New antimicrobial surfaces and intravascular catheters for the prevention of healthcare-associated infections

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If there is no struggle, there is no progress.
Frederick Douglass

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Résumé

Les infections associées aux soins de santé au niveau hospitalier demeurent un problème majeur de santé publique dans le monde entier. Ces infections sont responsables de séjours prolongés à l'hôpital, de coûts plus élevés pour le système de santé, de la nécessité d'utiliser des antibiothérapies à large spectre ou de seconde intention et d'une augmentation de la morbidité et de la mortalité. La contamination et/ou la colonisation des surfaces abiotiques de même que les cathéters intravasculaires sont des importants facteurs de risques pour ces infections.

De nombreuses mesures visant à réduire les contaminations des surfaces abiotiques et la colonisation des cathéters telles que la désinfections appropriées des locaux, l'antisepsie de la peau avant tout acte médical ou encore le lavement des mains avant et après des étapes critiques du soin médical ont été mises en place. Cependant, de telles mesures ont une efficacité limitée et peinent à supprimer complètement les infections associées aux soins. Pour cette raison, la conception de surfaces et des cathéters intravasculaires présentant des propriétés anti-bactériennes sont une stratégie intéressante. Dans ce contexte, cette thèse est focalisée sur la prévention en s'axant sur deux points très importants de la chaîne d'évènements conduisant aux infections nosocomiales.

Dans une première partie, nous avons évalué l'activité antimicrobienne des surfaces recouvertes d'un mince film de cuivre. A cet effet, nous avons utilisé une technique innovante, la pulvérisation cathodique magnétron pour produire des surfaces souples revêtues de cuivre. Par la suite, l'activité antimicrobienne de ces surfaces a été testée contre un large panel de microorganismes (multi)résistants aux antibiotiques dans des conditions d'obscurité et sous exposition à la lumière visible. Le mécanisme de mort cellulaire induite par ces surfaces fut aussi étudié.

Nous avons démontré que ces surfaces permettaient d'éradiquer les bactéries de manière rapide (en quelques minutes) dans l'obscurité, et ceci contre toutes les souches résistantes testées. Le mécanisme serait provoqué par le contact direct entre les cellules microbiennes et la surface filmée de cuivre. En effet, l'activité microbicide perdura en l'absence d'oxygène. De ce fait, les dérivés réactifs de l'oxygène ne peuvent pas être le prétendu mécanisme d'action comme il l'a parfois été supposé. En outre, l'activité des surfaces filmées de cuivre a été évaluée contre

les levures dans l'obscurité ainsi que sous exposition à la lumière visible. Ces expériences ont permis de mettre en évidence une accélération de la fongicidie par la photo-activation basée sur la réaction de l'oxyde de cuivre.

Dans une deuxième partie, la technique de pulvérisation cathodique a été utilisée pour produire deux types de cathéters intravasculaires, le premier revêtu de cuivre (cathéter de première génération) et le deuxième revêtu de cuivre et d'argent (cathéter de seconde génération). Dans cette continuité, nous avons évalué la capacité des cathéters de première et seconde génération à prévenir leur colonisation par le *Staphylococcus aureus* résistant à la méticilline (SARM), responsable des infections chez certains patients hospitalisés. Ces tests furent établis dans un modèle *in vitro* puis *in vivo* chez le rat, dans le but de simuler de façon la plus proche possible l'infection chez l'humain.

La première génération de cathéter (cathéter revêtu de cuivre) est parvenue *in vitro* à prévenir la colonisation par le SARM de manière significative. Néanmoins, *in vivo*, les cathéters revêtus de cuivre se sont avérés peu efficaces à prévenir l'infection. Pour cette raison, des cathéters de seconde génération furent testés. *In vitro*, les cathéters revêtus de cuivre et d'argent ont pu totalement empêcher la colonisation par le SARM. En outre, dans le modèle *in vivo*, l'incidence de l'infection fut réduite. Nous avons suggéré qu'une bactéricidie plus élevée serait engendré par une synergie entre le cuivre et l'argent. Cependant, bien que le taux d'infection fût réduit, il ne fut pas complétement aboli. Ceci pouvant s'expliquer par le phénomène d'absorption des protéines plasmatiques sur les cathéters, formant une couche qui empêcha le contact entre les cellules bactériennes et la surface de ce dernier.

En conclusion, nos résultats nous poussent à croire que la technologie de pulvérisation cathodique est une très bonne approche pour concevoir des surfaces ainsi que des cathéters intravasculaires à caractère antimicrobien afin de limiter le risque d'infections associées aux soins.

Mots-clés: Cuivre; Oxyde de cuivre; Argent; Technique de pulvérisation cathodique magnétron; Surface de polyester revêtue de cuivre; Cathéters intraveineux tridimensionnels; Infections associées aux soins de santé; Infections liées aux cathéters; Activité antimicrobienne; Pathogènes multi-résistants aux antibiotiques; Mort cellulaire induite par le contact; Les dérivés réactifs de l'oxygène; Lumière visible

Abstract

Healthcare-associated infections (HAIs) remain an important health issue around the world. The consequences of HAIs incidence include prolonged hospital stays, increased resistance of microorganisms to antimicrobials, higher costs for health systems and for the patients, and an increase in morbidity and mortality. Microbial and fungal contamination and colonization of abiotic surfaces and intravascular catheters (IVCs) are major risk factors that increase the incidence of HAIs.

A number of measures aimed at reducing the contamination of abiotic surfaces and catheter colonization have been implemented, such as surface cleaning and disinfection, hand hygiene and the use of sterile barrier precautions. However, such measures have limited efficacy and have failed to eradicate completely HAIs. For this reason, an attractive approach to overcome this issue is to design surfaces and IVCs which exhibit self-disinfecting activity. In that context, this thesis focused on these two important causes responsible for the high incidence of HAIs and their prevention.

First, the antimicrobial activity of copper-coated surfaces was assessed. To this aim, we used an innovative coating technique, direct current magnetron sputtering (DCMS), to generate flexible copper-sputtered polyester surfaces (Cu-PES). Then, the antimicrobial activity of these surfaces was tested under dark and visible light irradiation conditions against a broad spectrum of antimicrobial resistant (AMR) pathogens.

In addition, we investigated the mechanism of the killing process of microbial cells caused by those surfaces. It was shown that flexible Cu-PES have a rapid bactericidal activity (within minutes) under dark conditions against all tested AMR bacteria, which was likely induced by a contact killing process. Indeed, it was also demonstrated that the antimicrobial activity exerted by the Cu-PES persisted in the absence of oxygen. Therefore, the generation of reactive oxygen species (ROS) could not be the underlying mechanism of killing, as previously suggested. Then, the activity of Cu-PES was assessed against yeast under dark and visible light irradiation conditions. We showed that the photo-activation of those surfaces led to an acceleration of the fungicidal activity compared to the activity under dark conditions. The faster antifungal effect induced by visible light was due to the semiconductor Cu_2O/CuO charge separation.

Second, DCMS technology was used to generate copper (Cu)-coated IVCs (first generation of catheters) and silver-copper (Ag/Cu)-coated IVCs (second generation of catheters). The ability of these two generations of coated-IVC to prevent catheter colonization by methicillin-resistant *Staphylococcus aureus* (MRSA), a major culprit of catheter-related bloodstream infections in hospitalized patients, was assessed both *in vitro* and *in vivo*, in a rat model of intravascular catheter infection to closely mimic the infection occurring in humans.

First generation catheters (Cu-coated) showed a significant efficiency *in vitro* in preventing catheter colonization by MRSA. Nevertheless, Cu-coated catheters were less effective to prevent *in vivo* infection caused by MRSA. As the results of the activity of the first generation of catheters were unsatisfactory, a second generation coated-catheters (Ag/Cu-coated catheters) were tested. Ag/Cu-coated catheters completely prevented MRSA colonization *in vitro*. In addition, they reduced the rate infection *in vivo*. The higher antimicrobial activity was likely due to a synergistic effect between silver and copper. However, even if the infection rate *in vivo* was reduced, it was not fully prevented. This was explained by the adsorption of plasma proteins onto the catheters, which created a sheath that inhibited catheter-bacterial contact.

Overall, these findings suggest that DCMS technology is a suitable strategy to design novel antimicrobial surfaces and coated-IVC for preventing HAIs.

Keywords: Copper; Copper oxide; Silver; Direct Current Magnetron Sputtering; Copper-polyester surface; Tridimensional-coated intravenous catheters; Healthcare-associated infections; Catheter-related infections; Antimicrobial activity; Multidrug-resistant pathogens, Contact killing; Reactive Oxygen Species; Actinic light

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List of Abbreviations

Ag:	Silver
Ag^+:	Silver ions
$AgNO_3$:	Silver nitrate
AgNPs :	Silver nanoparticles
AgSD:	Silver sulfadiazine
AMP:	Antimicrobial peptide
AMR:	Antimicrobial-resistant
BC:	Before Christ
CAUTI:	Catheter-associated urinary tract infection
CDC:	Center for disease control and prevention
CFU:	Colony forming units
CHSS:	Chlorhexidine-silver-sulfadiazine
CLASBI:	Central line-associated bloodstream infection
CoNS:	Coagulase-negative staphylococci
Cu:	Copper
Cu-PES:	Copper-sputtered polyester
DCMS:	Direct current magnetron sputtering
DFI:	Diabetic foot infection
DNA:	Deoxyribonucleic acid
DRS:	Diffuse reflectance spectroscopy
ESBL:	Extended spectrum β-lactamase
HAIs:	Healthcare-associated infections
HCWs:	Healthcare workers
HPV:	Hydrogen peroxide vapor
ICP-MS:	Inductive coupled-plasma mass spectroscopy
IVC:	Intravascular catheter
MDR:	Multidrug-resistant
MRSA:	Methicillin-resistant *Staphylococcus aureus*
MW:	Molecular weight
PEG:	Polyethylene glycol
PES:	Polyester
PPE:	Personal protective equipment

PVD: Physical vapor deposition

ROS: Reactive oxygen species

RT: Room temperature

SARM: *Staphylococcus aureus* résistant à la méticilline

SBP: Sterile barrier precaution

SEM: Scanning electron microscopy

TEM: Transmission electron microscopy

TiO_2: Titanium dioxide

USA: United States of America

UV: Ultra-violet

VAP: Ventilator-associated pneumonia

VRE: Vancomycin resistant enterococci

WHO: World Health Organization

XPS: X-ray photoelectron spectroscopy

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General Background

“Never forget to wash your hands after having touched a sick person “

“I dismount from my animal, wash my hands, go forth to my patients”

Moses Ben Maimon (1135 Cordova-1204 Cairo)

Medieval philosopher

1. Healthcare-associated infections

Healthcare-associated infections (HAIs), formerly known as nosocomial infections, are defined by the World Health Organization (WHO) as “an infection occurring in a patient during the process of care in a hospital or other health care facility which was not present or incubating at the time of admission” (Allegranzi et al. 2011). The last definition given by the WHO points out that the occurrence of HAIs has moved beyond hospitals to other healthcare facilities, such as ambulatory settings and long-term care and home care facilities (Kleinpell et al. 2008).

HAIs are a major concern of public health around the globe (Allegranzi et al. 2011). Indeed, the Centers for Disease Control and Prevention (CDC) estimate at approximately two million the number of patients affected annually by HAIs in the USA (Klevens et al. 2007; Magill et al. 2014). In Europe, the overall incidence of HAIs on any given day was estimated at 7.1% of patients in acute care hospitals (European Center for Disease Prevention and Control 2012). HAIs lead to a significant mortality and morbidity that incurs a high cost due to the extended length of stay in care facilities (Zimlichman et al. 2013).

1.1. Microorganisms responsible of HAIs

HAIs are caused mostly by bacterial and fungal organisms (Sievert et al. 2013a). Bacteria are prokaryotic and fungi are eukaryotic microorganisms which differ according to their size and composition of their cell envelope. Bacteria are generally smaller than fungi. In bacteria, the main component of the cell wall is peptidoglycan, which is responsible for cellular rigidity. Peptidoglycan is a hetero-polymer comprised of glycans and amino acids that form a layer outside of the plasma membrane. The glycan strands consist in alternation of β1,4-linked N-acetylglucosamine (GlcNAc)

and N-acetylmuramic acid (MurNAc) residues that are cross-linked by short peptides (Vollmer and Bertsche 2008). According to the presence or absence of an outer membrane, bacteria can be classified as Gram-negative or Gram-positive, respectively (Figure 1). In fungi, the key components of the cell wall are β-glucan and chitin (Figure 1).

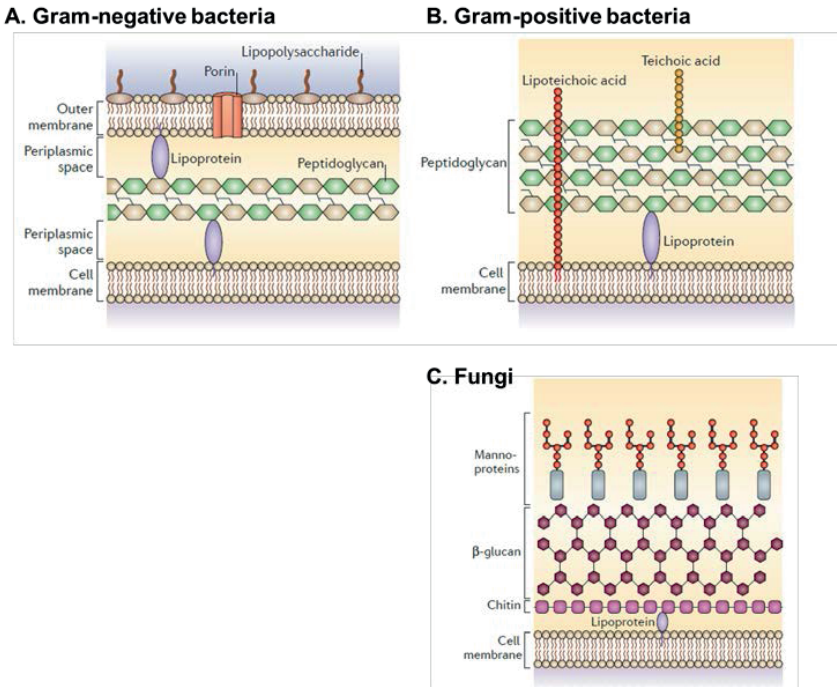

Figure 1. Structure of the cell wall of Gram-negative bacteria (A), Gram-positive bacteria (B) and fungi (C).
Reproduced with permission of Nature copyright 2015 (Brown et al. 2015).

The most pathogenic microorganisms leading to HAIs are described in Table 1. Among them are the Gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* the Gram-positive bacteria *Staphylococcus aureus and Enterococcus spp.* and the yeast *Candida* spp. (Hidron et al. 2008b). Of note, *S. aureus* has been surpassed by *E. coli* during the five past years as the most prevalent agent responsible for HAIs (Sievert et al. 2013b).

Importantly, many of the microorganisms isolated from HAIs can sometimes be antimicrobial-resistant (AMR), such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and Gram-negative bacteria producing extended spectrum β-lactamasees (ESBL) (Hidron et al. 2008b). The acronym "ESKAPE" has been introduced to describe the six major antimicrobial-resistant pathogens responsible of HAIs: *Enterococcus faecium, S. aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter* (Boucher et al. 2009). The increase in antimicrobial resistance among microorganisms causing HAIs, combined with a shortage of new antibiotics challenges the management of HAIs (Pendleton et al. 2013).

Table 1. Distribution of the prevalence of pathogens responsible for HAIs, as reported by the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014.

Pathogens	Number of infections (%)	Rank
Escherichia coli	15.4	1
Staphylococcus aureus	11.8	2
Klebsiella pneumoniae	7.7	3
Coagulase-negative staphylococci	7.7	4
Enterococcus faecalis	7.4	5
Pseudomonas aeruginosa	7.3	6
Candida albicans	6.7	7
Enterobacter spp	4.2	8
Enterococcus faecium	3.7	9
Candida glabrata	2	14

Adapted from (Weiner et al. 2016)

1.2. The source of infectious agents

Microorganisms responsible for HAIs have been localized in different habitats, such as human reservoirs and inanimate environmental surfaces. The human body contains around 10^{14} bacteria. This flora, known as commensal, is present on the skin, in the gastrointestinal tract and on mucous membranes, in humans. Usually, this flora is harmless in healthy individuals (Davis 1996) and even beneficial for precluding colonization by exogenously introduced pathogens (Donskey 2004). Nevertheless, these commensal microorganisms could become opportunistic pathogens under certain conditions and be responsible for disease in hospitalized patients.

HAIs can therefore originate from the patient's own flora (Pfaller 1996). Typically, this is often the case for the infections caused by *S. aureus,* which is present in the nose and on the skin of colonized individuals (van Belkum 2006). In addition, microorganisms of the gastrointestinal tract, such as Enterobacteriaceae, Enterococcus spp., *Clostridium difficile* or Candida spp., can be disseminated in the close environment of the patient due to incontinence and diarrhea, the so called “faecal veneer”, and then be transferred to another patient sharing the same room (Pfaller 1996; Mayer et al. 2003).

HAIs can also originate from the flora of healthcare workers (HCWs). Indeed, HCWs represent human reservoirs of *S. aureus* (Ben-David et al. 2008). For instance, Albrich and Harbath gathered epidemiology data to determine the prevalence of *S. aureus* in HCWs of different institutions in the world and showed that 24% and 4-6% of HCWs to be carriers of methicillin-susceptible *S. aureus* and MRSA, respectively (Albrich and Harbarth 2008). For instance, Pittet et al have described MRSA outbreaks related to colonized HCWs (Pittet et al. 2000). Moreover,

it has been shown that a successful decolonization of HCWs helped in controlling an endemic infection due to MRSA in hospitals (Friedrich et al. 1990).

Contaminated surfaces in the environment of the patient play a major role in the transmission of pathogens (Weber et al. 2013). It is well recognized that patients admitted to a room previously occupied by a patient colonized or infected with a pathogen are at an increased risk of developing colonization or infection with that same pathogen, such as *S. aureus* or *C. difficile* (Boyce 2007; Weber et al. 2013; Freedberg et al. 2016). Indeed, such microorganisms can persist on inanimate surfaces for a long time, and may transiently colonize the hands of HCWs and be transmitted from patient to patient (Kramer et al. 2006; Weber et al. 2013). In addition, personal protective equipment (PPE) of HCWs, such as coats or gowns, can potentially be contaminated due to the frequent contact between HCWs with patients and contaminated surfaces, and further contribute to the dissemination of microorganisms (Pilonetto et al. 2004; Banu et al. 2012).

1.3. Mode of transmission of microorganisms

There are two generally accepted routes of transmission for microorganisms in healthcare facilities:

- Airborne transmission, when the pathogen is transmitted by air and finally inhaled by a patient.
- Contact transmission by direct and indirect contact.

1.3.1. Airborne transmission

The airborne transmission of infecting microorganisms can take place through aerosols and droplets (Ferguson 2009). Aerosol transmission is described as when particles size of ≤5 µm are spread to a distance superior to 1 m, including *Aspergillus* spp. (spores) and rhinovirus (Gralton et al. 2011). Droplet transmission is when the size of the particle is larger and the distance of spreading is smaller.

Airborne pathogens can be transmitted directly from patient to patient. They can also be recovered from an inanimate surface and then transmitted to a new recipient after inhalation. In both cases, the patient could end with a respiratory tract infection. Then, the infecting pathogen can be disseminated to abiotic surfaces or to a new patient (Herfst et al. 2016).

1.3.2. Contact transmission

Contact transmission is the prevalent mode of transmission of healthcare-associated pathogens. Contact transmission can be direct, where there is physical contact between at least two persons to spread the infectious agents, or indirect. Indirect contact transmission is defined as a transfer of the infectious agent to a person from a contaminated inanimate object (Siegel et al. 2007). In this context, bed rails, bed surfaces and supply carts are considered “high-touch” surfaces and, if contaminated, they increase the risk of microbe transmission from patient to patient (Huslage et al. 2010). As stated above, the hands of HCWs are one of the major causes of pathogen transmission to patients either directly or indirectly (Bhalla et al. 2004b).

1.4. The susceptible host

The occurrence and the severity of an infection are closely related to the underlying host condition of the patient. Indeed, risk factors for developing an infection depend, for instance, on the age of the patient, the presence of antimicrobial therapy, any surgical procedure, their immunological status, the presence of an indwelling device and/or the length of hospital stay (Safdar and Maki 2002). For instance, the acidity of the stomach is responsible of the killing of most of the ingested microorganisms. As the age of the patient or treatment such as proton pump inhibitors could modify the gastric acidity, it has been observed that ingested opportunistic pathogens could colonize the patient (Donskey 2004). In addition, patients who underwent a surgical procedure (Sax et al. 2011; Marimuthu et al. 2016) or are immunocompromised (Kamboj and Sepkowitz 2009) are more prone to develop an HAI, even by microorganisms with low pathogenicity potential (Marcel et al. 2008).

The presence of an invasive device, such as intravascular catheters (IVCs), urinary catheters and endotracheal catheters, are a major cause of HAIs, such as central-line associated bloodstream infection (CLABSIs), catheter-associated urinary tract infections (CAUTI) and ventilator-associated pneumonia (VAP) (Gaynes and Edwards 2005). Since IVCs are a leading cause of healthcare-associated bloodstream infections, which have become costly for the health system and lead to important morbidity and mortality (12 to 25% in critically ill patients), (Raad et al. 2007; Leonidou and Gogos 2010), deserve further examination. The interaction between the source and mode of pathogen transmission, the underlying disease conditions of the patients, and the risk of developping a HAI is depicted in Figure 2.

