied to the biomass from the cultured plates obviously indicated a higher count on the control door handles than on the alloy-coated handles, as it was also clear from the actual colony counts.

MALDI-TOF MS analysis performed on randomly chosen isolates from copper-silver alloy coated and uncoated stainless steel surfaces installed in the Danish clinic revealed no marked difference among the surfaces in terms of surviving bacterial species. Most abundant were *Micrococcus luteus* and staphylococci (*S. hominis*, *S. epidermidis* and *S. capitis*) on both copper-silver alloy coated and uncoated door handles, although *Staphylococcus aureus* was found on stainless steel but not on copper-silver alloy coated door handles at FamilieLægerne Espergærde (Figure 1).

DNA from the direct swabs was tested by qPCR for presence of six pathogenic bacteria and two resistance genes, and none of the samples were positive. DNA from 16 of the washed plated test samples and from 24 control samples in Texas were tested for presence of *Staphylococcus aureus* by PCR. Three out of the 16 test samples (19%) and three out of the 24 reference samples (13%) were positive for the pathogen (Table II). The presence of *S. aureus* was also confirmed by the yellow discoloration on selective (mannitol salt) agar plates. No *Pseudomonas aeruginosa, Serratia marcescens, Streptococcus pyrogenes, Streptococcus agalactiae* were the detected at any of the samples based on biomass from cultured plates.

Resistance genes were detected in the bacterial biomass washed from the agar plates probably because of the much higher bacterial load as compared to the direct swabs. Seven out of the 16 test samples (44%) and 22 out of the 24 reference samples (92%) were positive for the *mecA* gene (Table II). At the last sampling, the *vanA* gene was detected in one biomass-plate sample from the control group. The greater occurrence of *mecA* gene the control group could be due to the larger bacterial counts on the plates and hence a larger biomass.

The EDS analysis on door handles before and after field-testing at FamilieLægerne Espergærde reveled a 5 ± 1 wt% relative difference in terms of copper and silver content, whereas there was basically no change in relative composition of copper and silver prior and after installation at the Southwest Regional Wound Care Center (Table III). Carbon and oxygen could be detected on the surfaces after the field tests, but the amount was significant only in the case of carbon (10.9 ± wt% and 12.3 ± 1.9 wt%). No significant surface oxidation was observed on the

surface before and after field-testing. It is likely that door usage and other environmental affecting factors may have contributed to reduce the copper content in the copper-silver alloy coated door handles at FamilieLægerne Espergærde, as compared to the ones at the Southwest Regional Wound Care Center. To our knowledge, the cleaning procedure should have not strongly influenced the surface chemistry, since door handles were not subjected to extensive disinfection in these environments. Only routine surface wiping was performed, and an ethanol-based disinfectant spray was used directly on the surfaces only once at the Southwest Regional Wound Care Center. However, considering the reduction in copper content in the copper-silver alloy coated door handles after the field test in the Danish clinic, the lifetime (durability) of the coating could be safely estimated to 72 weeks. At that point, the door handles should be recoated. During usage, a complementary cleaning procedure to remove dirt and filth (carbon presence on the surface) and ensure direct contact between bacteria and the alloy would be definitely recommended.

4. Conclusions

Copper-silver alloy coated door handles carried a lower bacterial load in terms of total aerobic plate count as compared to stainless steel or satin brass door handles. Therefore, it can be expected that this would lower the chances of transmission of bacteria. However, a selective higher antibacterial efficacy of copper surfaces against Gram-negatives and *S. aureus* as previously suggested was not observed [3]. In these conditions, the lifetime of the coating was estimated to a safe period of one year. After that, a re-coating intervention should be performed, and a cleaning procedure to remove surface contamination and ensure direct contact between surface and bacteria should be regularly performed during usage. Hence, this study poses a promising basis for a clinical trial where the infection rates (pre- and post-installation) would also be monitored, and for a future long-term implementation of this coating as antibacterial strategy.

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Table I. Average total aerobic plate count in Log CFU/100cm^2 from copper-silver coated and uncoated reference door handles at FamilieLægerne Espergærde and Southwest Regional Wound Care Center.

	Weekly samplings	**Average Log CFU/100cm^2**		***p*-value**
		Cu-Ag coated	Reference	
FamilieLægerne Espergærde	6	1.3 ± 0.4	2.4 ± 0.4	0.0008
Southwest Regional Wound Care Center	4	0.8 ± 0.3	1.7± 0.4	0.0068

Table II. Cycle threshold (Ct)-values for 16S rDNA from test (copper-silver alloy coated door handles) and control (sating brass door handles) DNA extracts, when DNA extraction was performed directly from the swabs and after the growth step on agar, as described in MM. In the latter case, occurrence (+/–) of *S. aureus*, *mecA* and *vanA* genes among the selected DNA extracts is also reported.