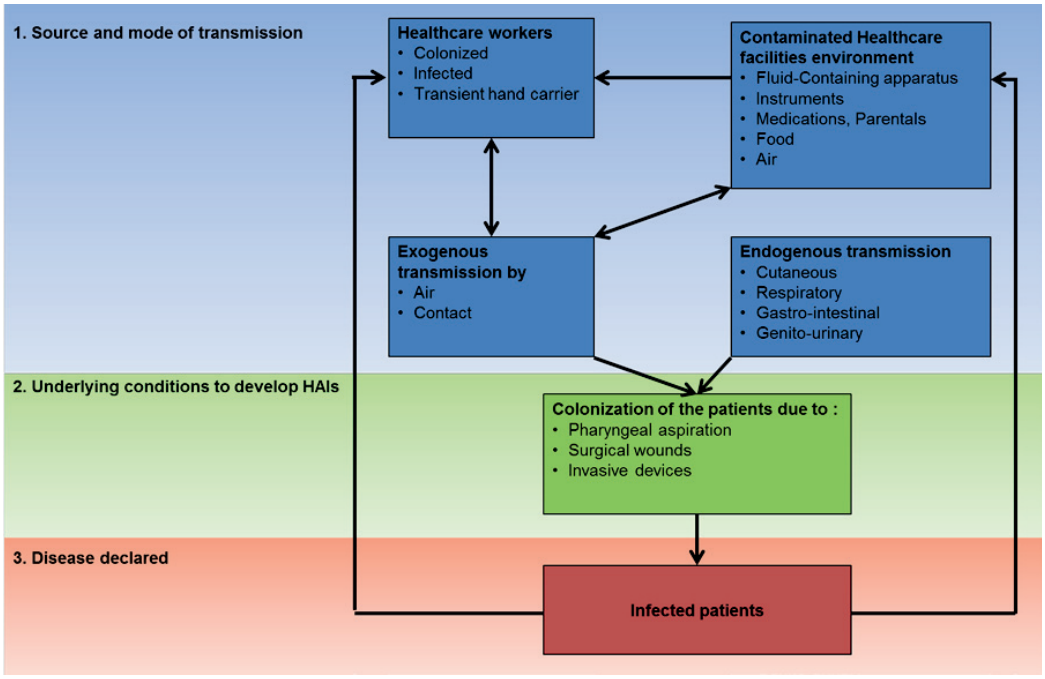

Figure 2. Interactions between the source of infectious agents, the mode of transmission and the host conditions resulting in HAIs.
Adapted from (Otter et al. 2011)

2. Intravascular catheter-related infections: a leading cause of HAIs

Intravascular catheter (IVC)-related infections are an important subset of HAIs (Klevens et al. 2007; Marcel et al. 2008; Sax et al. 2011) and are largely used in healthcare facilities (Raad et al. 2007). They have different applications, such as the administration of drugs and fluids, hemodialysis, or taking blood sample. IVCs include short-term (<14 days) and long-term use catheters inserted either peripherally (venous or arterial) or percutaneously or surgically tunneled (Raad et al. 2007; Leonidou and Gogos 2010; Shah et al. 2013).

IVC-related bloodstream infections comprise mainly CLASBIs. Such infections account for ca. 40% of all HAIs in United States (Sievert et al. 2013b). The high burden of IVC-related bloodstream infections depends on risk factors predisposing its colonization, such as:

- The type of device and its localization (e.g.: subclavian, jugular, femoral) (Parienti et al. 2015)
- The underlying conditions of the patient
- The type of care and the prevention measures taken during insertion (O'Grady et al. 2011a; Gahlot et al. 2014).

Three different pathways can be identified in the development of IVC infections (Figure 3). The first is the contact between the microorganisms present on the skin and the medical device during the time of insertion or later. This leads to the colonization of the external surface of the catheter by the microorganisms present at the insertion site (Eggimann et al. 2004). The transmission of microorganisms from the skin of the patient to the hub of the catheters from healthcare workers hands has also been reported. This leads to the colonization of the hub and the intraluminal surface of the catheter. In that context, colonization is mostly due to the frequent

opening of the hub (Shah et al. 20§13). Colonization of the external surface and the hub are the most preponderant when short-term catheters and long-term catheters are used, respectively. This mechanism is the most common pathway identified in the development of catheter-related infections.

The second pathway of catheter colonization is due to an hematogeneous seeding of microorganisms originating from another site of infection (e.g. gastro intestinal site) (Edgeworth 2009). This route of colonization is rare. Finally, although very rare, catheter colonization could be also due to the contamination of fluids or drugs administered intravenously (Edgeworth 2009; Mermel 2011).

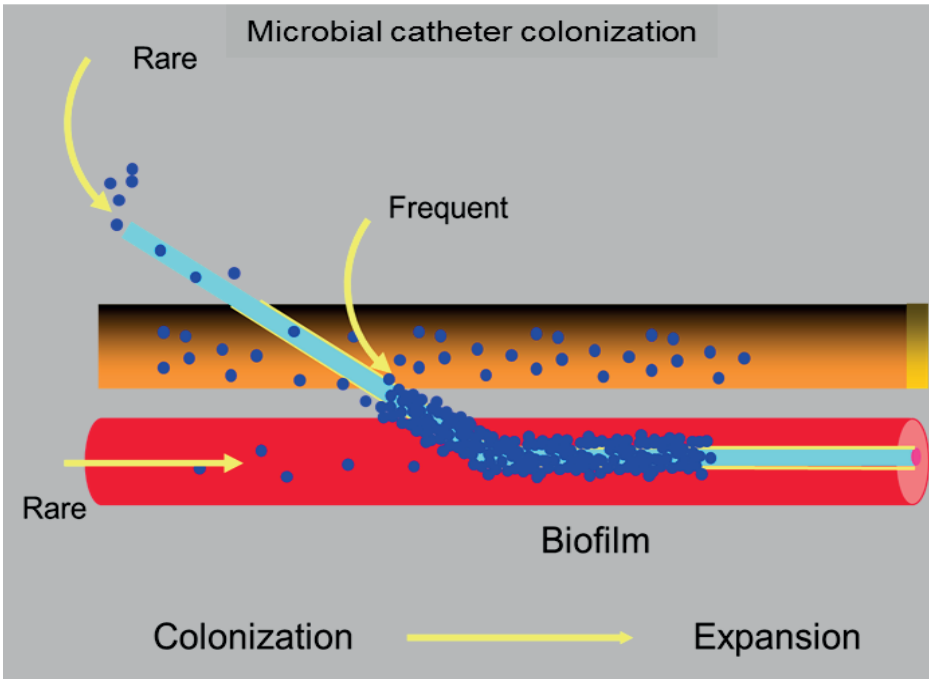

Figure 3. Routes of intravascular catheter colonization.
Courtesy of Pr. P. Moreillon

After colonization of the catheter, microorganisms attached to its surface readily form biofilms. Biofilms are a microbial community embedded in an extracellular polymeric matrix attached to a surface (Donlan 2001). Biofilm formation is enhanced by the presence of host proteins, including glycoproteins, such as fibrinogen, fibronectin, and collagen. Microorganisms in the biofilm are protected against the activity of antibiotics and the innate immune system. Furthermore, the layer of adsorbed glycoproteins may further enhance bacterial adherence, in particular that of *S. aureus* and coagulase-negative staphylococci (CoNS), to the catheter (Katsikogianni and Missirlis 2004; Donlan 2011; Tong et al. 2015).

3. Current strategies for the prevention and control of surface-associated infections and their limitations

Working towards the elimination of HAIs, and more particularly those of catheter-associated bloodstream infections, is a priority, and should be considered as a crucial challenge to improve the quality care for hospitalized patients. Guidelines for the prevention and control of HAIs have been proposed by several committees and institutions such as the Centers for Disease Control and Prevention in USA and the National Health Service in England (Pratt et al. 2007; Talbot et al. 2013). The principal strategies are summarized below.

3.1. Strategies applying to medical material and environmental surfaces

Medical material and environmental surfaces have the potential to expose patients to pathogens. Therefore, disinfection and/or sterilization of medical equipment and cleaning/disinfection of the environment are imperative to prevent the transmission of infectious organisms. In order to achieve an adequate elimination of the microorganisms, it is important to consider the type of patient-care items:

- Critical items (e.g. surgical instruments, urinary and vascular catheters, and implants) are required to be sterilized, as they may be in contact with a sterile tissue. Sterilization destroys all microorganisms including spores.
- Semi-critical items (e.g. respiratory therapy and anesthesia equipment, gastrointestinal endoscopes) demand a high level of disinfection as they might enter in contact with mucous membranes or non-intact skin. The activity of high-level disinfectants covers all microorganisms except spores.
- Non-critical items, i.e., those that can come in contact with intact skin, but not mucous membranes (e.g. bed rails, blood pressure cuffs, surfaces) need a low level

of disinfection. Low-level of disinfection is active against some bacteria, fungi and viruses, but not against mycobacteria and spores. (Abreu et al. 2013; Rutala and Weber 2013b).

Traditional sterilization and disinfection for critical to non-critical items involves the use of different methods (Boyce et al. 2011; Abreu et al. 2013; Rutala and Weber 2013b). The advantages and the limitations of these methods are summarized in Table 2.

Table 2. Advantages and limitations of sterilization and disinfections methods

Disinfectant methods	Advantages	Limits
Sterilization for critical items		
Steam	Nontoxic for patients, HCWs and environment Cycle easy to control and monitor Rapidly microbiocidal Penetrates medical packing	Deleterious for heat-sensitive instruments May leave instruments wet, causing them to rust
Hydrogen peroxide vapor (HPV)	Safe for the environment and HCWs Fast cycle time Compatible with heat and moisture sensitive items	Limited in applications (e.g. liquid, powders and medical devices with a lumen diameter <1mm) Requires synthetic packaging
Ethylene oxide gas	Penetrates medical packaging and many plastics Compatible with most medical materials Cycle easy to control and monitor	Potential hazards to staff and patients Lengthy cycle/aeration time Toxic, carcinogenomic and flammable
High-level of disinfection for semi-critical items		
Glutaraldehyde	Relatively inexpensive Excellent material compatibility	Respiratory irritation Coagulates blood and fixes tissue to surfaces
Hydrogen peroxide	No activation required No odor or irritation issues Fast acting high-level disinfectant	Requires removal of the patients and HCWs Material compatibility concerns Serious eye damage with contact
Ultra-Violet (UV) exposure	Air-purifying systems Active against spores Inactivation of microorganism on surfaces	Requires removal of the patients and HCWs High acquisition cost
Low-level of disinfection for noncritical items		
Alcohol	Effective against vegetative bacteria, fungi and viruses	Not active against spores
Chloro-compounds (e.g. sodium hypochlorite)	Effective in removing biofilms on surfaces Demand short time exposure	Corrosive to metals Might be inactivated by organic matter Formation of potential carcinogenic compounds Resistance
Quaternary ammonium compounds (e.g. benzalkonium chloride)	Surface compatible Antimicrobial also against enveloped viruses	Not sporicidal Could result in asthma Resistance

Adapted from (Abreu et al. 2013; Rutala and Weber 2013b; Rutala and Weber 2013a)

3.2. Strategies applying to healthcare workers and patients

The hands of hospital staff are one of the major causes of pathogen transfer from patient to patient (Boyce and Pittet 2002; Sax et al. 2007). Therefore, hand washing has been defined as compulsory at five times points, the so called “My five moments for hands hygiene”:

- Before patient contact
- Before an aseptic task
- After body fluid exposure
- After patient contact
- After contact with patient surroundings.

In the case of catheter insertion for example, hand hygiene should be performed before and after any manipulation of the catheter and the catheter site (O'Grady et al. 2011a). Recommended practices for hand hygiene include the use of alcohol-based hand rubs or the use of conventional soap (Boyce and Pittet 2002; Sax et al. 2007).

Other measures, such as cutaneous antisepsis, decontamination of the oropharynx and the digestive tract of patients, as well as nasal decontamination in *S. aureus* carriers, have also been shown to contribute to prevent HAIs (Bode et al. 2010). Finally, the use of personal protective equipment (e.g. gloves, gowns, and face mask), the use of maximum sterile barrier precaution (SBP) and the isolation of infected patients are also important measures to limit the spread of infections by HCWs and by the patient (Collins. 2008 Apr.). Such preventive measures have contributed to decrease the frequency of HAIs. Nonetheless important efforts are still mandatory to decrease the incidence of HAIs. One of the issues is in part related to the lack of compliance of HCWs to strictly apply infection control programs. For

example, in hospitals overcrowded with patients and understaffed personnel, compliance cannot be implemented effectively and this results in poor adherence to hand hygiene guidelines and a poor outcome in patient safety (Pittet 2001; Collins 2008). In addition, dressing changes can be associated with moisture and therefore promote microbial proliferation (Eggimann et al. 2004). Finally, it has also been reported that the use of SBP during catheter insertion is not totally effective and failed to prevent catheter colonization (Rijnders et al. 2003).

In conclusion, the implementation of infection control programs in many hospitals has been insufficient to eradicate HAIs. This highlights the need to consider new technologies and products to control HAIs and/or minimize their transmission.

4. Emerging strategies for the prevention of surface-associated infections using antimicrobial surfaces

Microbial surface contamination and colonization is known to be a major risk factor of HAIs (Katsikogianni and Missirlis 2004). Pathogens can persist on hospital surfaces, like plastic, polyester or stainless steel, since they do not have antimicrobial activity (Neely and Maley 2000; Kusumaningrum et al. 2003). In recent years, the development of surfaces coated with antimicrobial agents (e.g. antibiotics) or metals (e.g., titanium oxide, silver and copper) possessing antimicrobial activity, has been recognized as an important strategy for surface disinfection and to mitigate the risk of microbial cross-transmission in the hospital environment (Abreu et al. 2013; Campoccia et al. 2013).

The physico-chemical concept of antimicrobial surfaces is based on two main modes of action: first, repulsion, formerly known as antifouling system, and second, contact-killing (Hasan et al. 2013).

Antimicrobial surfaces are based on the alteration of their microstructure and can be processed by polymerization or coating (Hasan et al. 2013). Surface polymerization can be achieved by different ways, such as covalent bonding. For instance, quaternary ammonium compounds are suitable for immobilization on polymer surfaces. Their mechanism of killing is due to the accumulation of the compounds into the cell (Yao et al. 2008). An additional example of polymerization process is given by Lin et al. who chose to immobilize an antimicrobial agent (N-hexylated + methylated high molecular weight polyethylenimine) on woven textiles (cotton, nylon and polyester). Such surfaces were only able to kill ≤ 3 Log_{10} colony forming units (CFU) of a range of bacteria and fungi, (Lin et al. 2003). Since an antimicrobial agent is considered bactericidal or fungicidal when it kills at least

3 Log_{10} CFU of the initial inoculum, the reduction observed with such surfaces was suboptimal. Furthermore, surfaces with immobilized antimicrobial peptides (AMPs) have been described. The mechanism of action of AMPs is the disruption of negatively charged membranes. This depends on the physico-chemical properties of the AMPs such as the number of amino acids, their molecular weight (MW) and their cationic character (Costa et al. 2011). Nevertheless, some resistance mechanisms have been reported in Gram-positive and Gram-negative bacteria. In the case of *S. aureus*, the expression of an AMP sensor system would be involved in the regulation of resistance genes upon contact with AMPs (Li et al. 2007).

Surface coating is a common approach for the manufacturing process of antimicrobial surfaces and is the principal method used to impregnate antibiotics or metals. When in contact with the cell, the antimicrobial surface is toxic and causes cell death by releasing the antimicrobial agents. . One of the approaches is to incorporate antibiotics such as cefazolin, minocycline/rifampin or vancomycin on the surface (Darouiche et al. 1999). These surfaces are effective, but the potential of antimicrobial resistance is a major concern. Therefore, surfaces which are not coated with antibiotics will be preferred. The use of metals for coating can be an alternative strategy. The most frequent metals used to coat abiotic surfaces are titanium dioxide TiO_2, silver (Ag) and copper (Cu).

4.1. Titanium dioxide-coated surfaces

The photocatalytic properties of titanium dioxide (TiO_2) for surface disinfection are well known. Indeed, it has been shown that TiO_2 has a large range of antimicrobial activity against Gram-positive and Gram-negative bacteria, as well as fungi (Muranyi et al. 2010; Visai et al. 2011). TiO_2 is the most widely used photocatalyst material. It is a semiconductor defined by an energetic band, the band-gap, which is between the lower valence band (e_{vb}^-) that is filled by electrons and the higher conduction band (e_{cb}^-) (Foster et al. 2011). The mechanism of action of TiO_2 is based on the passage of electrons from the e_{vb}^- to the e_{cb}^- (Figure 4). Firstly, the promotion of electrons will lead to a hole in the positively charged valence-band (h_{vb}^+). Therefore, generated free electrons are able to migrate within the conduction band. In addition, the holes created may be filled by the migration of an electron from an adjacent molecule (e.g. water (H_2O), leading to the generation of reactive oxygen species (ROS) (Liou and Chang 2012). ROS, including superoxide (O_2^-), hydroxyl radicals ($^{\cdot}HO$) and hydrogen peroxide (H_2O_2), exert an antimicrobial effect by the disruption of the cell membrane and denaturation of DNA (Kim et al. 2013; Yoo et al. 2015).

The aforementioned reaction is possible due to the absorption of photons with equal or higher energy of the band-gap. In the case of anatase (one of the three mineral forms of TiO_2), the energy required is 3.2eV. This is the reason why an effective photo-activation of TiO_2 demands photons in a the ultraviolet (UV) range (Muranyi et al. 2010) (Figure 4). Despite some limitations, such as the activating energy (UV) necessary to promote the catalytic process and the lack of information about the durability of its antimicrobial effect (Visai et al. 2011), TiO_2-coated surfaces appear to be a promising approach against HAIs transmission.

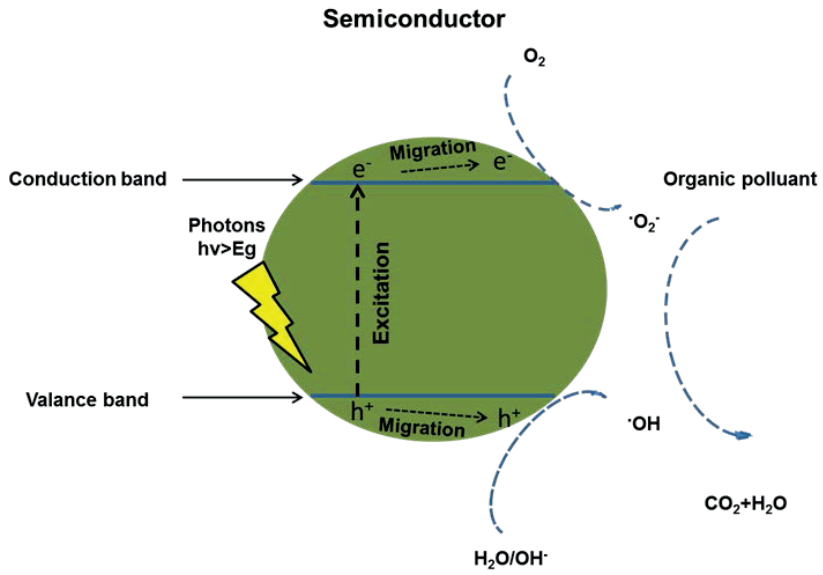

Figure 4. General mechanism of photocatalytic oxidation process.
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4.2. Silver-coated surfaces

Silver (Ag) has been used since ancient times for its medicinal properties and remains useful today for the prevention of water-associated infections and biofilm formation (Vonberg et al. 2008), as well as for the treatment of wound infections, especially in burn patients (Atiyeh et al. 2007). Different chemical forms of silver have been investigated for their antimicrobial activity, e.g., 0.5% silver nitrate ($AgNO_3$), silver chloride, 1% silver sulfadiazine (AgSD) and silver nanoparticles (Edwards-Jones 2009).

The renewed interest in silver is due the decline of antibiotic efficacy together with the emergence of metal-coated surfaces in material science. Silver or silver nanoparticles incorporated into solid surfaces resulted in excellent bacterial killing (Chen et al. 2006; Xiu et al. 2012). Citrate-capped/nanosilver particles on titanium surfaces have also exhibited a good antimicrobial activity against both planktonic and sessile *S. aureus* and *P. aeruginosa* (Flores et al. 2013). Moreover, silver

impregnated dressings have re-emerged as a viable treatment option for infections in burn patients. For instance, a silver-incorporated textile (Cliniweave®) showed a rapid reduction (within hours) of MRSA compared with non-impregnated fabrics (O'Hanlon and Enright 2009). Another approach to coat antibacterial textiles is to deposit Ag by sol-gel methods, as proposed by Xing et al. In this work, a cotton coat textile was treated with silica sols from water glass and then with a silver nitrate solution. The results showed that the treated textile had an excellent antimicrobial effect and which persisted after laundering against *E. coli* (Xing et al. 2007).

The antimicrobial activity of Ag is based on the release of Ag ions (Ag^+) from metallic silver, silver nanoparticles (AgNPs) and silver salts (Chernousova and Epple 2013). Ag^+ rapidly complexes protein with thiol and amino groups, or with nucleic acids and cell membranes, therefore leading to cell death (Edwards-Jones 2009). The activity of AgNPs depends on their size, which directly influences the surface area in contact with the cell (the greater the surface, the higher the antimicrobial activity) (Maillard and Hartemann 2013). The manufacturing of AgNPs has appeared as a new approach to increase the bioavailability of Ag ions and to extend their antimicrobial activity.

Four mechanisms have been suggested for their action (Dakal et al. 2016):

- The adhesion of AgNPs to the cell envelope, leading to its disruption.
- The internalization of AgNPs in the cell across the cell-wall and to the cytoplasma (the so-called Trojan-Horse mechanism).
- The generation of ROS.
- The modulation of phosphorylation cascades.

However, the challenge is how to deliver and maintain an optimal concentration of ionic silver (Ag^+), the active form of Ag, and how to deal with cytotoxic effects on fibroblasts and keratinocytes (Atiyeh and Hayek 2007).

4.3. Copper-coated surfaces

4.3.1. Copper history

Like silver, copper (Cu) has been used since ancient times for medical applications. In ancient times, copper was used for ornaments and rituals (Hong et al. 1996). Copper was the first metal used by humans, as it can be found in a native state in nature. The discovery of melting led to the emergence of the metallurgic age (3000-1200 BC). Indeed, one of the big advantages of copper was that it could be mixed with tin to form a more solid alloy, bronze (Grass et al. 2011). In 2007, copper consumption was evaluated at around 18 million tons, through a broad range of applications like construction, transport, telecommunications, and electrical wires, due to its conductivity and malleability (Radetzki 2009).

Medical applications of copper came with the Egyptian civilization (2600-2000 BC). Copper was used to sterilize drinking water, and as a medicine to heal infected wounds and burns. Later, around 1000 BC, holistic Ayurvedic medicine employed copper in medical devices. Hippocrates (400 BC) used copper to treat pulmonary

diseases. The use of copper in medicine became widespread in the 19th and early 20th centuries for the treatment of eczema or tubercular infections (Grass et al. 2011). Copper also represents one of the most attractive options to be used for surface coating due to its potent antibacterial, antifungal and antiviral activity. Similar to silver, medical applications of copper are regaining the attention of researchers after the rise of infections due to antibiotic resistant pathogens during the last few decades (Grass et al. 2011).

4.3.2. Copper as a bioelement

Copper, is an essential component for humans and microorganisms, and is involved in numerous metabolic processes, i.e., respiration, iron transport, oxidative stress protection, blood clotting and pigmentation, and as cofactor for reduction-oxydation enzymatic reactions (Solioz et al. 2010). In humans, copper must be attained through food sources, where shellfish and organ meats are the richest sources. The average absorption of copper for an adult ranges between 0.6 and 1.6 mg/day (Linder and Hazegh-Azam 1996). Dissolved copper is poisonous for the cell, which is why it is tightly bound to proteins like metallo-proteins (Dupont et al. 2011). In humans, the alteration of copper transport is also responsible for two genetic diseases: Menkes syndrome, that affects copper levels in the body, that leads to copper deficiency, and Wilson disease, where copper overload can lead to toxicosis (Linder and Hazegh-Azam 1996).

In bacteria, fungi, and viruses, trace levels of copper are also required for their metabolism, but an overload of copper will also lead to toxicity. The level of copper ions in these microorganisms is, therefore, tightly regulated. (Magnani and Solioz 2007).

4.3.3. Copper homeostasis in bacteria

To regulate the level of copper and avoid toxic effects, bacteria developed several mechanisms: extracellular sequestration, relative impermeability of the cellular membrane to copper, scavenging system by metallothionein proteins and export of excess copper. In bacteria, the best characterized systems of copper transport across the cell membranes are those of the Gram-negative *E. coli* and the Gram-positive *Enterococcus hirae* (Magnani and Solioz 2007). In *E. coli*, the excess of copper is managed by the copper-ATPase CopA. This protein is localized in the inner membrane of the bacteria and exports the toxic Cu(I) from the cytoplasm to the periplasm compartment (Rensing and Grass 2003). Another element mediating copper homeostasis is the multi-copper oxidase CueO involved in copper detoxification. Indeed, CueO oxidizes Cu(I) to the less toxic Cu(II) (Grass and Rensing 2001). In *E. hirae*, the homeostatic system is managed by the *copYZAB* operon, which encodes the protein products CopY and CopZ that act as repressors or activators of the *cop* operon, respectively, and the copper export ATPases, CopA and CopB (Solioz and Stoyanov 2003; Solioz et al. 2010). The copper-homeostasis gene regulation in bacteria has been recently reviewed in detail (Rademacher and Masepohl 2012).

4.3.4. Copper as an antimicrobial agent

The exact killing mechanism of microorganisms by copper is not completely understood (Grass et al. 2011). Copper is a transition metal, which can exist under two redox states, Cu(I) and Cu(II) (Dupont et al. 2011). Due to its redox properties, it has been suggested that the antimicrobial activity of copper is associated with the generation of ROS, by the catalysis of the Fenton-like reaction (see reaction 2 below) (Jomova and Valko 2011).

$$Cu^{2+} + O_2^{-} \text{ – } O_2 + Cu^{+} \quad (1)$$

$$Cu^{+} + H_2O_2 \text{ – } HO^{\cdot} + HO^{-} + Cu^{2+} \quad (2)$$

The reactive hydroxyl radical ($HO^{\cdot}$) reacts with lipids, proteins and DNA (Halliwell and Gutteridge 1992) and would lead to cell death. Nevertheless, since microorganisms attempt to keep a low level of intracellular H_2O_2, it is unlikely that the reaction (2) would induce cell death (Magnani and Solioz 2007). Furthermore, studies have shown that, under anaerobic conditions, the antimicrobial activity of copper was higher than in aerobic conditions (Outten et al. 2001; Macomber and Imlay 2009).

Another suggested killing mechanism of copper is based on the damage induced by Cu(I) and also by Cu(II) on iron-sulfur cluster proteins by binding to Thiol groups and/or displacing iron ions from sulphide ligands (Magnani and Solioz 2007). Macomber and Imlay demonstrated *in vitro* that the fumarase A, an iron-sulfur cluster protein, was altered after exposition to copper ions and in oxygen-independent conditions (Macomber and Imlay 2009).

Finally, the antimicrobial activity of copper also appears to be explained by “contact killing”. Contact killing is defined as “damages on the cell membrane occurring after the contact of the microbe with a copper surface, plus an increase of

intracellular copper ions into the cell" (Weaver et al. 2010; Espirito Santo et al. 2011; Grass et al. 2011; Quaranta et al. 2011). Indeed, Mathews et al. indicated that preventing the physical contact between the Gram-positive *E. hirae* and copper surface by an inert polymer significantly decreased the antimicrobial activity of copper (Mathews et al. 2013). The level of dissolved copper has also been shown to be an important factor for contact killing (Molteni et al. 2010).

4.3.5. Copper as a self-cleaning surface

The antimicrobial activity of copper and copper alloys have been registered at the U.S. Environmental Protection Agency (EPA) since 2008 (Molteni et al. 2010). Copper-impregnated hospital materials, such as Cu-impregnated dressings, Cu-polymeric materials and Cu-gloves, have shown bactericidal activity against healthcare-associated organisms such as MRSA, vancomycin-resistant enterococci (VRE) and *A. baumannii*, as well as fungicidal activity against Candida spp. (Borkow and Gabbay 2004; Gant et al. 2007; Mehtar et al. 2008; Eser et al. 2015). Importantly, these products did not have skin-sensitizing properties (Borkow et al. 2010b). The safety and the "self-sterilizing" properties of copper fabrics suggest that they could be implemented in the hospital setting to reduce the transmission of infectious microorganisms by healthcare workers. In the last few years, several studies have reported the use of Cu-coated surfaces in clinical settings (Casey et al. 2010; Karpanen et al. 2012; Schmidt et al. 2012; Schmidt et al. 2013). Overall, these studies demonstrate that the use of Cu-coated surfaces reduces bacterial viability by 80-90% in comparison to control surfaces (Schmidt, MG. 2012). Moreover, recent studies indicate that patients cared for in Intensive Care Unit (ICU) rooms equipped with copper alloy surfaces have a significantly lower incidence (50%) of HAIs than

patients treated in standard rooms (Salgado et al. 2013; Schmidt et al. 2016). The antimicrobial properties of Cu-coated surfaces are long lasting and do not diminish by cleaning or oxide formation on the surface (Grass, AEM 2011). Importantly, emergence of bacterial resistance to Cu-impregnated surfaces has not been detected, probably because contact killing is a very rapid process (Grass, AEM 2011). However, the minimum Cu content on surfaces required for antimicrobial activity is still a controversial matter (O'Gorman and Humphreys 2012).

5. Current strategies for the prevention and control of catheter-related HAIs and their limitations

The use of IVCs in patients is often associated with the development of bloodstream infections (CLASBIs), particularly in intensive care units (ICU) (Eggimann, P. Lancet 2000). IVC-related infections are most commonly caused by bacteria from the cutaneous flora, including *S. aureus*, and especially MRSA. (Hidron et al. 2008a; Burton et al. 2009). Different measures have been developed so far to prevent IVC-related infections. As stated above, priority is given to hygiene during catheter maintenance for the prevention of surface-associated HAIs, as well as hand hygiene prior to insertion. Chlorhexidine skin antisepsis and maximal barrier precautions like cap, mask and sterile equipment use during IVC insertion are mandatory (O'Grady et al. 2011a).

Additional strategies to prevent IVCs infection include the use of antiseptic- or antibiotic- impregnated catheters and silver-impregnated catheters.

Antiseptic-impregnated catheters include chlorhexidine-catheters, first generation chlorhexidine-silver sulfadiazine (CHSS) (impregnated only on the external surface) and second-generation CHSS (impregnated on external and internal surfaces). A study by Sherertz et al. showed no benefit of chlorhexidine-impregnated catheters at preventing bloodstream infections in intensive care unit patients (Sherertz et al. 1996). Other studies, in contrast, have demonstrated that the use of antiseptic-impregnated catheters resulted in a significant reduction in the rate of IVC infections as compared to standard catheters (Ramritu et al. 2008; Lorente et al. 2016). For instance, Ramritu et al. have observed that in the adult intensive care unit, the relative risk (RR) of catheter colonization and blood stream infection was lower (RR: 0.66) with the use of chlorhexidine-impregnated catheters compared to the use of uncoated catheters.

Antibiotic-impregnated catheters include principally minocycline/rifampin-, cefazolin- and vancomycin-impregnated catheters. Catheters with a combination of chlorhexidine/minocycline/rifampicin have also been investigated in an *in vitro* study (Raad, 2012). The use of antimicrobial-coated catheters resulted in a significant reduction in the rate of IVC infections as compared to standard catheters. For instance, the use of minocycline/rifampin-impregnated catheters (impregnated in both external and internal surfaces) was superior against staphylococci compared to first generation CHSS catheters (Raad et al. 1996). In an *in vitro study*, Raad et al. have also shown that the efficacy of chlorhexidine/minocycline/rifampin-treated catheters against MRSA, vancomycin-resistant enterococci (VRE) and *Candida* spp. was superior and more prolonged to that of chlorhexidine- and minocycline/rifampin-treated catheters (Raad et al. 2012).

Silver-coated catheters include silver sulfadiazine (SSD) and silver/platinum/carbon incorporated into the catheter. The efficacy of silver-coated catheters appears controversial. While some randomized studies have shown a significant decrease in catheter contamination and catheter-related infections (Andes et al. 1998; Corral et al. 2003), other prospective trials have failed to show any benefit in decreasing catheter colonization and CLBSIs (Chen et al. 2014).

Despite the evidence of the effectiveness of current strategies intended to prevent catheter-related HAIs, several concerns are associated with them. In the case of hygiene measures, the main issue is a lack of compliance (Allegranzi et al. 2013). Concerning the use of antiseptic-, antibiotic- and silver-impregnated catheters, it has been shown that minocycline/rifampin-impregnated catheters are not effective against *P. aeruginosa* or *Candida* spp. (Hanna et al. 2006; Raad et al. 2012). The insufficient activity against MRSA, the risk of selecting chlorhexidine-, minocycline- or

rifampin-resistant bacteria (Batra et al. 2010) and the development of allergic reactions (Guleri et al. 2012) are also major problems. Finally, an additional issue is that the current impregnating methodology offers limited durability of the different coatings (Darouiche et al. 2005; Hanna et al. 2006).

6. Magnetron sputtering and surface characterization

6.1. Magnetron sputtering

Different technologies have been used to deposit metals on textiles, polymer films and plastics used in hospitals. The deposition of thin-films on substrates has been carried out by physical vapor deposition (PVD). PVD is an environmentally friendly technology that works by vaporizing the active antibacterial compound through heating in the PVD-unit and condensing the film on colder sections of the PVD-unit (Kern and Schuegraf 2001).

Magnetron sputtering (Figure 5) is one of the favored processes of PVD for thin film deposition of metals or alloys on a substrate (Helmersson et al. 2006). In the classical sputtering process, a target, also called the cathode, is bombarded by energetic ions generated in a glow discharge plasma in the vicinity of the target. Atoms of the target will be ejected and directed to the substrate (Alfonso 2012). By Direct Current Magnetron Sputtering (DCMS), neutral argon atoms are introduced in the magnetron chamber unit under a vacuum. The applied voltage leads to the ionization of argon atoms and generates plasma, consisting of electrons/atoms/ions from the target. The high energy argon ions dislodge atoms/ions from the metallic or oxide target. These atoms/ions are directed to the substrate (see Figure 5) (Kelly and Arnell 2000). The DCMS induces an increase of ions in a dense plasma in the target vicinity that leads to an higher rate of deposition (Alfonso 2012; Shahidi et al. 2015). The sputtering yield, defined as the number of atoms dislodged from the target by Ar^+, reaches 1-2% for Cu-sputtered by DCMS at an electronic density of 10^{14}-10^{15} e-/m^3 (Helmersson et al. 2006).

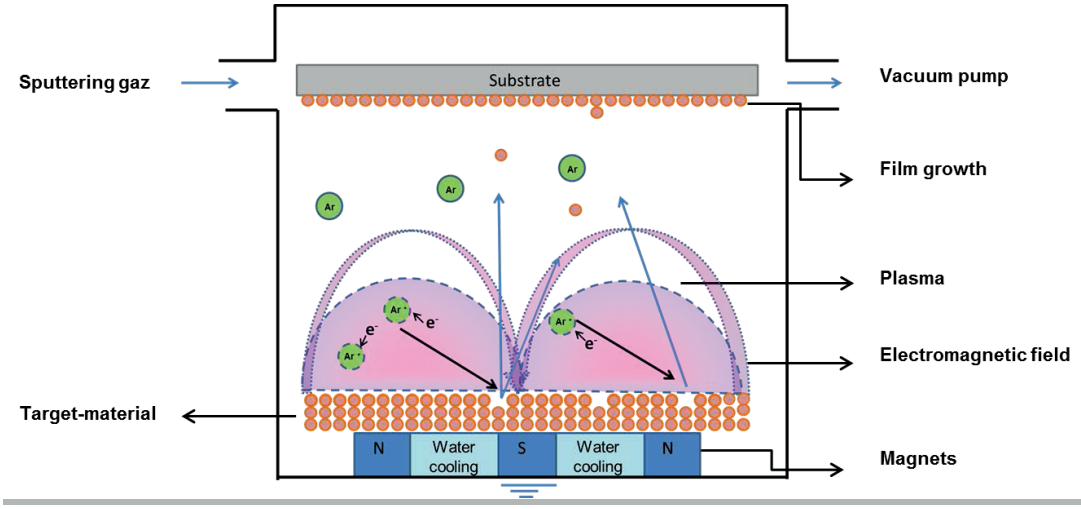

Figure 5. Scheme of the mechanism of sputtering.
Ar stands for argon; Ar^+ stands for argon ions: e^- stands for electrons

6.2. Surface characterization

The principal techniques used to characterize the coated-surfaces are X-ray photoelectron spectroscopy (XPS) analysis and inductive coupled-plasma mass spectroscopy (ICP-MS) (McGuire et al. 1999).

6.2.1. X-ray photoelectron spectroscopy (XPS) analysis

XPS allows surface analysis and the determination, in a non-destructive way, of the surface composition, as well as the electronic environment to a depth of 0 to 2 nm (Turner and Schreifels 2000). XPS spectroscopy is induced by X-rays, leading to the excitation of surface electrons in the substrate. The kinetics and the number of electrons are monitored, which leads to the identification of the elements of the substrate (McGuire et al. 1999).

6.2.2. Inductive coupled-plasma mass spectroscopy (ICP-MS)

The principle of inductively coupled-plasma mass spectrometry is based on the pumping of a liquid sample into a spray chamber. The liquid sample is converted into an aerosol, which is subsequently injected into the plasma and then submitted to vaporization, atomization, and ionization. The excitation of the outer electron of a ground state atom leads to an emission of a particular photon for each element with a defined wavelength. This allows the monitoring of the release of the elements of the coatings during and after the chemical reactions in the ppb range (Thomas 2004).

Research Objectives and Thesis Outline

Bacterial and fungal colonization of abiotic surfaces and intravascular catheters are major causes of healthcare associated infections (HAIs). However, current measures for preventing HAIs have limited efficacy. The global aim of this thesis was to investigate innovative copper (Cu)-coated surfaces and Cu- and silver (Ag)/Cu-coated catheters, in order to prevent their colonization by antibacterial-resistant pathogens and azole-resistant yeast responsible for HAIs.

The specific objectives of this thesis work are:

(I) To evaluate the *in vitro* antimicrobial activity of Cu-sputtered polyester against a broad range of antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE), vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA, respectively), vancomycin-resistant *Enterococcus* (VRE), extended spectrum beta-lactamase (ESBL)-producing enterobacteriaceae, carbapenem-resistant *P. aeruginosa*, quinolone-resistant *A. baumannii* and azole-resistant *Candida* spp.

(II) To evaluate by electronic microscopy the damages and conformational changes to the bacterial and yeast cell structures leading to loss of viability.

(III) To evaluate Cu-coated and Ag/Cu-coated catheters for the prevention of catheter colonization by methicillin-resistant *S. aureus* (MRSA), *in vitro* and *in vivo* in a model of intravascular catheter infection in rats.

Experimental Work

Chapter I.

Evaluation of Copper-Sputtered Polyester Activity Against Multidrug-Resistant Pathogens

This chapter is based on the following publications:

Bactericidal Activity and Mechanism of Action of Copper-Sputtered Flexible Surfaces Against Multidrug-Resistant Pathogens
Myriam K. S. Ballo, S. Rtimi, S. Mancini, J. Kiwi, C. Pulgarin, J. M. Entenza and A. Bizzini
Applied Microbiology and Biotechnology, 2016

And

Fungicidal Activity of Copper-Sputtered Flexible Surfaces Under Dark and Actinic Light Against Azole-Resistant *Candida albicans* and *Candida glabrata*
Myriam K. S Ballo, S. Rtimi, J. Kiwi, C. Pulgarin, J. M. Entenza and A. Bizzini
(Submitted to the Journal of Photochemistry and Photobiology B)

A. Bactericidal Activity and Mechanism of Action of Copper-Sputtered Flexible Surfaces Against Multidrug-Resistant Pathogens

Preface to Article A

In this first article, we were interested in preventing the contamination of polyester (PES) surface environments by developing antimicrobial coated-textiles, using copper (Cu) as an antimicrobial agent and a new coating-technology, Direct Current Magnetron Sputtering (DCMS). DCMS allows the deposition of uniform Cu-films on a substrate, which show good adhesion, and mechanical resistance (Helmersson et al. 2006). Previous work in our laboratory showed that such surfaces have a good bactericidal activity against MRSA (Rio et al. 2012). Based on these results, we extended this work to a larger panel of multidrug-resistant (MDR) Gram-positive and Gram-negative pathogens. In addition, we suggest the mechanism of bactericidal activity mediated by Cu-PES.

Cu-PES inactivated MDR bacteria within minutes. This was likely induced by a contact killing process rather than by the generation of reactive oxygen species (ROS). Indeed, what is generally accepted so far is that Cu-generated ROS are the leading cause of cell death. Indeed, hydrogen peroxide (H_2O_2) produced through the Fenton-like reaction would lead to the production of superoxide (O_2^-) and hydrogen radicals ($HO^{\cdot}$) that would damage the cell by altering lipids, proteins, DNA and other biomolecules (Simpson et al. 1988; Borkow and Gabbay 2009; Lemire et al. 2013) . However, we provided evidence that the antimicrobial activity is due to the intrinsic Cu-cytotoxicity that persisted in the absence of oxygen, therefore challenging the ROS-induced killing hypothesis.

Abstract of Article A

Using direct current magnetron sputtering (DCMS), we generated flexible copper polyester surfaces (Cu-PES) and investigated their antimicrobial activity against a range of multidrug-resistant (MDR) pathogens, including eight Gram-positive isolates (three methicillin-resistant *Staphylococcus aureus* [MRSA], four vancomycin-resistant enterococci, one methicillin-resistant *Staphylococcus epidermidis*) and four Gram-negative strains (one extended-spectrum β-lactamase-producing [ESBL] *Escherichia coli*, one ESBL *Klebsiella pneumoniae*, one imipenem-resistant *Pseudomonas aeruginosa*, and one ciprofloxacin-resistant *Acinetobacter baumannii*). Bactericidal activity (≥3 $\log_{10}$ CFU reduction of the starting inoculum) was reached within 15-30 min exposure to Cu-PES. Antimicrobial activity of Cu-PES persisted in the absence of oxygen against both Gram-positive and Gram-negative bacteria containing elevated levels of catalases. This indicates that reactive oxygen species (ROS) do not play a primary role in the killing process. The decrease in cell viability of MRSA ATCC 43300 and *Enterococcus faecalis* V583 correlated with the progressive loss of cytoplasmic membrane integrity both under aerobic and anaerobic conditions, therefore suggesting that Cu-PES mediated killing is primarily induced by the disruption of the cytoplasmic membrane function. Overall, we present here novel antimicrobial copper surfaces with improved stability and sustainability and provide further insights into their mechanism of killing.

Keywords: Copper; Antimicrobial activity; Multidrug-resistant pathogens, Healthcare-associated infections; Contact killing; Reactive Oxygen Species

1. Introduction

Healthcare-associated infections (HCAIs) are a worldwide problem. In fact, HCAIs are an important cause of morbidity and mortality associated with increased duration of hospitalization and costs (Allegranzi et al. 2011). The problem becomes more critical when HCAIs are caused by multidrug-resistant (MDR) pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) or extended-spectrum β-lactamase-producing (ESBL) enterobacteriaceae, as available treatments are scarce (Sievert et al. 2013a). Copper has widely recognized antimicrobial activity, and its use has been recently proposed to curb the spread of healthcare-associated infections (Salgado et al. 2013). However, issues related to the stability, the operational lifetime of the Cu coatings and the amount of metal required for production thereof have cast doubts on their applicability.

The hospital environment plays an important role in the incidence of HCAIs. Pathogens are able to survive for days or even months on inanimate surfaces (Kramer et al. 2006). Textiles can be a reservoir for bacteria that can be transferred to a susceptible patient by a simple touch through the hands of healthcare workers or visitors (Borkow and Gabbay 2008; Dancer 2009).

Important measures to prevent the spread of microorganisms, such as the personal protective equipment of healthcare workers (*e.g.* gloves and gowns), are not totally effective in eradicating transmission of MDR pathogens and new approaches should be considered (Abreu et al. 2013; Campoccia et al. 2013). For example, the prevalence of nosocomial infections with MDR bacteria in the hospital environment could be limited by the use of self-disinfecting antibacterial surfaces and fabrics able to reduce the bacterial load (Humphreys 2014). In this context, the potential of

coating copper (Cu) on textiles has been recently gaining attention (Borkow and Gabbay 2008; Humphreys 2014).