		16s rDNA (direct extr.) Ct-values			**16s rDNA (growth step) Ct-values**					***S. aureus* (growth step) +/-**				**mecA (growth step) +/-**				**vanA (growth step) +/-**			
		# sampling			# sampling					# sampling				# sampling				# sampling			
	Room	1	2	avg	3*	4	5	6	avg	3	4	5	6	3	4	5	6	3	4	5	6
Test	Exam 2	24.1	25.5		≥30	≥30	16.2	12.9		NA	NA	NA	-	+	NA	NA	+	-	NA	NA	-
	Exam 4	23.6	25.4		≥30	≥30	16.3	19.1		NA	NA	NA	NA	-	NA	NA	NA	-	NA	NA	NA
	Exam 6	24.1	25.2		20.5	≥30	17.6	17.9		-	NA	-	NA	-	NA	-	NA	-	NA	-	NA
	Exam 8	24.0	25.3		≥30	≥30	16.5	18.0		NA	NA	-	NA	NA	NA	-	NA	NA	NA	-	NA
	Exam 10	24.1	25.0		18.6	19.1	18.0	14.3		-	-	+	-	NA	-	+	+	NA	-	-	-
	Exam 12	23.9	24.0		19.9	17.8	19.6	16.4		-	+	NA	-	NA	+	NA	+	NA	-	NA	-
	Exam 14	25.0	24.4		≥30	≥30	20.6	13.2		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Men's West	25.1	23.8		≥30	15.8	22.6	16.5		NA	-	NA	NA	NA	-	NA	NA	NA	-	NA	NA
	Lab Extraction	25.1	23.8		≥30	≥30	19.1	18.1		NA	NA	-	-	-	NA	-	-	-	NA	-	-
	Lab Reaction	25.2	23.8		15.4	19.9	18.5	20.7		+	-	NA	NA	NA	-	NA	NA	NA	-	NA	NA
average		**24.4**	**24.6**	**24.5**	**18.6**	**18.1**	**18.5**	**16.7**	**18.0**												
Control	Exam 1	24.0	25.3		16.4	17.3	12.5	14.0		-	NA	NA	-	+	NA	NA	+	-	NA	NA	-
	Exam 3	23.9	25.2		18.1	≥30	15.5	13.4		-	NA	NA	NA	+	NA	NA	NA	-	NA	NA	NA
	Exam 5	24.0	25.5		15.0	15.9	14.1	14.8		-	NA	-	-	+	NA	+	+	-	NA	-	-
	Exam 7	23.9	24.8		14.0	19.1	16.1	15.8		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Exam 9	23.8	26.1		14.2	14.5	16.9	13.9		NA	-	-	-	NA	+	-	+	NA	-	-	-
	Exam 11	23.8	25.2		≥30	17.3	14.2	16.5		NA	-	-	NA	NA	+	+	NA	NA	-	-	-
	Men's East	25.2	23.9		11.8	15.4	16.5	12.5		-	+	-	-	+	+	+	+	-	-	-	+
	Women's East	25.4	24.1		≥30	14.7	15.7	13.9		NA	-	-	NA	NA	+	+	NA	NA	-	-	NA
	Women's West	25.1	24.0		12.5	15.1	≥30	13.1		-	-	NA	+	+	+	NA	+	-	-	NA	-
	Lab Detection	25.2	23.7		15.4	19.6	16.6	13.3		-	-	+	-	+	-	+	+	-	-	-	-
average		**24.4**	**24.8**	**24.6**	**14.7**	**16.6**	**15.3**	**14.1**	**15.2**												

Table III. Relative weight percentage (wt%) values of copper and silver with respect to the their total content in copper-silver alloy coated door handles before installation and after field-testing at FamilieLægerne Espergærde and the Southwest Regional Wound Care Center.

		Rel. Cu wt%	Rel. Ag wt%
FamilieLægerne	Before field-testing	61 ± 4	39 ± 4
Espergærde	After field-testing	56 ± 3	44 ± 3
SWR Wound Care	Before field-testing	64 ± 2	36 ± 2
Center	After field-testing	66 ± 1	34 ± 2

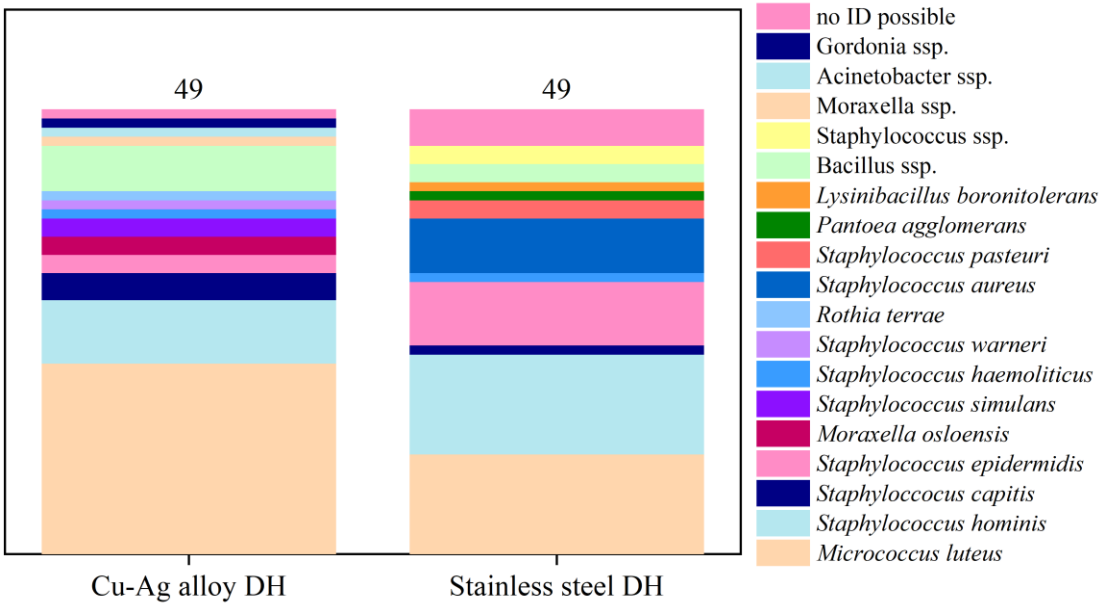

Figure 1. Species abundance of 98 isolates recovered from copper-silver alloy coated and uncoated door handles at FamilieLægerne Espergærde and analyzed by MALDI/TOF. The species are ordered according to their abundance in the column stacks and correspondingly in the legend. Identification to the species level was not possible for Bacillus ssp. and score values below 2.00, thus the genus is reported. It was not possible to recover and identify one isolate from stainless steel door handle due to lack of growth.