Cu and alloy surfaces have been reported to kill bacteria, fungi and viruses. Their antimicrobial properties are now well established, and copper has been recently registered at the U.S. Environmental Protection Agency as the first solid antimicrobial material (Grass et al. 2011). Several studies have been conducted to evaluate the antimicrobial efficacy of surfaces composed of Cu or Cu alloys, both *in vitro* and in the clinical setting (Salgado et al. 2013; Schmidt et al. 2013). However, issues regarding the variation in the constituents of Cu alloys and the stability of Cu-impregnated surfaces have raised concerns about the practicality of using Cu as self-sanitation material on flexible surfaces (Torres et al. 2010). Thus, coatings presenting an improved stability and a suitable amounts of Cu may represent an improvement to the present state of the art (O'Gorman and Humphreys 2012). Recently developed uniform, adhesive, Cu-nanoparticle films obtained by Direct Current Magnetron Sputtering (DCMS) appear to satisfy these requirements (Castro et al. 2010). Moreover, we have recently shown that Cu-nanoparticles sputtered on flexible surfaces possess rapid bactericidal activity against a number of MRSA isolates (Rio et al. 2012). Here, we extend this study to a broad range of Gram-positive and Gram-negative MDR pathogens typically involved in HCAIs. Furthermore, we provide insights into the killing process promoted by copper surfaces, which is not initiated by ROS.

2. Materials and Methods

Microorganisms and growth conditions. The microorganisms used in this study are described in the Table 3. Bacteria were stored at -80°C in cryovial bead preservation systems (Microbank; Pro-Laboratory Diagnostics, Richmond Hill, Canada) and streaked onto blood agar plates before each experiment. Single colonies were picked from the blood agar plate, inoculated in either brain-heart infusion broth (BHI; for *S. aureus* or enterococci) and tryptic soy broth (TSB; for all the other bacterial strains) and incubated overnight at 37°C. Chloramphenicol 30 µg/ml was added where indicated. Growth media were purchased from Oxoid (Basingstoke, United Kingdom).

Table 3. Strains used in this study

Strains	Description	References
Gram-positive		
MRSA ATCC 43300	Methicillin-resistant *S. aureus*	(Rio et al. 2012)
VRSA 510	Vancomycin-resistant *S. aureus*	(Kosowska-Shick et al. 2010)
VISA Mu50	Vancomycin-intermediate *S. aureus*	(Hiramatsu et al. 1997)
E. faecalis UCN-41	Vancomycin-resistant	(Entenza et al. 2010)
E. faecalis V583	Vancomycin-resistant	(Sahm et al. 1989)
E. faecium D366	Vancomycin-resistant	(Entenza et al. 2010)
E. faecium VR1	Vancomycin-resistant	This study
MRSE AW67	Methicillin-resistant *S. epidermidis*	This study
Gram-negative		
ESBL *E. coli* 8543	Extended Spectrum β-Lactamase producer	This study
ESBL *K. pneumoniae* 8534	Extended Spectrum β-Lactamase producer	This study
P. aeruginosa UR1156	Imipenem-resistant	This study
A. baumannii H12555	Ciprofloxacin-resistant	(Zanetti et al. 2007)
Others		
E. coli pACYC184	Containing empty vector	(Kullik et al. 1995)
E. coli pGS058	Containing KatG-overexpressing plasmid	(Mancini and Imlay 2015)

Preparation and characterization of Cu-PES. Samples were prepared by sputtering for 160 seconds Cu onto polyester fabric (4 cm^2) by DCMS as previously described (Rio et al. 2012). The Cu was DC-sputtered at temperatures < 130°C on a non-thermal resistant substrate like PES. The Cu-content of Cu-PES was evaluated by X-ray fluorescence (XRF) using a PW2400 spectrometer (PANalytical; Almelo, The Netherlands). DCMS sputtered Cu-PES samples were determined to present loadings of 0.11 % Cu by weight/weight PES.

Antimicrobial activity of Cu-PES. The antimicrobial activity of Cu-PES was evaluated by direct transfer of the samples onto agar-plates, as previously described (Rio et al. 2012). Briefly, overnight cultures were washed 2 times with 0.9% NaCl and serially diluted to the selected concentration. Then, unsputtered-PES (control) and Cu-PES (4 cm^2) were loaded with 20 µl of the tested microorganism, corresponding to an inoculum of 10^6-10^7 CFU. The samples were then incubated at room temperature in a humidified chamber in the dark. At preselected times, the polyester samples (with bacteria facing the agar) were transferred on the agar plates. At preselected times, the polyester samples (with bacteria facing the agar) were transferred on the agar plates. Falcon tubes (BD Biosciences, San Jose, CA, USA) were filled with 50 ml of water and successively applied (0.07 N/cm^2 pressure) onto the polyester for 1 min to induce the transfer of bacteria on the agar plates. After a 24 h incubation period at 37°C, the number of colonies was counted. The detection limit of this procedure was 1 $\log_{10}$ CFU. Antimicrobial activity was defined as being ≥ 3 $\log_{10}$ CFU from the initial inoculum. All experiments were carried out in triplicate.

Live/Dead staining to evaluate membrane damage. Unsputtered and Cu-PES samples were inoculated with 10^8 CFU of MRSA 43300 and incubated for 2 h in a humidified chamber at room temperature. Bacteria were subsequently detached from the polyesters through vigorous vortexing for 3 min in 2 ml saline solution. Bacterial staining was performed using LIVE/DEAD® *Bac*light™ Bacterial Viability kit (Life technologies, OR, USA). SYTO9® is a green nucleic acid fluorescent dye which can cross membranes of both live and dead bacterial cells. Propidium iodide is a red nucleic acid fluorescent dye which can enter only cells with damaged membranes. Therefore, cells with damaged membranes will fluoresce red, while cells with intact membranes will fluoresce green. Cells were observed using an automated inverted

microscope (Leica DMI 4000 B, Heerbrugg, Switzerland). Total magnification used was 63x oil immersion.

Evaluation of the impact of reactive oxygen species (ROS) on Cu-PES killing. The impact of ROS on Cu-PES killing was evaluated by assessing the antimicrobial activity of the surfaces under both aerobic and anaerobic conditions and at various levels of catalase expression. First, anaerobic experiments were performed using MRSA ATCC 43300 and *Enterococcus faecalis* V583 in anaerobic glove box (COY, Michigan, USA) under an atmosphere of 85% N_2, 10% H_2 and 5% CO_2. Anaerobic atmosphere was monitored by a CAM-12 (COY, Michigan, USA) disposable anaerobic indicator. Media and samples (unsputtered and Cu-PES) were pre-incubated in the anaerobic chamber 48 h before use. Second, to evaluate the impact of catalases towards the Cu-PES antimicrobial activity, *E.faecalis* V583 (grown in BHI supplemented or not with 10 µM hemin), *Escherichia coli* pGS058 (harboring a catalase (KatG)-overexpressing plasmid and its parent strain *Escherichia coli* pACYC184 (with empty plasmid) were used.

Visualization of bacteria exposed to Cu-PES by transmission electronmicroscopy (TEM). Microorganisms (10^6 CFU) were loaded on unsputtered and Cu-PES. After 24 h of incubation, bacteria were fixed with a glutaraldehyde solution (EMS, Hatfield, USA) in 2.5% Phosphate Buffer (PB) (Sigma-Aldrich, St Louis, USA). Samples were rinsed in PB buffer and post-fixed with a solution containing 1% osmium tetroxide (EMS, Hatfield, PA, USA) and 1.5% of potassium ferrocyanide (Sigma-Aldrich). Samples were then dehydrated in a graded acetone series (Sigma Aldrich) and embedded in Epon resin (Sigma-Aldrich). Ultrathin sections of 50 nm were cut by Leica Ultracut (Leica Mikrosysteme GmbH, Vienna,

Austria) and deposited on a copper grid (EMS, Hatfield, USA) coated with a polystyrene film (Sigma-Aldrich). Sections were post-stained with 4% uranyl acetate (Sigma-Aldrich). Micrographs were taken with a FEI CM100 (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 80kV with a TVIPS TemCam-F416 digital camera (TVIPS GmbH, Germany).

Statistical analysis. Differences in viable counts between bacteria exposed to unsputtered-PES and Cu-PES, expressed as mean ± standard deviation, were compared using the unpaired Student's t test. All statistical analyses were performed with GraphPad Prism software (version 6.05 for Windows; GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Differences were considered significant when $P < 0.05$ by use of two-sided significance levels.

3. Results

Antimicrobial activity of Cu-PES against a broad range of MDR pathogens. Cell viability of MDR pathogens was assessed after 15, 30 and 60 min incubation on unsputtered and Cu-PES (Fig. 6). As expected, unsputtered polyester did not show antimicrobial activity. In contrast, Cu-PES samples exhibited a high and rapid activity against both Gram-positive and Gram-negative pathogens. For staphylococci, a reduction of the initial inoculum of 2 to 4 $\log_{10}$ CFU after 15 min, and of ca. 5 $\log_{10}$ CFU after 30 min was observed. No detectable growth was observed at 60 min (Fig. 6a). For enterococci, cell counts fell below the detection limit (>5 $\log_{10}$ CFU killing of the initial inoculum) after just 15 min of exposure (Fig. 6b). Similarly, Gram-negative bacterial strains rapidly lost viability and no CFU was observed on the plates after 15 min exposure (Fig. 6c).

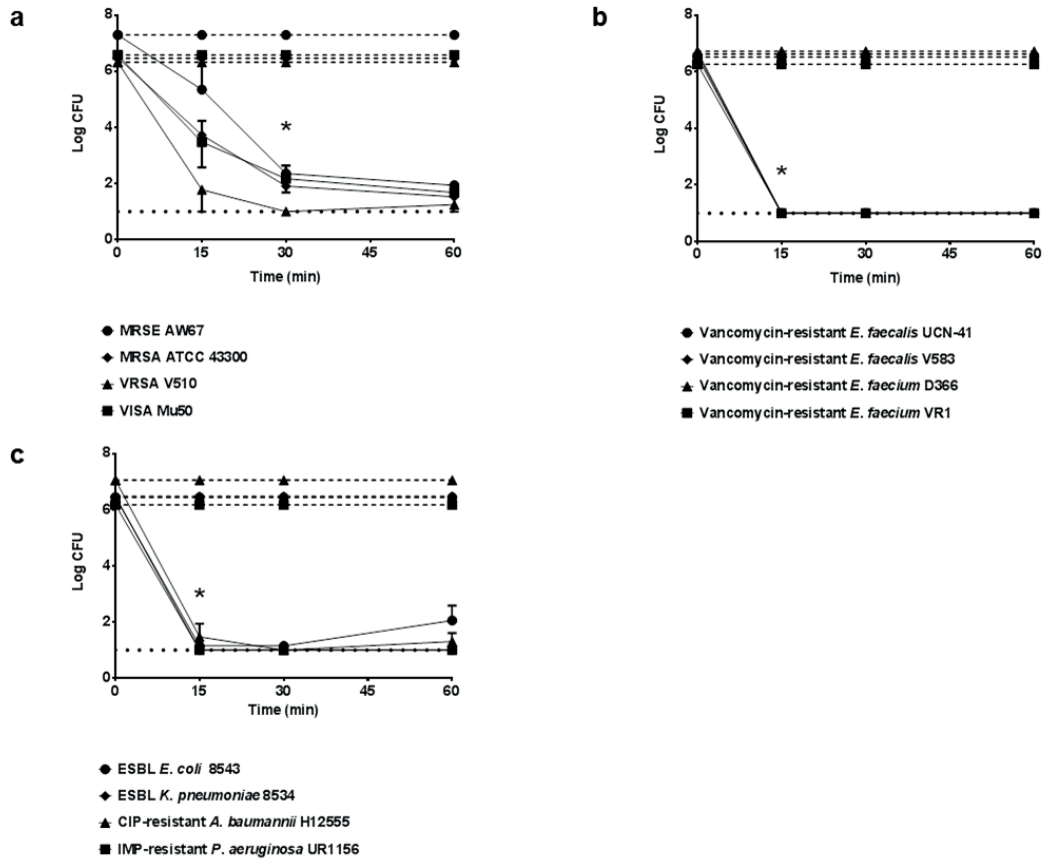

Figure 6. Antimicrobial activity of Cu-PES against a range of MDR pathogens.

Copper-sputtered polyester (Cu-PES) exhibited rapid broad-range antimicrobial activity against MDR-pathogens. 10^6–10^7 CFU of 12 different MDR-pathogens were exposed to unsputtered-PES (dashed lines) and Cu-PES (solid lines). Loss of viability was assessed after 15, 30 and 60 min incubation at room temperature in the dark. Dotted lines represent the limit of detection. * indicates a $P< 0.005$ compared to unsputtered-PES.

Mechanism of Cu-PES killing

***ROS are not primarily responsible for the Cu-PES killing* process**. Oxidative damage of the cytoplasmic membrane has been suggested to be the predominant cause of bacterial killing on Cu-surfaces (Hong et al. 2012; Warnes et al. 2012; San et al. 2015a). This model implicates the copper-induced formation of hydroxyl radicals, which trigger lipid peroxidation and subsequent loss of membrane integrity (Girotti 1998). Hence, since hydroxyl radicals are produced through successive univalent reduction events of molecular oxygen, this process should not take place under anaerobic conditions. To test this hypothesis, the antimicrobial activity of Cu-PES against MRSA ATCC43300 and *E. faecalis* V583 was evaluated in an anaerobic chamber. No significant difference in the killing rates of bacteria grown under anaerobic and aerobic conditions was observed (Fig. 7), suggesting that ROS do not have a primary role in the Cu-PES killing.

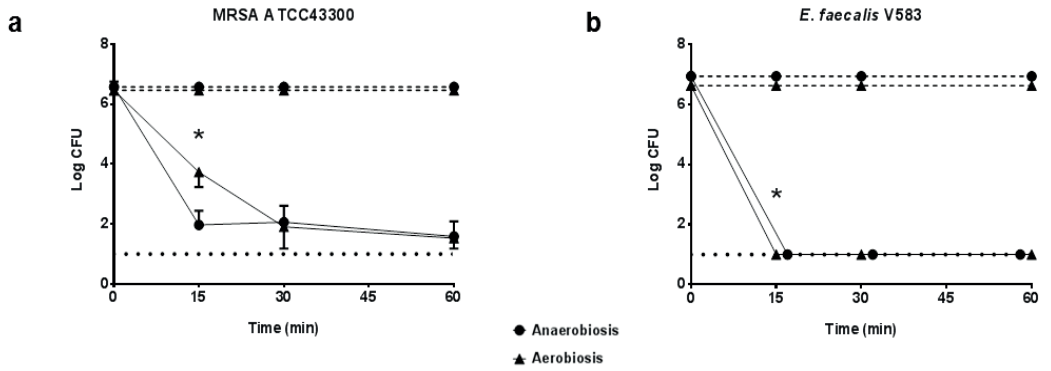

Figure 7. Reactive oxygen species (ROS) are not involved in the copper-sputtered polyester (Cu-PES) killing mechanism.
The viability of (a) MRSA ATCC 43300 and (b) *E. faecalis* V583 exposed to unsputtered-PES (dotted lines) and Cu-PES (solid lines) was assessed after 15, 30 and 60 min of incubation under aerobic and anaerobic conditions at room temperature in the dark. Dotted lines represent the limit of detection.
* indicates a $P < 0.005$ compared to unsputtered-PES.

Intracellular catalase does not protect bacteria from Cu-PES killing. Warnes et al. have proposed oxidative DNA damage to be the underlying mechanism on copper surfaces killing (Warnes et al. 2010). According to this model, ionic Cu^{+1} released from the copper surfaces is taken up by cells and, in the presence of H_2O_2, leads to the generation of deleterious hydroxyl radicals, which in turn induce DNA damage. In this context, induction of catalase by the bacteria, the front-line defense against H_2O_2, would be expected to reduce the bactericidal activity of Cu-PES. To test this hypothesis, *E. faecalis* grown in the presence of hemin, which induces the production of functional catalase (Frankenberg et al. 2002), and an *E. coli* containing an overexpressed catalase G (*E. coli* pGS058) were used (Mancini and Imlay 2015). As shown in Fig. 8a and 8b, no appreciable difference in the killing rates was observed, indicating that catalases do not protect bacteria from Cu-PES killing.

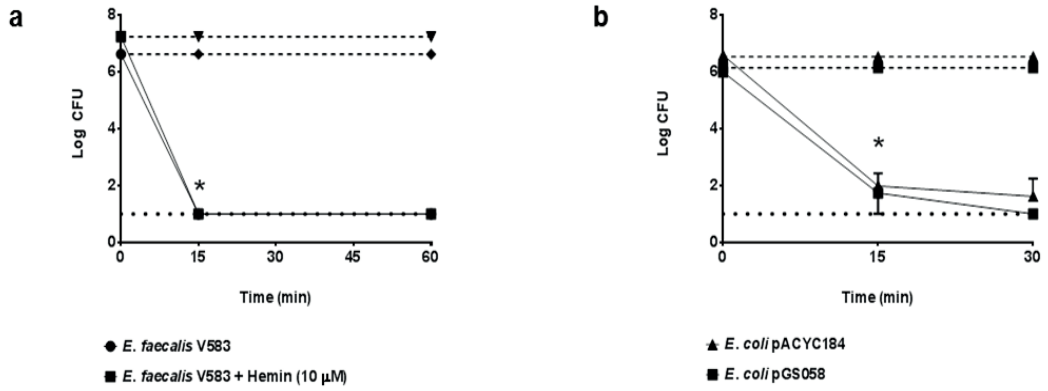

Figure 8. Catalase does not prevent nor attenuate bacterial killing by copper-sputtered polyester (Cu-PES).
Time-kill curves of (a) *E. faecalis* V583 cultivated overnight in hemin-depleted or hemin-containing media to induce the expression of functional catalase, and (b) *E. coli* pACYC184 (empty plasmid) and *E. coli* pGS058 (catalase [KatG]-overexpressing plasmid) on unsputtered-PES (dashed lines) or Cu-PES (solid lines) after 15 and 30 min incubation at room temperature in the dark. Dotted lines represent the limit of detection. * indicates a $P< 0.005$ compared to unsputtered-PES.

Killing is triggered by loss of cell membrane integrity. Previous works have shown that the biocidal activity of Cu surfaces against diverse bacteria is associated with an early disruption of the membrane function (Hong et al. 2012; Warnes et al. 2012; San et al. 2015a). We investigated whether the antimicrobial activity of Cu-PES against MRSA ATCC 43300 and *E. faecalis* V583 correlated with loss of cell membrane integrity under both aerobic and anaerobic conditions using Live/Dead staining. Figs. 9a and 9b show that the number of bacteria stained red on Cu-PES increased over time. After 60 min of exposure, when no viable CFU were found on the agar plate (Fig. 6a and 6b), the totality of bacterial cells were stained red, regardless of the presence or absence of oxygen. These results indicate that substantial damage of the cell membrane is triggered by Cu-PES both under aerobic and anaerobic conditions.

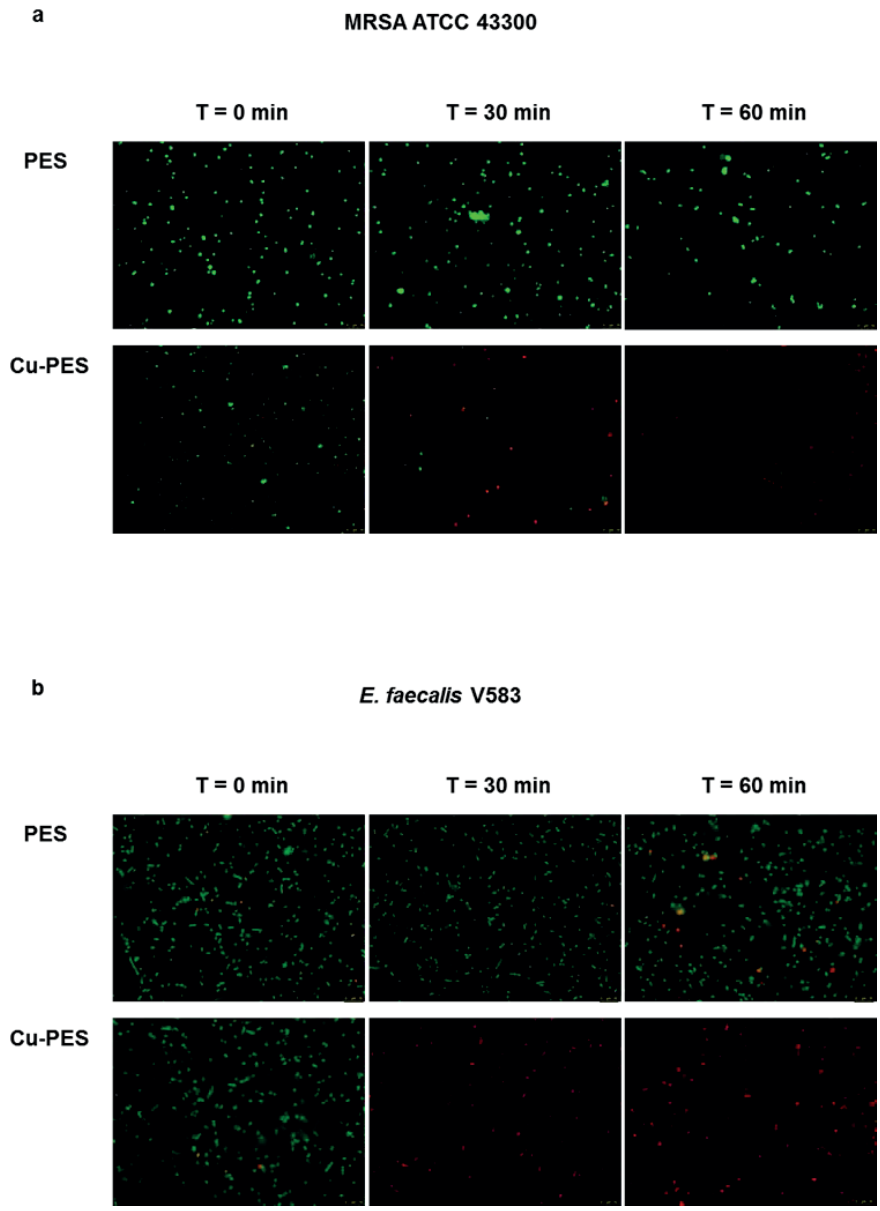

Figure 9. Progressive loss of cell membrane integrity of MRSA ATCC 43300 and *E. faecalis* V583 upon exposure to copper-sputtered polyester (Cu-PES). Bacterial cells (10^6 CFU) were exposed to unsputtered-PES and Cu-PES. At the indicated time points, bacterial cells were stained with the Live/Dead BacLight Assay as described under materials and methods. Cells with damaged membranes fluoresce in red while cells with intact membranes fluoresce in green. Similar results were observed in anaerobic and aerobic conditions.

Ultrastructural cell changes *after* ***Cu-PES exposure***. TEM analysis of MRSA ATCC 43300 and *E. coli* ESBL 8543 was performed to investigate whether the antimicrobial activity of Cu-PES was associated with changes in bacterial cell morphology. The results are depicted in Fig. 10. Both MRSA and *E. coli* not exposed to Cu-PES had a preserved ultrastructure. The cell wall and the cytoplasm displayed a homogeneous electronic density (See Fig. 10a and 10b). By contrast, after 24 h of exposure to Cu-PES, both MRSA and *E. coli* cells showed visible damage of the cell envelope and in the cytoplasm (Fig. 10c and 10d). In addition, bacteria adhered to the deposited copper layer on the polyester fibers. This is consistent with the concept that the contact of bacteria with the copper surfaces is required for their antimicrobial activity (Mathews et al. 2013).

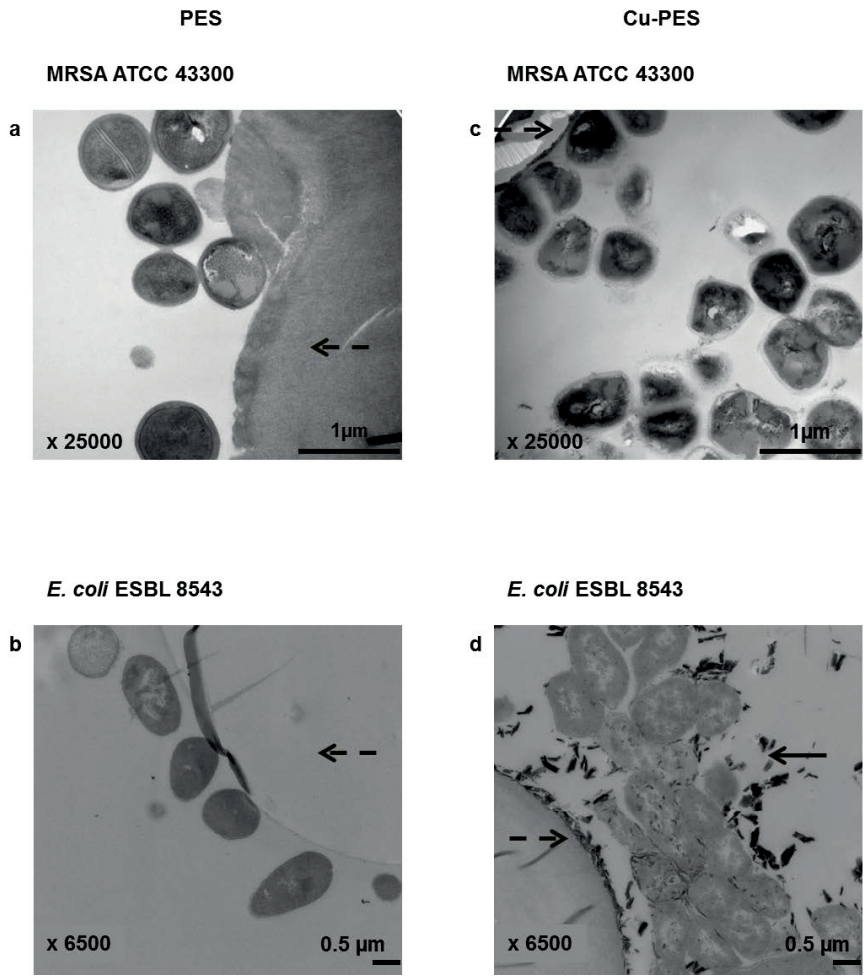

Figure 10. TEM Images of MRSA and *E.coli* exposed 24h with PES and Cu-PES.

TEM images of MRSA ATCC 43300 and *E. coli* ESBL 8543 exposed for 24 h to unsputtered-polyester (PES; a and b) or to copper-sputtered PES (Cu-PES; c and d). Dashed arrows indicate fibers of the polyester and plain arrows Cu-nanoparticles surrounding bacteria. Macroscopic alterations of bacterial morphology appeared after protracted exposure to Cu-PES.

4. Discussion

The prevention of HCAIs remains a major concern. To face this challenge, effective prevention measures have been implemented in hospital settings. Nevertheless, in addition to conventional known practices, alternative approaches should also be sought in order to limit the spread of MDR organisms in healthcare facilities. For instance, the use of Cu-impregnated surfaces and clothing might prove an effective approach to reduce the bacterial load in the hospital environment (Borkow and Gabbay 2008). Cu-coated preparations, however, require significant amounts of copper, present low uniformity, little adhesion and are not mechanically stable over time (Irie et al. 2008).

The Cu-PES presented in this work represents a progress in the field of antibacterial surfaces since the sputtered surfaces are uniform, present good adhesion, are mechanically stable, remain active over time (Kusiak-Nejman et al. 2011) and will not be removed by contact, such as colloidal coatings on PES (Zhang et al. 2012). Moreover, we also showed that Cu-PES possess a broad range antimicrobial activity (within 30 min) against a wide panel of MDR Gram-positive and Gram-negative isolates. This study extends our previous work showing that Cu-PES rapidly killed MRSA strains and provides further evidence that DCMS Cu-sputtered surfaces are effective in reducing pathogen loads in the hospital environment (Rio et al. 2012).

TEM images showing bacterial cell adhesion onto Cu-PES immediately upon challenge suggest that Cu-PES-mediated microbicidal action is exerted by contact, as previously described (Espirito Santo et al. 2011; Mathews et al. 2013). Two different models to explain the process of metal copper contact mediated killing have been recently proposed. One model suggests the cell membrane as the primary

target of toxicity (Hong et al. 2012; Warnes et al. 2012; San et al. 2015a). The second model considers genomic DNA degradation as the underlying mechanism of killing by Cu-surfaces (Warnes et al. 2010; Weaver et al. 2010). This latter hypothesis is still controversial. Indeed, a recent work showed that copper does not catalyse significant DNA damage *in vivo* (Macomber et al. 2007). Moreover, several studies have recently shown that neither higher DNA mutagenicity rate nor extensive fragmentation occurred in Gram-positive, Gram-negative or yeast exposed to copper surfaces (Espirito Santo et al. 2011; Quaranta et al. 2011; Warnes and Keevil 2011; Hong et al. 2012), suggesting that genomic DNA degradation is a consequence, rather than a cause, of metal contact killing.

Both models invoke the involvement of ROS, such as hydroxyl radicals, formed by the Cu(I)-dependent Fenton reaction, as follows:

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + OH^{\cdot}$$

In fact, hydroxyl radicals can damage biomolecules such as DNA, proteins and lipids. Copper-dependent peroxidation of unsaturated fatty acids of the cytoplasmic membrane has often been claimed to be the first step leading to the killing of Gram-positive and Gram-negative bacteria (Hong et al. 2012). According to this model, copper-induced hydroxyl radical formation leads to lipid peroxidation, which results in immediate membrane depolarization followed by inhibition of respiration and ultimately cell death. Consistent with this hypothesis, we also observed a rapid loss of cytoplasmic membrane integrity in *S. aureus* MRSA ATCC43300 and *E. faecalis* V583, which was correlated with the loss of bacterial viability.

The claim that copper-induced ROS trigger the disruption of the cytosolic cell membrane can be challenged on the basis of the present findings. We have shown here that overexpression of the catalase, the predominant scavenger of H_2O_2, did not

reduce the antimicrobial activity of Cu-PES against *E. faecalis* and *E. coli* under aerobic conditions. Moreover, we have demonstrated that anaerobiosis did not affect the killing of Gram-positive bacteria *S. aureus* MRSA ATCC43300 and *E. faecalis* V583 by Cu-PES, and that disruption of the cytosolic membrane also occurred under anaerobic conditions, which correlated with the progressive loss of bacterial viability. These results are consistent with those of Espirito Santo *et al.* (2008) who showed that anaerobiosis did not influence the killing of *E. coli* exposed to copper surfaces (Espirito Santo et al. 2008). Hence, we conclude that although the loss of cytoplasmic membrane integrity likely represents the underlying mechanism of metal-contact killing, such a process is not initiated by oxidative damage exerted by ROS.

What else could be then the target of copper toxicity and induce disruption of the cytosolic membrane? Recent studies have shown that Cu^{2+}-ions markedly modified the number of free SH-groups of the membrane bound ATPase responsible for K^+ transport of *E. coli* and *Enterococcus hirae*, leading to conformational changes in this protein complex and a decrease in its activity (Kirakosyan and Trchounian 2007; Kirakosyan et al. 2008; Vardanyan and Trchounian 2010). The resulting opening of channels of nonspecific conductivity for cations has been proposed to be responsible of change in cytosolic membrane permeability and ultimately cell death. This intriguing model, which might well fit with the fast killing of Cu surfaces, clearly deserves further investigation.

In conclusion, here we present evidence that novel copper surfaces have rapid bactericidal activity against a broad range of MDR bacteria, suggesting that these surfaces could be a valuable method to prevent HCAIs. DCMS technology allowed copper to be incorporated in a stable and cost-effective manner (Lazary et al. 2014), into a variety of frequently touched textiles, such as clothing and bedding (Borkow

and Gabbay 2004). Cu-PES are unexpected to be harmful for medical staff and patients as Cu is a rare sensitizer and Cu values required for bacterial inactivation are below the toxicity level threshold allowed for mammalian cells (Rtimi et al. 2014) . Furthermore, we show that ROS do not play a primary role in the metal contact killing process. This finding contradicts the current model whereby oxidative damage of the cytoplasmic membrane constitutes the underlying mechanisms of killing induced by copper surfaces.

B. Fungicidal Activity of Copper-Sputtered Flexible Surfaces Under Dark and Actinic Light Against Azole-Resistant *Candida albicans* and *Candida glabrata*

Preface to Article B

This second article presents the effect of visible light on the activity of Cu-PES flexible surfaces against azole-resistant *Candida* spp. The present experiments were derived from the results previously reported for bacterial inactivation by Cu-PES in the dark, as well as in pilot studies with *Candida* spp. in the same conditions. These results showed that Gram-negative bacteria are killed more rapidly than Gram-positive bacteria, and that Gram-positive bacteria are killed more rapidly than yeast. Using the semiconductor nature of copper oxide (CuO), we assessed whether photo-induced Cu-PES, using the visible light, improves the fungicidal activity of these surfaces in comparison with their activity in the dark.

The results of the present experiments showed that exposure of Candida spp. to Cu-PES under visible light was followed by a more rapid fungicidal activity (closer to that of bacteria) than in the dark. This is evidence for the semiconductor behavior of CuO. The activity of Cu-PES correlated with changes in the cell envelope and cytoplasm membrane of the yeast, as assessed by transmission electron microscopy (TEM).

Abstract of Article B

We investigated the antifungal activity of Cu-sputtered polyester surfaces (Cu-PES) against azole-resistant *Candida albicans* and *Candida glabrata* under dark conditions and by low intensity visible light irradiation (4.65 mW/cm^2). The surface properties of Cu-PES photocatalysts were characterized by diffuse reflectance spectroscopy (DRS) and X-ray fluorescence (XRF). Under dark, Cu-PES showed a fungicidal activity (≥ 3 $\log_{10}$ CFU reduction of the initial inoculum) against both *C. albicans* DSY296 and *C. glabrata* DSY565 that lead to a reduction of the starting inoculum by 3.1 and 3.0 $\log_{10}$ CFU, respectively, within 60 min of exposure. Under low intensity visible light irradiation, Cu-PES exhibited an accelerated fungicidal activity against both strains with a reduction of 3.0 and 3.4 $\log_{10}$ CFU, respectively, within 30 min of exposure. This effect was likely due to the semiconductor Cu_2O/CuO charge separation. The decrease in cell viability of the two *Candida* strains under dark and light conditions correlated with the progressive loss of membrane integrity. These results indicate that Cu-PES represent an effective strategy for preventing the colonization of surfaces by yeast and that actinic light can improve its self-disinfecting activity.

Keywords: Copper oxide; Cu-PES; Fungicidal activity; Actinic light; Candida spp.

1. Introduction

Healthcare-associated infections (HAIs) are a growing health problem at the worldwide scale (Allegranzi et al. 2011). The hospital environment is a reservoir of pathogens and plays a major role in the incidence of HAIs. Indeed, many healthcare-associated pathogens can survive for a long time on inanimate surfaces, such as door handles, medical equipment or textiles, and may possibly be transmitted to a susceptible patient by a simple touch through the hands of healthcare workers or visitors (Bhalla et al. 2004a; Kramer et al. 2006; Otter et al. 2013).

Candida spp., among them *Candida albicans* and *Candida glabrata*, are commensals of the human mucous membranes and digestive tract, but can also be commonly found on abiotic surfaces in the hospital setting, as well as on health care worker's hands (Traore et al. 2002; Khodavaisy et al. 2011). Candida spp. are the most important fungal agents responsible of HAIs, especially in immune-compromised hosts (Vazquez et al. 1993; Vazquez et al. 1998; Vazquez 2010). The incidence of invasive infections due to *Candida* spp. in hospitals has increased during the last decades, and their treatment has been complicated by the rise of resistance to first line agents of the azole or echinocandin drug classes (Pfaller 2012; Kullberg and Arendrup 2015b). Surface disinfection and cleaning are recommended by international guidelines as a procedure for preventing HAIs (Dancer 2009). Despite considerable progress, however, efficient *Candida* spp. elimination from contaminated surfaces remains an unresolved clinical problem (Fernando et al. 2013). To counteract HAIs due to *Candida* spp., there is a need for development of novel

antimicrobial materials with antifungal properties (Borkow and Gabbay 2008; Campoccia et al. 2013).

The advanced oxidation processes (AOP's), by photocatalysis, have emerged in recent years as a promising approach to be used in bacterial inactivation (Kiwi and Pulgarin 2010). Titanium dioxide (TiO_2) is the most widely used photocatalyst material with a band gap of 3.2 eV, absorbing 4-5% of the visible light above 400 nm and UV-light-under 400 nm. UV < 340 nm is effective for inactivation of microorganisms via cell membrane disruption and DNA damage (Kim et al. 2013; Yoo et al. 2015). Nevertheless, the absorption of TiO_2 mainly in the UV-region is a drawback when the utilization of the widely available solar light is desired to inactivate pathogens (Egerton 2014).

Previous studies have shown that TiO_2 surfaces doped with Copper (Cu) can be photoactivated with visible light of up to 700 nm (Rtimi et al. 2015; Stucchi et al. 2016). Cu-doped TiO_2 (1.7 eV) surfaces have been reported to display bactericidal activity under visible light irradiation against both Gram-positive and Gram-negative bacteria (Karunakaran et al. 2010; Baghriche et al. 2012; Rtimi et al. 2015; Leyland et al. 2016). Cu and Cu-alloy surfaces have intrinsic antibacterial and antifungal activities (Borkow and Gabbay 2009; Grass et al. 2011). In recent years, the coating-technology by DCMS has become more widespread to prepare antibacterial textiles/polymer films. Copper-sputtered polyester (Cu-PES) presents a progress in the field of antimicrobial surfaces over previously used coating, such as colloidal coatings on PES (Zhang et al. 2012) Indeed, Cu-PES presents an adhesive, uniform and stable film on polyester. Furthermore, Cu-PES remains active over time (Castro et al. 2010; Mejia et al. 2010). Since they are mechanically stable, the

Cu-coating cannot be removed by contact during the microbial inactivation process (Castro et al. 2010). Rapid bactericidal activity of Cu-PES has been reported against a variety of nosocomial bacterial pathogens (Rio et al. 2012; Ballo et al. 2016).

Cu deposited on PES is present in the form of CuO (Copper II oxide) and Cu_2O (Copper I oxide) (Kusiak-Nejman et al. 2011). Previous studies have shown that CuO is a semiconductor with lower band gap energy (1.9 eV) than TiO_2 and can therefore absorb visible light (Gawande et al. 2016). The focus of this study was the inactivation by Cu-PES of *Candida* spp. both in the dark and under actinic light conditions used currently in hospitals in the visible region.

2. Materials and Methods

Microorganisms and growth conditions. *C. albicans* DSY296 and *C. glabrata* DSY565 (Coste et al. 2006; Ferrari et al. 2011) were used in this study. Both strains were stored at -80°C in cryovial bead preservation systems (Microbank; Pro-Laboratory Diagnostics, Richmond Hill, Canada) and streaked onto blood agar plates before each experiment. Cultures were prepared by inoculating a single colony from the blood agar plate into Sabouraud dextrose broth (Oxoid, Basingstoke, United Kingdom) followed by overnight incubation under agitation (180 rpm) at 37°C.

Cu-PES preparation and characterization. Polyester (PES) was obtained from the Swiss Federal Laboratories for materials Science and Technology (EMPA). Test clothe sample No 407 was a polyester Dacron polyethylene terephthalate, type 54 spun, 130 microns thick, plain weave ISO 105-F04. Cu-PES samples were prepared by sputtering Cu for 160 seconds onto polyester (4 cm^2) at temperature < 130°C by DCMS, as previously described (Rio et al. 2012). Coating layers of CuO (Copper II oxide) and Cu_2O (Copper I oxide) were deposited on PES (Kusiak-Nejman et al. 2011). The Cu-content of Cu-PES was evaluated by X-ray fluorescence (XRF) using a PW2400 spectrometer (PANalytical; Almelo, The Netherlands). The optical absorption of Cu on the sputtered polyesters samples was assessed by diffuse reflectance spectroscopy (DRS) with a Lambda 900 UV-VIS-NIR spectrometer (Perkin Elmer; Waltham, MA) within the wavelength range of 200-800 nm.

Killing activity of Cu-PES in the dark and under actinic light. The activity of Cu-PES was evaluated by direct transfer of samples onto agar plates, as previously described [30]. Briefly, overnight cultures of *C. albicans* DSY296 and *C. glabrata* DSY565 were washed 2 times with 0.9% NaCl and serially diluted to the required

concentration. Then, unsputtered-PES (control) and Cu-PES were loaded with 20 µl of the tested strains, corresponding to a final inoculum of ca. 107 CFU. Inoculated coupons were then incubated for 15, 30, or 60 min in a humidified chamber at room temperature (20-23^{o}C), in the dark or under actinic light. To measure the activity of PES and Cu-PES under light, a light box equipped with tubular actinic lamps (Master TLD-18W/865, Philips, The Netherlands) with a visible emission spectrum between 400 and 700 nm and generating a light intensity of 4.65 mW/cm^2, was used. The temperature inside the light box was maintained at 20°-23°C by a ventilation system. At each time point, agar plates were separated in four quadrants and the polyester coupons placed in each quadrant with fungi facing the agar. Falcon tubes (BD Biosciences, San Jose, CA) filled with 50 ml of water were successively applied (0.07 N/cm^2 pressure) onto the polyester for 1 min to induce the transfer of fungi on the agar plates. After 48 h of incubation at 37°C, the viable count was determined. The detection limit of this procedure was 1 log10 CFU. Fungicidal activity was defined as a reduction of ≥ 3 $\log_{10}$ (99.9%) CFU of the initial inoculum. Experiments were performed in duplicate and repeated at least three independent times.

Contrast phase and fluorescence microscopy. The effects of Cu-PES on Candida structure were assessed by contrast phase and fluorescence microscopy. Cu-PES and unsputtered-PES samples were inoculated with 10^8 CFU of either *C. albicans* DSY296 or *C. glabrata* DSY565 and incubated for 15, 30 and 60 min in a humidified chamber at room temperature in the dark and under actinic light. The yeast cells were subsequently detached from the coupons through vigorous vortexing for 3 min in 1 ml of NaCl. Cell staining was performed using the LIVE/DEAD® BaclightTM viability kit (Life technologies, Carlsbad, CA). This kit contains SYTO9™ and propidium iodide. SYTO9® is a green nucleic acid fluorescent dye which can cross

membranes of both live and dead microorganism cells and stains cells green. Propidium iodide is a red nucleic acid fluorescent dye that only enters cells with damaged membranes and stains cells red. Thus, cells with intact membranes will fluoresce green while cells with damaged membranes will fluoresce red. Cells were observed using an automated inverted microscope (Leica DMI 4000 B, Heerbrugg, Switzerland).

Visualization of yeast exposed to Cu-PES by transmission electron microscopy (TEM). The effect of Cu-PES samples on yeast cell morphology was assessed by TEM using *C. glabrata* DSY565 as a test organism. Briefly, cells of *C. glabrata* DSY565 (10^7 CFU) were loaded on unsputtered- and Cu-PES. After 24 h of exposure at room temperature in the dark, cells were fixed with a glutaraldehyde solution (EMS, Hatfield, PA) in 2.5% Phosphate Buffer (PB) (Sigma-Aldrich, St Louis, MO). Samples were rinsed in PB buffer and post-fixed with a solution containing 1% osmium tetroxide (EMS) and 1.5% of potassium ferrocyanide (Sigma-Aldrich). Samples were dehydrated in acetone series (Sigma-Aldrich) and embedded in Epon resin (Sigma-Aldrich). Ultrathin sections of 50 nm were cut by Leica Ultracut (Leica Mikrosysteme GmbH, Vienna, Austria) and deposited on a copper grid (EMS) coated with a polystyrene film (Sigma-Aldrich). Sections were stained with 4% uranyl acetate (Sigma-Aldrich). Micrographs were taken with a FEI CM100 transmission electron microscope (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 80kV with a TVIPS TemCam-F416 digital camera (TVIPS GmbH, Gauting, Germany).

3. Results

X-ray fluorescence (XRF) and diffuse reflectance spectroscopy (DRS) of Cu-PES. The Cu-content of Cu-PES samples was determined by XRF and presented a loading of 0.11 % Cu weight/weight PES. The spectral range for Cu(I)/Cu(II)-species, measured by DRS, ranged between 200 and 800 nm, therefore allowing a considerable absorption of the actinic light emitted between 400 and 700 nm (Figure 11). The optical absorption between 500 and 600 nm is due to the inter-band transition of Cu(I) and the absorption between 600 to 720 nm is attributed to the exciton band and the Cu(II) d-d transition (data not shown).

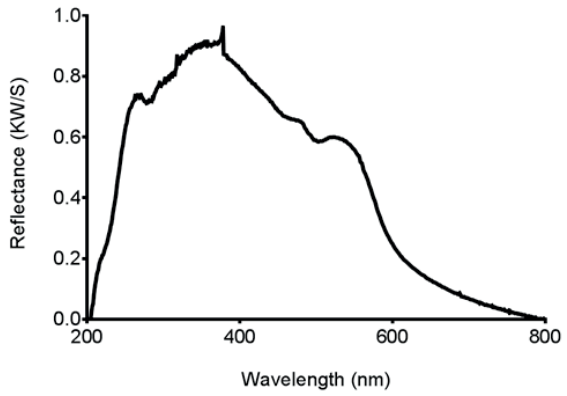

Figure 11. Diffuse reflectance spectroscopy (DRS) of copper-sputtered polyesters (Cu-PES).
Cu was sputtered by Direct Current Magnetron Sputtering (DCMS) on PES for 160 sec.

Activity of Cu-PES under dark and under visible light irradiation. Figure 12A shows the Cu-PES inactivation of *C. albicans* DSY296. Under dark conditions, Cu-PES was fungicidal (reduction of the initial inoculum by 3.1 $\log_{10}$ CFU) within 60 min. Under visible light, the fungicidal activity of Cu-PES (3.0 $\log_{10}$ CFU reduction) was already achieved within 30 min.

Similar results were observed in the case of *C. glabrata* DSY565 (Figure 12B). Indeed, Cu-PES under dark exhibited fungicidal activity (3.0 $\log_{10}$ CFU reduction) after 60 min. The fungicidal activity of Cu-PES was also reached more rapidly under visible light, with a loss in the starting inoculum of 3.4 $\log_{10}$ CFU within 30 min and a reduction of 3.9 $\log_{10}$ CFU at 60 min. Of note, control experiments showed that the survival of *Candida* cells was not affected under dark or actinic irradiation conditions upon exposure to unsputtered-PES.

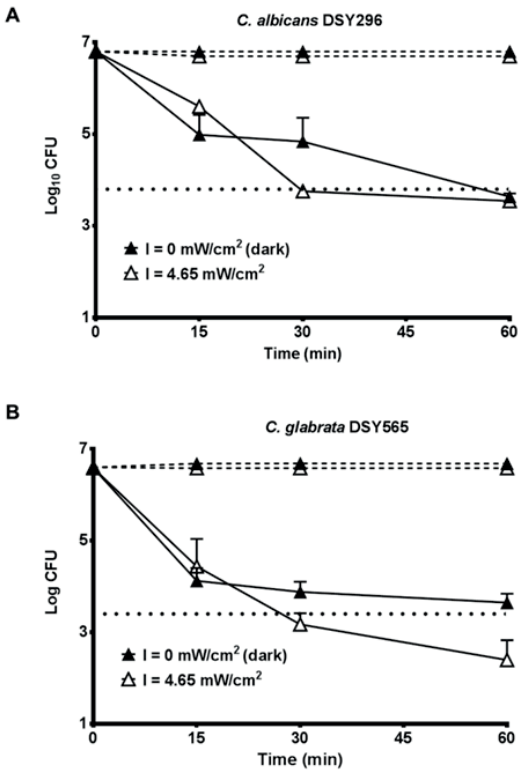

Figure 12. Antimicrobial activity of Cu-PES under light and dark conditions. Loss of viability induced by unsputtered- (dashed lines) or copper-sputtered polyesters (solid lines) against *C. albicans* DSY296 (A) and *C. glabrata* DSY565 (B) in dark (closed symbols) and visible light (open symbols) conditions. The horizontal dotted lines represent a 3 $\log_{10}$ CFU reduction of the initial inoculum sizes (fungicidal activities). Data represent the mean ± standard deviation of three independent experiments.

Fluorescence microscopy reveals the loss of membrane integrity. Contrast phase and fluorescence microscopy analysis of *C. albicans* DSY296 and *C. glabrata* DSY565 were performed to investigate whether the fungicidal activity of Cu-PES under light and dark conditions was associated with changes in yeast cell morphology and cell membrane integrity (Figure 13). Contrast phase microscopy images showed that cells of both *Candida* strains in contact with unsputtered-PES samples preserved their initial membrane integrity. In contrast, cells in contact with Cu-PES presented damage to the cell membrane and displayed a tendency to agglomerate to each other. Figure 13A and Figure 13B show that for both *Candida* isolates, the number of yeast stained red (i.e., dead) after Cu-PES contact increased with time, therefore indicating progressive cell membrane damage.

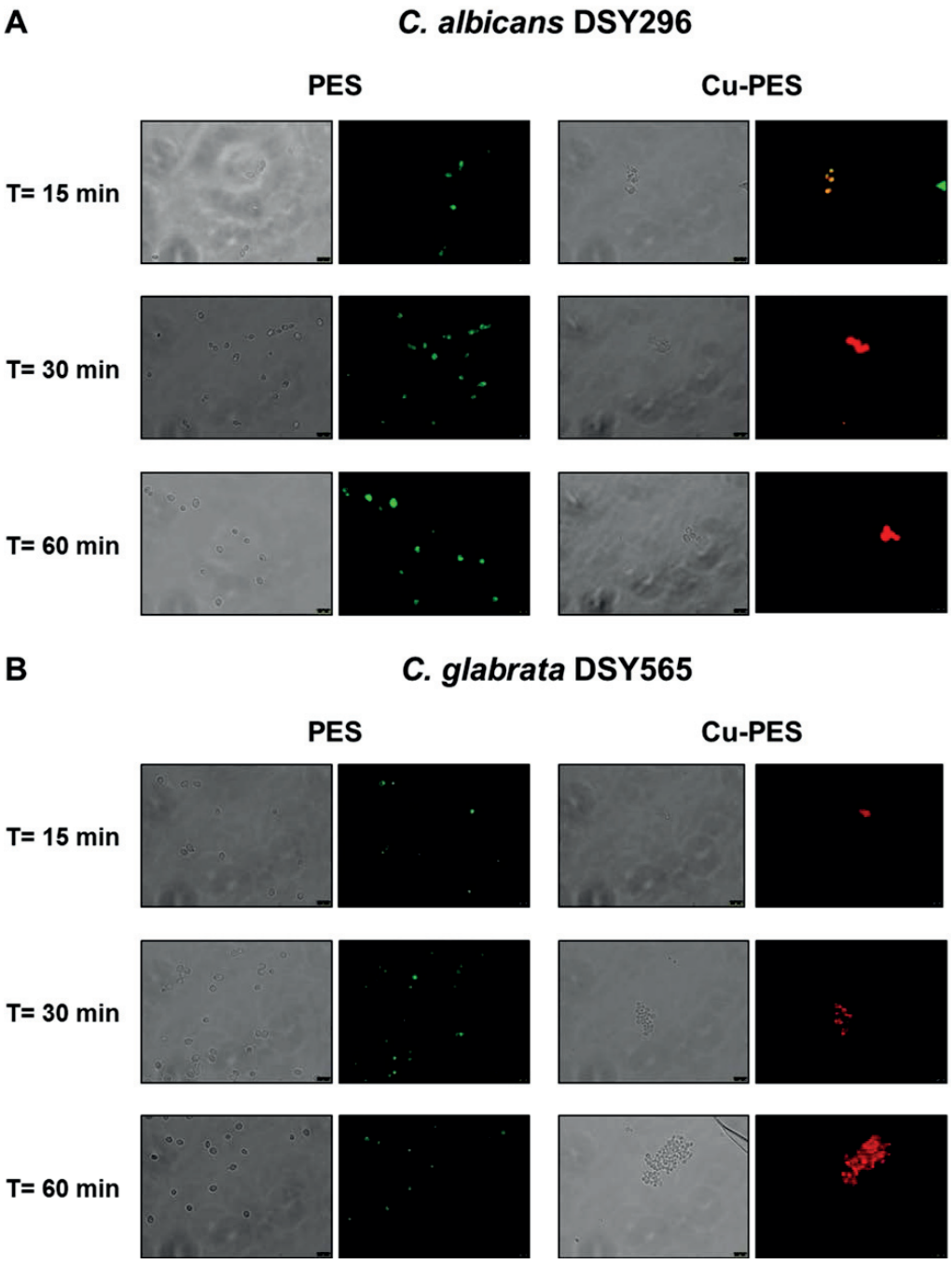

Figure 13. Progressive loss of cell membrane integrity of *C. albicans* and *C. glabrata.*

Fluorescence microscopy images of *C. albicans* (A) and *C. glabrata* (B) cells exposed to unsputtered-polyesters (PES) or copper-sputtered polyesters (Cu-PES) under light irradiation. Green fluorescence indicates live yeast cells and red fluorescence shows dead cells. Bars, 10 µm.

Visualization of ultrastructure changes after Cu-PES exposure. The effect of the Cu-PES contact with *C. glabrata* DSY565 was investigated by TEM (Figure 14). *C. glabrata* DSY565 exposed to unsputtered-PES exhibited normal morphologic features, such as a thick and uniform cell wall (Figure 14A). In contrast, after 24 h of exposure to Cu-PES, yeast showed visible damage of the cell envelope with an undefined cell wall, marked invagination of the plasma membrane and a non-homogeneous cytoplasm (Figure 14B).

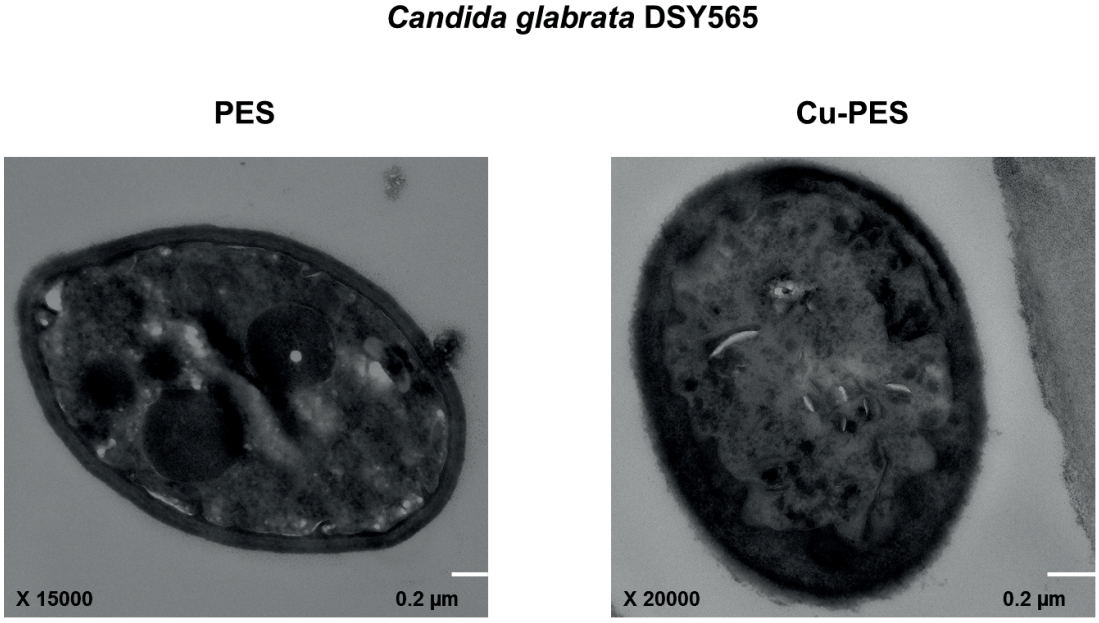

Figure 14. TEM images of *Candida glabrata* DSY565 after 24 h of contact with unsputtered-polyester (PES; sample A) or with copper-sputtered PES (Cu-PES; sample B).
Changes in the cell envelope and cytoplasm can be observed. The plain arrow indicates an abnormal and irregular cell wall, and the dashed arrow shows an invagination of the membrane.

4. Discussion

Over the last decade, metallic Cu on objects, such as door handles, bed rails, or toilet seats (Espirito Santo et al. 2011; Grass et al. 2011), and Cu-impregnated flexible materials, such as hospital gowns, bed sheets or clothing (Borkow and Gabbay 2004; Borkow and Gabbay 2008; Borkow and Gabbay 2009; Rio et al. 2012), have been demonstrated to efficiently decrease the bioburden of microorganisms in healthcare facilities. Cu-coated surfaces, however, require significant amounts of Cu, present low uniformity, little adhesion and are not mechanically stable over time (Irie et al. 2008). Thus, the existing Cu-coating technology needs to be improved. Moreover, there is a need of Cu-coated surfaces active against a broad range of microorganisms including bacteria and yeasts. Particular problems are encountered with surfaces contaminated with fungi that might cause difficult-to-treat infections (Neely and Orloff 2001; Kullberg and Arendrup 2015a). Here we investigated the activity of Cu-impregnated flexible surfaces against *Candida* spp. and the effect of visible light on its antimicrobial activity. Several important findings were observed.

First, we showed that Cu-PES possess fungicidal activity against *Candida* spp. This result extends our previous findings showing that Cu-PES possess an antibacterial activity against a wide panel of multidrug-resistant Gram-positive and Gram-negative bacteria under dark conditions (Rio et al. 2012; Ballo et al. 2016). However, in comparison with the substantial (>5 $\log_{10}$ CFU reduction) and rapid (15-30 min) bactericidal activity of Cu-PES under dark reported previously by our laboratory (Ballo et al. 2016), the fungicidal activity against *Candida* was reduced (ca. 3 $\log_{10}$ CFU) and slower (within 60 min).

This difference may be attributed to the fact that the cell envelope surrounding the eukaryotic yeast cells is structurally more complex (composed by 1,3-β glucan and chitin) and thicker than that found in bacteria (70-100 nm vs 20-80 nm, respectively) (Latge 2007).

Several reports have been published recently indicating that the microbiocidal activity of Cu and Cu-coated surfaces is due to the disruption of the cell membrane function (Hong et al. 2012; Santo et al. 2012; Warnes et al. 2012; Ballo et al. 2016). In microorganisms, the cell envelope is typically composed of a cell wall surrounding the plasma membrane. Accordingly, a thicker cell wall would protect the plasma membrane of the yeast against the biocidal effect of copper. The lower biocidal activity against yeast as compared with bacteria has also been observed with silver (Ag)-nanoparticles (Panáček et al. 2009).

We next showed that the fungi reduction due to Cu-PES against both *C. albicans* and *C. glabrata* isolates was accelerated under visible light (>400nm) compared to dark experiments, which is in agreement with previous work (Rtimi et al. 2015). The rupture of the cell membrane has also been established to be the mechanism for the antimicrobial activity of semiconductor catalysts (Leyland et al. 2016). This effect is likely related to an increased production of CuO. Before cell contact, the majority of Cu exists as Cu_2O, and there is a significant oxidation of Cu_2O to CuO that follows contact with microorganisms, as investigated by X-ray photoelectron spectroscopy (XPS) (Castro et al. 2010; Kusiak-Nejman et al. 2011; Rtimi et al. 2014). The variation from Cu^+ to Cu^{2+} is indicative of redox catalysis and, as previously

demonstrated, Cu^{2+} plays a major role in antimicrobial activity (Nan et al. 2008).

While the primary target of Cu-PES under dark conditions seems to be the plasma membrane, similar results were found when Cu-PES was photo-activated. Indeed, Live/Dead® staining showed that the contact of Cu-PES with yeast under light also led to the disruption of membrane integrity. Similar to this study, Mitoraj *et al.* have shown that the structure and the thickness of the cell wall might help microorganisms to resist more efficiently to the stress induced by oxidative semiconductor photocalysts (Mitoraj et al. 2007).

Finally, we showed that Cu-PES altered the ultrastructure of the yeast after contact using TEM, as reported previously for bacteria (Quaranta et al. 2011; Mathews et al. 2013; Rtimi et al. 2015; Ballo et al. 2016). As the reduction mediated by Cu-PES seems to be related to the structural composition of the microorganism envelope (San et al. 2015b), further investigations are required to assess whether Cu-PES might also be able to inactivate filamentous fungi (e.g., *Aspergillus* spp.) or spore-forming (e.g. *Clostridium* spp.) microbes, which are also a problem in healthcare facilities.

This study focused on the activity of Cu-impregnated flexible surfaces against *Candida* spp. and the effect of visible light on its antimicrobial activity. Stable, uniform and adhesive film of Cu on PES samples were shown to induce fungicidal activity against *Candida* spp within a short period of time under actinic light. These results further support the potential of Cu-PES as a valid surface to prevent microbial infection in hospital environments.

Chapter II.

Antimicrobial Activity of Coated-Intravascular Catheters

This chapter is based on unpublished studies and the following publication:

Evaluation *In Vitro* and *In Vivo* of an Innovative Silver-Copper Nanoparticle Coating of Catheters To Prevent Methicillin-Resistant *Staphylococcus aureus* Infection.
Myriam K. S. Ballo, S. Rtimi, C. Pulgarin, N. Hopf, A. Berthet, J. Kiwi, P. Moreillon, J. M. Entenza, A Bizzini
Antimicrobial Agents and Chemotherapy, 2016

C. An *In Vitro* and *In Vivo* Model for the Evaluation of Cu Catheters Activity Against Methicillin-Resistant *Staphylococcus aureus*

Preface to Article C

In this article, we extended the sputtering DCMS technology acquired in the preparation of 2D Cu-PES samples to the DCMS sputtering of Cu 3D polyurethane catheters. Medical grade polyurethane intravascular catheters pre-treated with inert titanium nitride (TiN), were sputtered with Cu. We then assessed the ability of the copper coating to prevent catheter colonization by methicillin-resistant *Staphylococcus aureus* (MRSA) *in vitro* (in the absence or the presence of plasma) and *in vivo*, in a model of intravascular catheter infection in rats. The results showed a difference between an excellent activity of the Cu-coated catheters to inhibit MRSA colonization *in vitro* and the lack of prevention of infection *in vivo*. The decreased activity of the Cu-coated catheters observed *in vitro* in the presence of plasma could explain the results obtained *in vivo*.

Abstract of Article C

In this study, copper- (Cu)-coated catheters were investigated for their efficacy in preventing methicillin-resistant *Staphylococcus aureus* (MRSA) colonization *in vitro* and *in vivo*. Cu was sputtered for 120 sec on polyurethane catheters by DCMS. *In vitro*, Cu-coated and uncoated catheters were immersed in PBS or rat plasma and exposed to 10^6-10^8 CFU/ml of MRSA ATCC 43300. *In vivo*, Cu-coated and uncoated catheters were placed in the jugular vein of rats. Thereafter, MRSA (10^7 CFU/ml) was inoculated in the tail vein. Catheters were removed 48 h later and cultured.

In vitro, Cu-coated catheters pre-incubated in PBS and exposed to 10^6-10^7 CFU/ml, prevented the adherence of MRSA (11-16% colonization) compared to uncoated catheters (100% colonization; $P< 0.005$). Cu-coated catheters retained their activity (0-44% colonization) when pre-incubated in rat plasma, while the colonization of uncoated catheters remained at 100% ($P< 0.005$). Cu-coating protection diminished with a higher inoculum of 10^8 CFU/ml in both PBS and plasma (67-83% colonization). *In vivo*, Cu-coated catheters caused neither a reduction in catheter colonization compared to uncoated catheters (71% vs 75%, respectively), nor the incidence of positive blood culture (75% vs 57%, respectively). The decreased activity of Cu-coated noticed *in vitro* when pre-exposed to plasma and the reduced activity of Cu-coated *in vivo* suggested that plasma proteins could preclude the preventive effect of Cu-coated catheters.

In vitro studies suggest that Cu-coated catheters have a potential for preventing MRSA infections. Further research is required to analyze whether this activity can be improved and to assess the influence of plasma proteins in the prevention of MRSA colonization.

Keywords: Copper; Tridimensional-coated intravenous catheters; Methicillin-Resistant *Staphylococcus aureus* ; Direct Current Magnetron Sputtering

1. Introduction

The use of intravascular catheters (IVCs) represents a remarkable advance in medical practice. Nevertheless, when IVCs are placed in patients for variable periods of time, there is a risk to develop catheter related infection (CRI) (Edgeworth 2009). The development of such infections is based on the adhesion of pathogens onto the surface of catheters. Pathogens usually originate from the skin flora or are transmitted by the hands of healthcare staff (Mermel 2011; Zhang et al. 2011). IVC-related infections are predominantly caused by staphylococci, comprising *Staphylococcus aureus*, and are particularly problematic in case of methicillin-resistant *S. aureus* (MRSA) (Burton et al. 2009; Bonnal et al. 2015; Tong et al. 2015).

Guidelines are established to control and/or limit the occurrence of IVC-related infections (O'Grady et al. 2011b), such as hand hygiene, maximal barrier precautions during IVC insertion or skin antisepsis by alcohol disinfection (Valles et al. 2008). Also, the use of antiseptic- or antibiotic- impregnated dressings or catheters can help to prevent IVC infections (Danese 2002; Falagas et al. 2007). However, these measures are hindered by a lack of compliance of healthcare workers (Furuya et al. 2011). Furthermore, the antimicrobial activity of coated-catheters has been demonstrated to reduce *S. aureus* IVC infections and subsequent BSI, but not totally (Raad et al. 1996; Raad et al. 1997; Darouiche et al. 1999). Concerns arise regarding the long-term use of coated catheters (Raad et al. 1996; Logghe et al. 1997; Walder et al. 2002; Hanna et al. 2006). The prevention of infections caused by MRSA is still insufficient and the probability of selecting resistant pathogens is a great risk (Batra et al. 2010; Guleri et al. 2012) . Therefore, it is required to implement new strategies to prevent IVC infections.

Modification of the catheter surfaces is a key approach to improving their efficiency (Liu et al. 2004). We first sputtered polyurethane catheters with titanium nitride (TiN) and subsequently with copper (Cu) using DCMS. TiN is widely used to improve the quality of the surface of implants used in biomedical application, such as ventricular assist devices (van Hove et al. 2015). TiN has been selected due to its biocompatibility properties in humans and has a reduced effect on red blood cells hemolysis (Komotori et al. 2001). Cu was used due to its antimicrobial activity (Weaver et al. 2010; Salgado et al. 2013). The objective of this study is to provide a biocompatible and antimicrobial intravascular catheter and evaluate its capability to prevent MRSA ATCC43300 colonization both *in vitro* and *in vivo*.

2. Materials and Methods

Preparation of Cu-coating on polyurethane catheters. Medical grade polyurethane catheters (Optiva® W; Smiths Medical, Rossendale, UK) were coated with TiN and Cu using the DCMS technique, as previously described (Rtimi et al. 2016). Cu-coated catheters were then sterilized overnight with ethanol 70% and packed individually. Only the external surface of the catheters was coated.

Bacterial strain and growth conditions. MRSA ATCC 43300 was grown for 18 h in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and then diluted to the desired inoculum concentration in PBS. The tested inocula were confirmed by serial dilution and plating on agar plates.

***In vitro* activity of Cu-coated catheters.** For *in vitro* assays, catheters were cut into 1-cm length portions and immersed in either 1 ml of PBS or 1 ml of rat plasma at 4^{o}C for 18 h. Plasma was used to test the effect of plasma proteins on the surface of uncoated and Cu-coated catheters, thus simulating more closely the conditions in blood. Catheters were then removed, washed two times in PBS and seeded by immersion for 90 min at 37°C, with a gentle shaking (100 rpm) in 2 ml of PBS containing increasing amounts of MRSA ATCC 43300 (from 10^6 to 10^8 CFU/ml). Following incubation, catheters were removed, washed two times in PBS, placed into 1 ml Trypsine-EDTA for 1 h at 37°C with gentle shaking (100 rpm), sonicated (5 min at 100 Hz), vortexed (30 sec), and the suspension serially diluted and plated onto blood agar plates. The catheters were finally processed by the roll plate technique on blood agar. The plates were then incubated for 48 h at 37^{o}C to count CFUs. All experiments were carried out in triplicate. Catheters exhibiting MRSA growth on the

plates were defined as colonized. The limit of detection was 1 $\log_{10}$ CFU/catheter for the dilution technique and 1 colony for the roll-plate method.

***In vivo* activity of Cu-coated catheters.**

(i) Rat model of IVC infection. All animal experiments were reviewed and approved by the Cantonal Committee on Animal Experiments of the State of Vaud, Switzerland (authorization 2971).

A rat model of IVC infection was used. Briefly, female Wistar rats (180-200 g) were anesthetized with a mixture of ketamine (75 mg/kg) and midazolam (5 mg/kg) given intraperitoneally. Using an aseptic technique, the left external jugular vein was isolated. Then, Cu-coated or uncoated-catheters were inserted through the pectoralis major into the left external jugular vein and advanced into the superior vena cava. The catheters were held in place by ligating the proximal end to the muscle with a sterile silk ligature and buried subcutaneously. This technique avoids the ligation of the jugular vein and permits blood flow around the extraluminal catheter surface. The incision was closed with surgical staples.

(ii) Determination of the bacterial infectivity on uncoated catheters. Before conducting experiments with Cu-coated catheters, we determined the lowest inoculum size of MRSA ATCC 43300 infecting around 80% of uncoated control catheters (infective dose 80% or ID_{80}). Briefly, uncoated catheters were placed as described above. Immediately after catheter implantation, groups of rats were challenged via the tail vein with increasing inoculum sizes (from 10^5 to 10^8 CFU/ml) of MRSA ATCC 43300. Forty-eight hours later, catheters were sterilely removed, their extra-venous portion was cut away and the remaining intravascular portion was

sealed at both ends and placed in a sterile tube with 2 ml of PBS. Since only the outer portion of the catheters was coated with Cu, it was important to seal both ends to avoid contamination with possible intra-luminal infecting bacteria. Catheters were then processed as described above for *in vitro* studies. Bacterial counts of $>10^3$ CFU/catheter were considered as catheter colonization, according to clinical microbiology criteria for catheter colonization (Cleri et al. 1980; Linares et al. 1985; Brun-Buisson et al. 1987; Mermel et al. 2009; Chauhan et al. 2012). Blood samples and spleens were removed and cultivated quantitatively on agar plates.

Uncoated catheters were also placed in animals that did not receive bacterial inoculation in order to exclude the possibility of spontaneous colonization.

(iii) Activity of Cu-coated catheters. Cu-coated catheters and uncoated catheters were placed in the jugular vein of rats as described above. Immediately after catheter implantation, animals were inoculated in the tail vein with 10^7 CFU/ml, i.e. the ID_{80} of MRSA ATCC 43300 (see below). Forty-eight hours later, catheters were removed and processed as described above. Blood and spleen cultures were also performed.

3. Results and Discussion

***In vitro* activity of Cu-coated catheters.** Fig. 15A shows that 9/9 (100%) uncoated catheters pre-incubated in PBS were colonized when exposed to 10^6, 10^7 or 10^8 CFU/ml of MRSA. The colonization rate of Cu-coated catheters (9 catheters per group) was significantly reduced to 16% and 11% when exposed to 10^6 or 10^7 CFU/ml of MRSA, respectively ($P< 0.05$; Chi-square test). Nevertheless, when exposed to 10^8 CFU/ml, 4/6 (67%) of Cu-coated catheters were colonized. A significant difference between uncoated- and Cu-coated catheters was also observed with regards to mean number of viable colonies on colonized catheters ranging from 4.0 ± 0.1 to 5.7 ± 0.5 CFU per uncoated catheter versus 1.0 ± 0.1 to 3.0 ± 0.5 CFU per Cu-coated catheters ($P< 0.0001$; Student's *t* test) (Fig. 15B). Therefore, it was assumed that the prevention effect of Cu-coating is dose dependent, as at the concentration of 10^8 CFU/ml of MRSA we reach a threshold where the reduction of catheter colonization was less marked.

When pre-incubated in rat plasma (Fig. 15C), the rate of colonized uncoated catheters (n= 9) was 100% when exposed to 10^6, 10^7 or 10^8 CFU/ml of MRSA. Thus, there was no a significant difference with the colonization rate of uncoated catheters pre-incubated in PBS (Fig 15A). In contrast to uncoated catheters, 4/9 (44%) of Cu-coated catheters were colonized when exposed to 10^7 CFU/ml of MRSA (P=0.03; Chi-square test). Furthermore, the colonization rate was higher, 89%, when Cu-coated catheters were exposed to 10^8 CFU/ml. The number of microorganisms retrieved from colonized control uncoated catheters ranged from 3.2 ± 0.5 to 5.0 ± 0.4 $\log_{10}$ CFU/catheter, and was significantly higher than the number of organisms adhering to Cu-coated catheters (1.0 ± 0.7 to 2.3 ± 0.5 $\log_{10}$ CFU/catheter; $P< 0.05$; Student's *t* test) (Fig. 15D). Thus, *in vitro* studies showed that the Cu-coating was

effective in preventing catheter colonization by MRSA both in PBS-incubated and plasma-incubated devices at a concentration <10^8 CFU/ml. Above this concentration, the activity threshold was reached and diminished the prevention activity of Cu-coated catheters. Moreover, the presence of plasma affects Cu-coating efficacy.

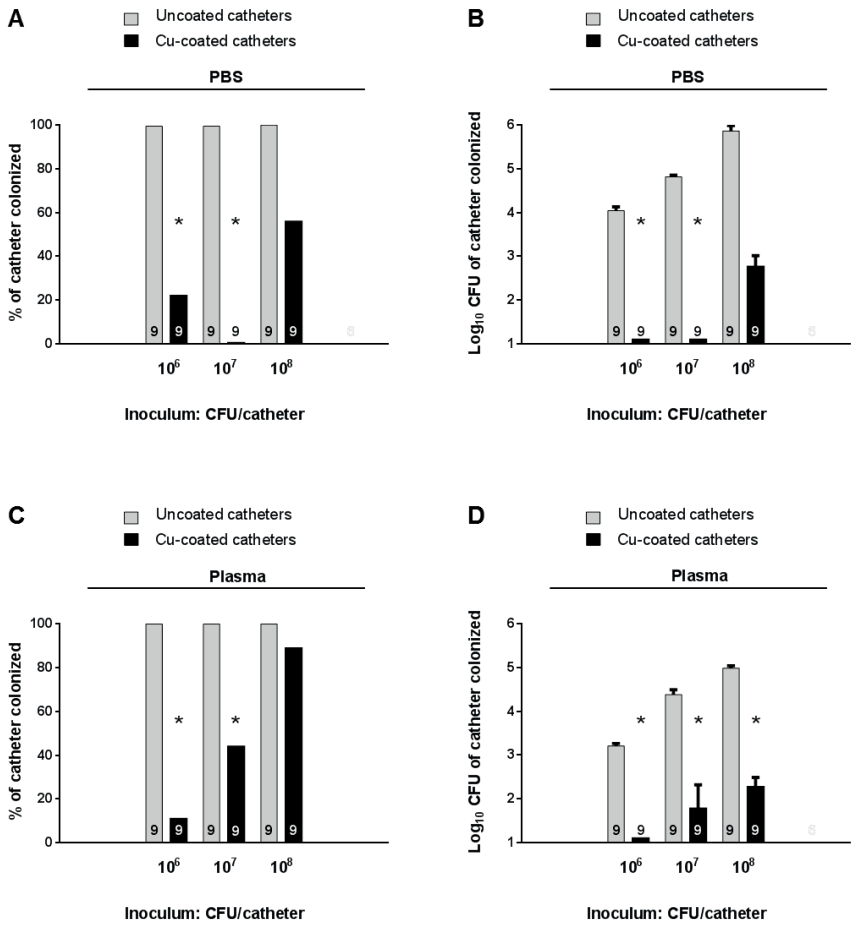

Figure 15. ***In vitro*** **MRSA ATCC 4300 colonization (A and C) and bacterial densities (B and D) of uncoated- and Cu-coated catheters.**
The number of catheters is indicated at the bottom of each column.
*,*P*<0.05 compared to uncoated catheters.

Rat model of IVC infection.

(i) **Bacterial infectivity in uncoated catheters**. No catheter colonization was observed in animals challenged with 10^5 CFU/ml and only 1 of 5 (20%) of animals injected with 10^6 CFU/ml showed infection. The inoculum sizes that caused significant infection in uncoated catheters were 10^7 CFU/ml (3 of 4 animals; 75%) and 10^8 CFU/ml (5 of 5 animals; 100%). Therefore, the 10^7 CFU/ml inoculum defined as the ID_{80}, was used in the comparative study. Blood and spleen cultures were all positive for MRSA. Catheter cultures showed no infection when harvested from animals that had not been challenged with bacteria.

(ii) Evaluation of Ag/Cu-coated catheters activity. Animals with control uncoated- and Cu-coated catheters were inoculated with 10^7 CFU/ml of MRSA ATCC 43300. Fig. 16A shows that colonization in Cu-coated catheters (71%) was not different from that of uncoated catheters (75%). The number of viable colonies on uncoated-catheters was smaller than what retrieved from Cu-coated colonized catheters, but not significantly (4.5 ± 0.4 and 5.7 ± 0.8 $\log_{10}$ CFU/catheter, respectively). In addition, blood cultures in rats with uncoated catheters were more often positive (3/4; 75%) than in animals with Cu-coated catheters (4/7; 57%) but this difference was also not significant. Spleen cultures were all positive.

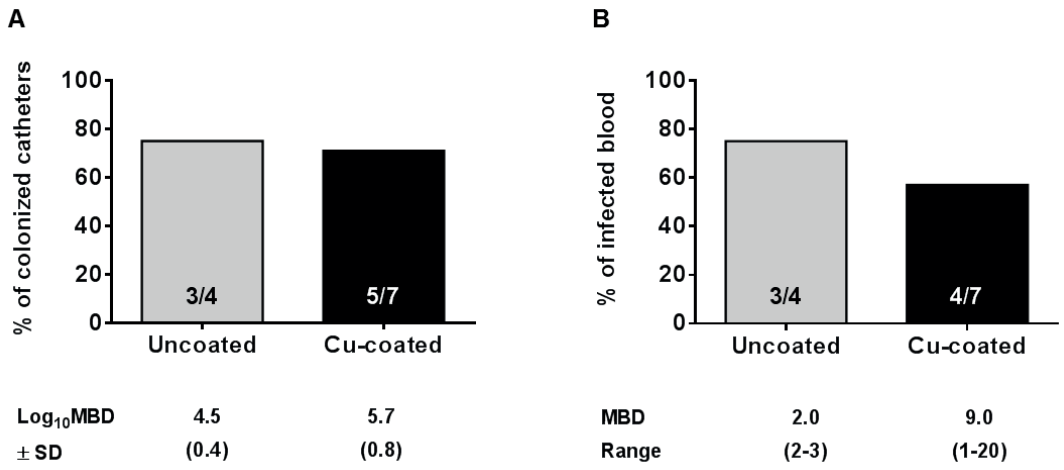

Figure 16. *In vivo* colonization of uncoated- and Cu-coated polyurethane catheters (A) and blood cultures (B).
Infection was evaluated in rats 48 h after intravenous catheter insertion and challenge with MRSA ATCC 4300. The numbers of animals with colonized catheters or blood/total number of animals are indicated at the bottom of each column. MBD stands for median bacterial densities.

Thus, compared to the good activity *in vitro*, the *in vivo* antimicrobial efficiency of Cu-coated catheters was impaired. The poor activity of Cu-coating in preventing catheter colonization *in vivo* compared with their efficacy *in vitro*, may arise from the fact that catheters are placed in a dynamic flow system *in vivo*. In addition, *in vitro* studies with plasma proteins could have impaired the effect of Cu by diminishing the bioavailability of copper ions. Indeed, plasma proteins have a great affinity for Cu-ions, which could impair its antimicrobial activity (Cabrera et al. 2008a; Linder 2016).

In conclusion, stable DCMS Cu-sputtered films provided a stable antimicrobial activity of Cu-coated catheters *in vitro*. These results support the use of Cu-coating for the prevention of IVC infection, but further investigations are needed to better understand the reduced activity showed *in vivo*.

C. Evaluation *In Vitro* and *In Vivo* of an Innovative Silver-Copper Nanoparticle Coating of Catheters To Prevent Methicillin-Resistant *Staphylococcus* aureus Infection.

Preface to Article D

In this article, we pursue our experiments on the prevention of catheter infection by methicillin-resistant *S. aureus* (MRSA). In order to improve the efficacy of copper-coated catheters described in article C, catheters previously treated with inert titanium nitride (TiN) were coated with a mixture of silver (Ag) and copper (Cu) nanoparticles using the DCMS technology described previously. The reason for using the Ag/Cu combination was based on the Ag/Cu synergistic activity against bacteria, including staphylococci (McLean et al. 1993) and *Pseudomonas aeruginosa* (Huang, HI. 2008). Different Ag/Cu concentrations were assayed in pilot studies and, finally, a concentration ratio of 67/33 atomic ratio, which showed the best antibacterial activity, was retained for further evaluation.

Ag/Cu-coated catheters were tested *in vitro* and *in vivo* for the prevention of MRSA colonization and infection in the same conditions as for Cu-coated catheters (Article C). *In vitro*, Ag/Cu-coated catheters led to virtually no colonization by MRSA and, as compared to Cu-coated catheters, were less affected in their efficacy by prior exposure to plasma. In the *in vivo* model of intravascular catheter infection in rats, Ag/Cu-coated catheters decreased both the catheter colonization rate, although not significantly, and the percentage of positive blood cultures. Scanning electron microscopy (SEM) on explanted catheters revealed the presence of a fibrin sheath covering the surface of the catheters that explained the incomplete prevention of infection.

Abstract of Article D

In this study, silver/copper (Ag/Cu)-coated catheters were investigated for their efficacy in preventing methicillin-resistant *Staphylococcus aureus* (MRSA) infection *in vitro* and *in vivo*. Ag and Cu were sputtered (67/ 33% atomic ratio) on polyurethane catheters by Direct Current Magnetron Sputtering. *In vitro*, Ag/Cu-coated and uncoated catheters were immersed in PBS or rat plasma and exposed to 10^4-10^8 CFU/ml of MRSA ATCC 43300. *In vivo*, Ag/Cu-coated and uncoated catheters were placed in the jugular vein of rats. Close by, MRSA (10^7 CFU/ml) was inoculated in the tail vein. Catheters were removed 48 h later and cultured.

In vitro, Ag/Cu-coated catheters pre-incubated in PBS and exposed to 10^4-10^7 CFU/ml, prevented the adherence of MRSA (0-12% colonization) compared to uncoated catheters (50-100% colonization; $P< 0.005$), Ag/Cu-coated catheters retained their activity (0-20% colonization) when pre-incubated in rat plasma while colonization of uncoated catheters increased (83-100%; $P< 0.005$). Ag/Cu-coating protection diminished with 10^8 CFU/ml in both PBS and plasma (50-100% colonization). *In vivo*, Ag/Cu-coated catheters reduced the incidence of catheter colonization compared to uncoated catheters (57% vs 79%, respectively; $P= 0.16$) and bacteremia (31% vs 68%, respectively; $P< 0.05$). Scanning electron microscopy of explanted catheters suggests that the suboptimal activity of Ag/Cu catheters *in vivo* was due to the formation of a dense fibrin sheath over their surface.

Ag/Cu-coated catheters have a potential for preventing MRSA infections. Their activity might be improved by limiting plasma protein adsorption on their surface.

Keywords: Copper; Silver; Intravenous catheters; Methicillin-Resistant *Staphylococcus aureus*

1. Introduction

The use of intravenous catheters (IVCs) in patients is often associated with the development of bloodstream infection (BSI), metastatic abscesses and infective endocarditis (Edgeworth 2009; Roig et al. 2012) among other infections. These infections contribute to substantial health-care associated morbidity, prolonged hospital stay and increased costs (Pittet et al. 1994; O'Grady et al. 2011b).

IVCs become colonized predominantly by microorganisms present on the skin, most often at the time of intravascular insertion (Bjornson et al. 1982; Mermel 2011). IVC-related infections are most commonly caused by staphylococci, including *Staphylococcus aureus*, and are particularly problematic in case of methicillin-resistant *S. aureus* (MRSA) (Burton et al. 2009; Bonnal et al. 2015; Tong et al. 2015).

Measures have been developed so far to prevent IVC-related infections (O'Grady et al. 2011b). Priority is given to hygiene including maximal barrier precautions during IVC insertion such as skin antisepsis by alcohol disinfection (Valles et al. 2008), hand hygiene, strict aseptic insertion procedures and sometimes the use of antiseptic- or antibiotic- impregnated dressings or catheters (Veenstra et al. 1999; Falagas et al. 2007).

The use of aseptic procedures help in preventing insertion-related IVC infections (Eggimann et al. 2000; O'Grady et al. 2011b). However, these measures are limited by practical compliance problems (Furuya et al. 2011). A number of clinical trials have demonstrated that antimicrobial-coated catheters reduce *S. aureus* IVC infections and subsequent BSI, but not completely (Raad et al. 1996; Raad et al. 1997; Darouiche et al. 1999). Moreover, several concerns are associated with their use, including limited durability of the coating (Raad et al. 1996; Logghe et al. 1997; Walder et al. 2002; Hanna et al. 2006), insufficient activity against resistant

organisms such as MRSA, the risk of selecting resistant bacteria (Batra et al. 2010) and serious side effects (Guleri et al. 2012). Therefore, the need for efficient prevention strategies of IVC infection warrants the present investigation.

Silver (Ag) and copper (Cu) are effective against a broad spectrum of microorganisms (Jung et al. 2008; Grass et al. 2011). In this study we used Direct Current Magnetron Sputtering (DCMS) to coat polyurethane catheters with an innovative combination of Ag and Cu nanoparticles (Rtimi et al. 2016a). The DCMS technique coats uniform and thin film metals on surfaces (Rtimi et al. 2016a). Then, we investigated the activity of Ag/Cu-coated catheters in preventing MRSA adherence and colonization *in vitro* and infection *in vivo*, in a rat model of intravenous implantation that reflects the pathogenesis of IVC infection in humans. We also tested Ag and Cu nanoparticles for the risk of skin toxicity, using an *ex vivo* model of human skin irritation.

In this study we show that Ag/Cu-coating of catheters completely prevented catheter colonization by MRSA *in vitro*. *In vivo*, the catheter colonization rate was reduced but not completely suppressed. This relative lower *in vivo* activity was likely attributed to the adsorption of plasma proteins on the catheter surface, generating a sheath hindering the contact of the Ag/Cu film with the staphylococci. Such a phenomenon raises concerns as it might also affect other antibacterial prosthetic coatings.

2. Materials and methods

Preparation of Ag/Cu coating of polyurethane catheters. Medical grade polyurethane catheters (Optiva® W; Smiths Medical, Rossendale, UK), pre-treated with inert titanium nitride, were coated with Ag and Cu nanoparticles (67/33% atomic ratio, respectively) using the DCMS technique (Rtimi et al. 2016a). The Ag/Cu-coated catheters were then sterilized overnight with ethanol 70% and packed individually. The DCMS coating method yielded a thin (80 nm) layer of nanoparticles of Ag and Cu onto the surface of the catheters. Thus, the coating method did not significantly affect the diameter of the catheters. Only the external surface of the catheters was coated.

Bacterial strain and growth conditions. MRSA ATCC 43300 was used as a model organism because MRSA is frequently responsible for catheter-related BSI and represents a difficult to treat organism (Burton et al. 2009). MRSA ATCC 43300 was grown for 18 h in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) and then diluted to the desired inoculum concentration in PBS. The tested inocula were confirmed by serial dilution and plating on agar plates.

***In vitro* activity of Ag/Cu-coated catheters.** For the *in vitro* assay, catheters were cut into 1-cm length portions and immersed in 2 ml of either PBS or rat plasma at 4^{o}C for 18 h. Plasma was used to test the effect of plasma proteins on the surface of uncoated and Ag/Cu-coated catheters, thus simulating more closely the conditions in blood (Mermel 1993; Yu et al. 1994). Catheters were then removed, washed two times in PBS and seeded by immersion for 90 min at 37°C, with a gentle shaking (100 rpm), in 2 ml of PBS containing increasing amounts of MRSA ATCC 43300

(from 10^4 to 10^8 CFU/ml). Following incubation, catheters were removed, washed two times in PBS, placed into 1 ml Trypsine-EDTA for 1 h at 37°C with gentle shaking (100 rpm), sonicated (5 min at 100 Hz), vortexed (30 sec), and the suspension serially diluted and plated onto blood agar plates. The catheters were finally processed by roll plate technique on blood agar. The plates were then incubated for 48 h at 37^{o}C to count CFU. All experiments were carried out in triplicate and repeated at least 2-times. Catheters exhibiting MRSA growth on the plates were defined as colonized. The limit of detection was 1 $\log_{10}$ CFU/catheter for the dilution technique and 1 colony for the roll-plate method.

Evaluation of the skin toxicity of Ag/Cu nanoparticles. Tissues for this study were obtained from the DAL Biobank at the University Hospital of Lausanne (CHUV), under anonymus donation, in accordance with its regulation. The study was approved by the Ethics Commission of the State of Vaud, Switzerland (Protocol 264/12).

The potential toxicity of Ag and Cu nanoparticles for the skin was evaluated *ex vivo* in a human skin irritation model, using a 6 flow-through diffusion cell system (PermeGear®; SES Analytical System, Bechenheim, Germany) (Miles et al. 2014).

Briefly, human abdominal full thickness skin was dermatomed to a thickness of 0.8 mm (Acculan II, B. Braun/Aesculap, Sempach, Switzerland) and mounted on the flow-through diffusion cell, between the donor and the receptor chambers. Skin integrity was verified by measuring transepidermal water loss (VapoMeter wireless, Delfin Technologies Ltd., Kuopio, Finland). Then, the donor chamber was filled with 200 µl of PBS containing 2 ppb of Ag and 20 ppb of Cu, as quantified by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). These concentrations very the maximal amount that could be released from the catheters after intensive washing

(Rtimi et al. 2016b). The Ag/Cu suspension was then continually applied on skin samples via the donor chamber during 24 h. PBS was used as non-irritant solution control. After 24 h, the skin sample was removed, washed with saline, fixed in formaldehyde, and stained with standard hematoxylin and eosin for histological assessment. The histological slides were analyzed using a Zeiss Axio imager.Z2 microscope and 10 section images were randomly selected across each slide. Each image was analyzed for changes to the epidermis (spongiosis and vacuolization of the basal layer) and cell morphology. Skin samples from 3 different donors were used.

***In vivo* activity of Ag/Cu-coated catheters.**

(i) Rat model of IVC infection. All animal experiments were reviewed and approved by the Cantonal Committee on Animal Experiments of the State of Vaud, Switzerland (authorization 2971).

A rat model of IVC infection was used. Briefly, female Wistar rats (180-200 g) were anesthetized with a mixture of ketamine (75 mg/kg) and midazolam (5 mg/kg) given intraperitoneally. Using an aseptic technique, the left external jugular vein was isolated. Then, Ag/Cu-coated or uncoated-catheters were inserted through the pectoralis major into the left external jugular vein and advanced into the superior vena cava. The catheters were held in place by ligating the proximal end to the muscle with a sterile silk ligature and buried subcutaneously. This technique avoids the ligation of the jugular vein and permits blood flow around the extraluminal catheter surface. The incision was closed with surgical staples.

Statistical analysis. Both the percent of colonized catheters, as determined by ≥1 CFU and ≥3 $\log_{10}$ CFU per catheter, respectively, and blood cultures were evaluated. The bacterial load per catheter and blood culture was also determined. All values were reported as mean ± standard error of the mean. Whenever appropriate, statistical comparisons were performed using the Chi-square test or the Student's *t* test. A value of $P < 0.05$ was considered significant by using two-tailed significance levels. All statistical analyses were performed with the GraphPad Prism 6.0 program (www.graphpad.com).

3. Results

***In vitro* activity of Ag/Cu-coated catheters.** Fig. 17A shows that 3/6 (50%), 6/6 and 6/6 (100%) uncoated catheters pre-incubated in PBS and exposed to 10^4, 10^5 or 10^6 CFU/ml of MRSA, respectively, were colonized, while Ag/Cu-coated catheters (6 catheters per group) were exempt of bacteria ($P< 0.05$; Chi-square test). In colonized uncoated catheters, the number of adherent MRSA ranged from 1.3 ± 0.1 to 3.6 ± 0.3 $\log_{10}$ CFU/catheter ($P< 0.005$; Student's *t* test). When exposed to 10^7 and 10^8 CFU/ml, 6/6 (100%) uncoated catheters were colonized compared to 1/8 (12%) and 3/6 (50%) Ag/Cu-coated catheters ($P< 0.05$; Chi-square test). A significant difference between uncoated- and Ag/Cu-coated catheters was also observed with regards to mean number of viable colonies on colonized catheters (4.3 ± 0.6 and 5.7 ± 0.9 versus 2.1 ± 0.9 and 2.6 ± 0.9 $\log_{10}$ CFU/catheter, respectively; $P< 0.01$; Student's *t* test) (Fig. 17B).

When pre-incubated in rat plasma (Fig. 17C), the colonization rate of uncoated catheters increased to 83% (5/6) when exposed to 10^4 CFU/ml while the activity of Ag/Cu-coated catheters was retained at 10^4 to 10^7 CFU/ml inocula. However, compared to the activity in PBS, Ag/Cu catheters showed slightly reduced activity, albeit not significantly, at 10^7 CFU/ml. When exposed to 10^8 CFU/ml, the efficacy of Ag/Cu catheters was abolished (100% colonization) and was significantly lower ($P= 0.045$; Chi-square test) than the activity in PBS. The number of microorganisms retrieved from colonized control uncoated catheters ranged from 1.3 ± 0.5 to 5.7 ± 0.4 $\log_{10}$ CFU/catheter, and was significantly higher than the number of organisms adhering to Ag/Cu-coated catheters (2.2 ± 0.7 to 2.6 ± 0.5 $\log_{10}$ CFU/catheter; $P< 0.05$; Student's *t* test) (Fig. 17D).

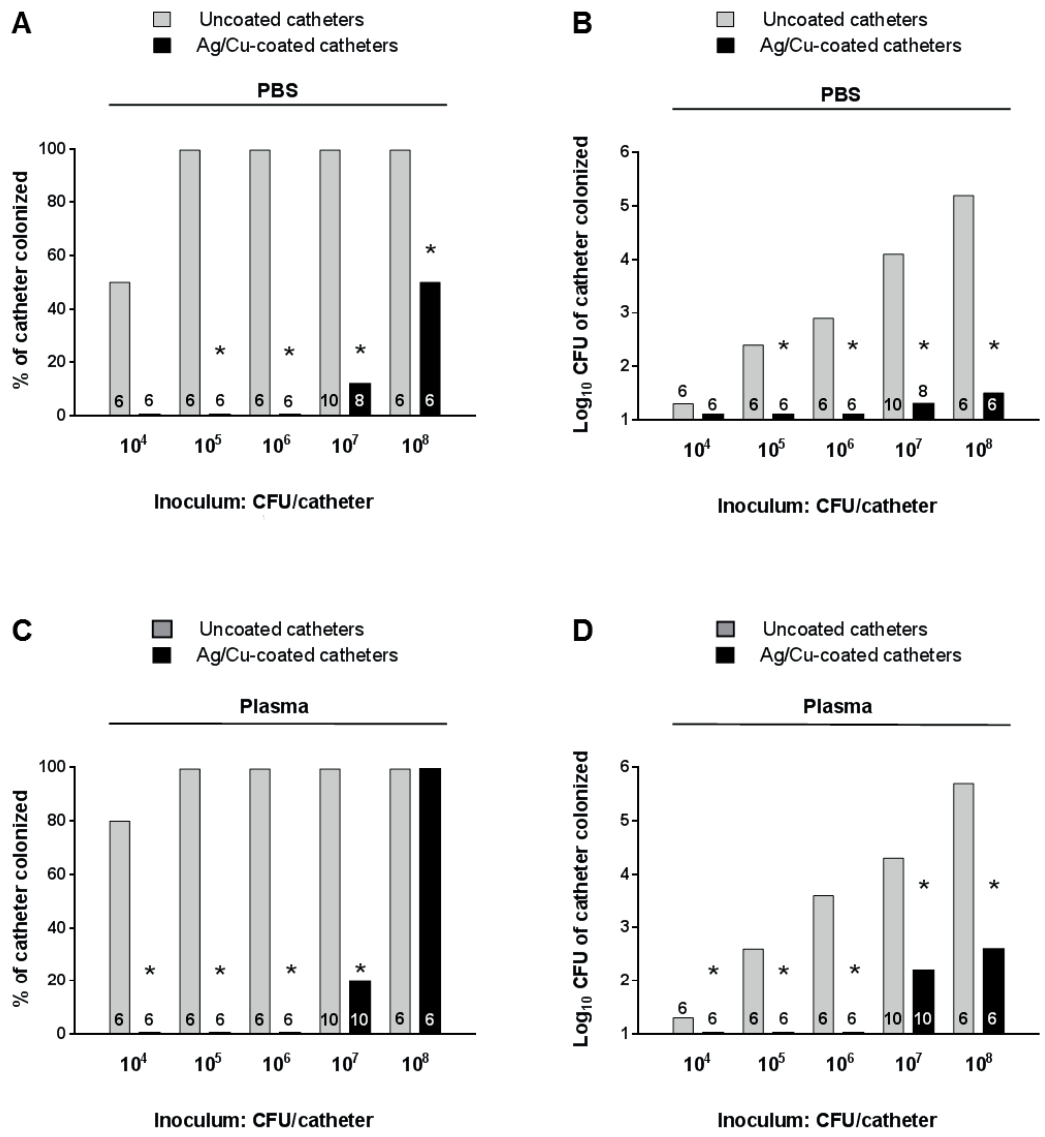

Figure 17. ***In vitro*** **MRSA ATCC 4300 colonization and bacterial densities on uncoated- and Ag/Cu-coated.**

The number of catheters is indicated at the bottom of each column.
*,*P*<0.05 compared to uncoated catheters.

Evaluation of skin irritant effect of Ag and Cu nanoparticles. Fig. 18 shows that histopathological analysis of human skin exposed for 24 h to Ag and Cu nanoparticles revealed no morphological changes compared to exposure to PBS. Thus, Ag and Cu nanoparticles did not cause adverse effects on skin under the experimental conditions.

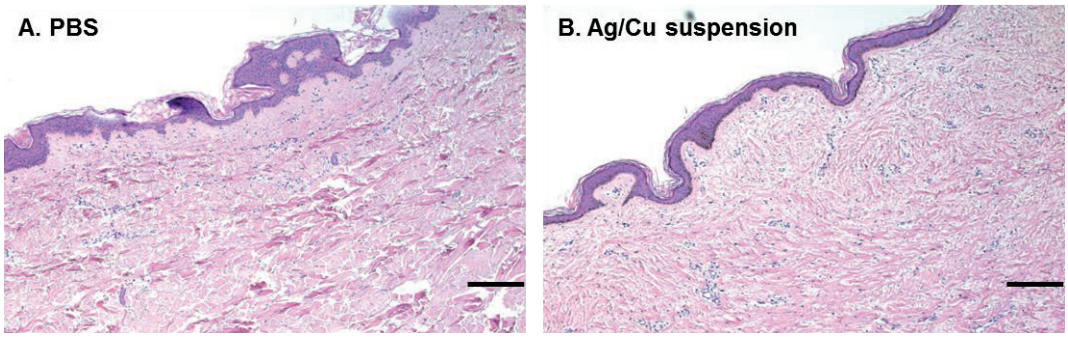

Figure 18. Histological cross-section of human skin exposed to PBS (A) or to Ag and Cu nanoparticles (B).
No irritation signs were observed. Black bars 200μm.

Rat model of IVC infection.

(i) **Bacterial infectivity in uncoated catheters**. No catheter colonization was observed in animals challenged with 10^5 CFU/ml and only 1 of 5 (20%) of animals injected with 10^6 CFU/ml showed infection. The inoculum sizes that caused significant infection in uncoated catheters were 10^7 CFU/ml (4 of 5 animals; 80%) and 10^8 CFU/ml (5 of 5 animals; 100%). Therefore, the 10^7 CFU/ml inoculum, defined as the ID_{80}, was used in the comparative study. Blood and spleen cultures were all positive for MRSA. Catheter cultures showed no infection when harvested from animals that had not been challenged with bacteria.

(ii) Evaluation of Ag/Cu-coated catheters activity. Animals with control uncoated- and Ag/Cu-coated catheters were inoculated with 10^7 CFU/ml of MRSA ATCC 43300. Fig. 19A shows that catheter colonization rate was higher in uncoated catheters (15/19; 79%) than in Ag/Cu-coated catheters (11/19; 57%), but the difference was not statistically significant. No reduction was observed in the number of viable colonies on uncoated- and Ag/Cu-coated infected catheters (5.3 ± 0.9 and 5.1 ± 1.1 $\log_{10}$ CFU/catheter, respectively). In contrast, blood cultures in rats with uncoated catheters were more often positive (13/19; 68%) than in animals with Ag/Cu-coated catheters (6/19; 31%; *P*= 0.023; Chi-square test). Moreover, the number of circulating bacteria in blood cultures from rats with colonized uncoated catheters (41.6 ± 16.8 CFU/ml of blood) was superior to that from rats with colonized Ag/Cu catheters (2.4 ± 1.5 CFU/ml of blood), a difference that was close to the level of significance (*P*= 0.059; Student's *t* test). Spleen cultures were all positive.

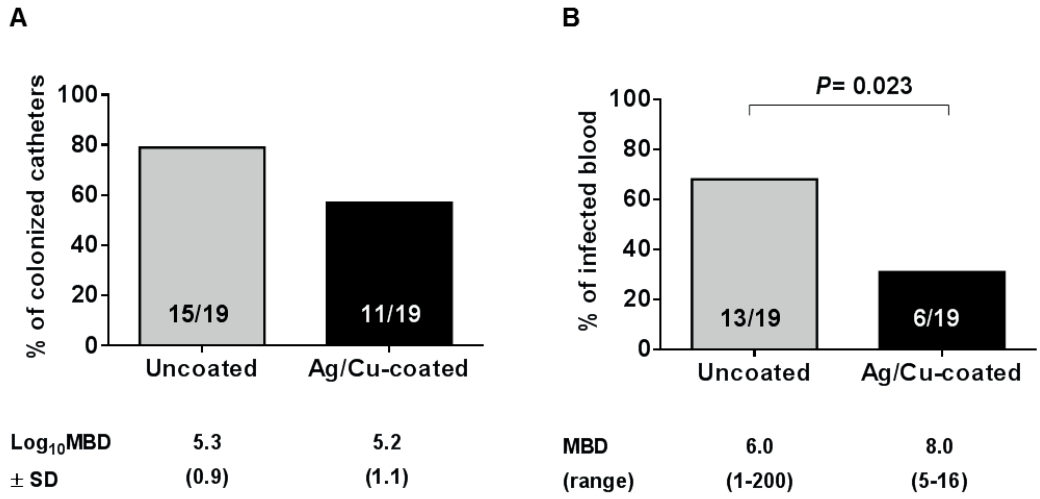

Figure 19. ***In vivo*** **colonization of uncoated- and Ag/Cu-coated polyurethane catheters (A) and blood cultures (B).**
Infection was evaluated in rats 48 h after intravenous catheter insertion and challenge with MRSA ATCC 4300. The numbers of animals with colonized catheters or blood/total number of animals are indicated at the bottom of each column. MBD stands for median bacterial densities.

SEM studies. The surfaces of uncoated- and Ag/Cu-coated catheters were examined by SEM after 48 h of implantation in animals non-inoculated or inoculated with MRSA. Representative electron micrographs (Fig. 20) showed a sheath adhering to the surface of both types of catheters (Fig. 20A and 20B). Interestingly, sheath of Ag/Cu-coated catheters appeared to be thicker than that of uncoated catheters. Moreover, SEM on Ag/Cu-coated catheters from inoculated animals showed bacteria embedded within a more complex matrix sheath than that of uncoated catheters (Fig. 20C and 20D).

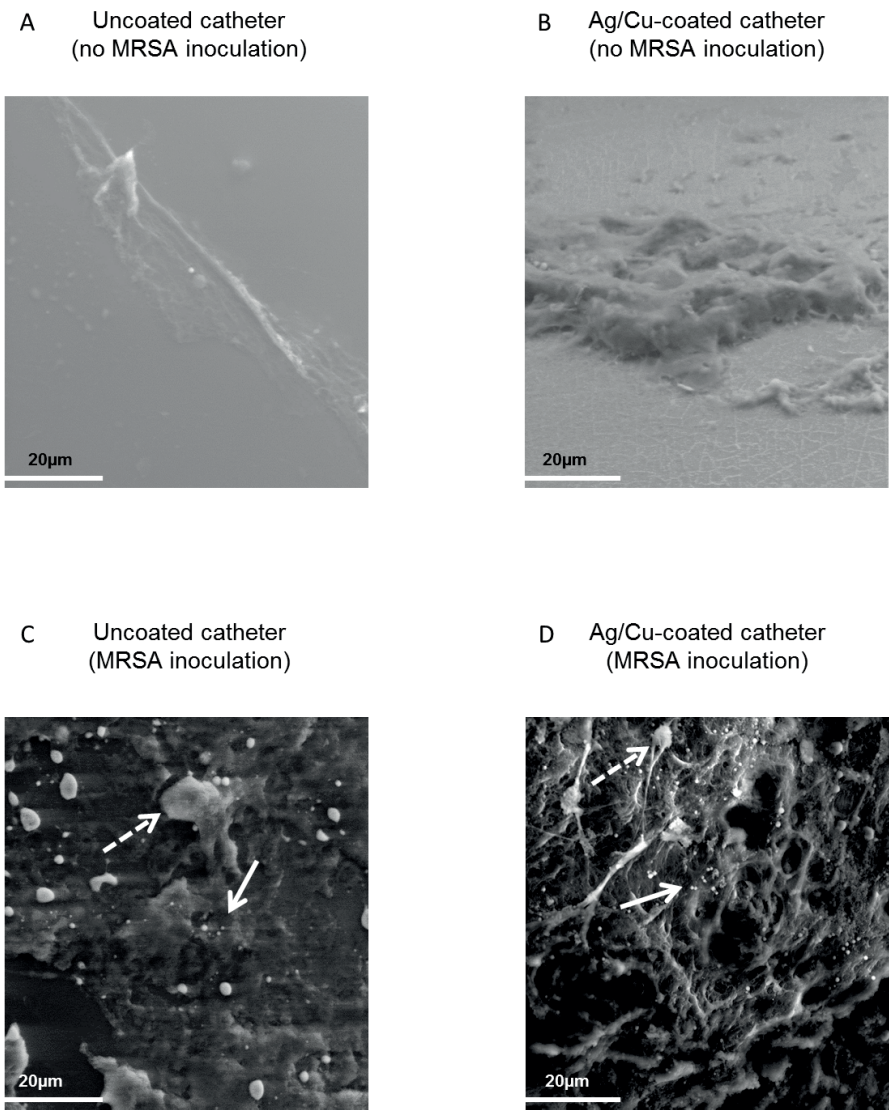

Figure 20. Scanning electron microscopy representative micrographs of sheath formation on the surface.

Scanning electron microscopy representative micrographs of sheath formation on the surface of uncoated (A) and Ag/Cu-coated catheters (B) and view of the matrix sheath inside (C and D) containing fibrinous structures, bacteria and blood cells. Catheters were explanted 48 h post-insertion from the jugular vein of rats non-inoculated or inoculated with MRSA ATCC 43300. Plain arrows point bacteria. Dashed arrows point blood cells.

Short-term implanted catheter colonization and SEM studies. Since most of Ag/Cu-coated catheters explanted 48 h after inoculation were colonized and a thick sheath was observed over their surfaces, we investigated whether their efficacy was hindered as a result of the sheath formation. Therefore, additional experiments were performed in which catheters from animals (five per group) challenged or not with 10^7 CFU/ml of MRSA ATCC 43300 were processed 30 min after implantation. Both uncoated and Ag/Cu-coated catheters were devoid of bacteria and covered with small amounts of fibrin, irrespective of bacterial inoculation. In inoculated animals, all blood and spleen were positive (data not shown).

To test whether a higher inoculum might be more infectious after 30 min further experiments were performed. In these studies, animals were challenged with 10^8 CFU/ml of MRSA (instead of 10^7 CFU/ml) and the catheters were removed and processed 30 min later. In spite of the higher inoculum no catheter colonization was detected while, again, all blood and spleen were positive (data not shown). These results strongly suggest that the formation of a protein sheath on the catheter surface was necessary to initiate bacterial colonization.

4. Discussion

In the present study we coated medical grade polyurethane catheters with an innovative combination of Ag and Cu nanoparticles, using DCMS technology, and investigated their *in vitro* and *in vivo* efficacy in preventing colonization and infection due to MRSA, which is a common cause of IVC-related bacteremia and endocarditis in humans (Tong et al. 2015).

In vitro experiments showed that Ag/Cu-coating was effective in preventing catheter colonization by MRSA (>80% prevention) both in PBS-incubated and plasma-incubated devices. When tested *in vivo* in the catheter-implanted model Ag/Cu-coating became less effective. It decreased catheter colonization by only 22%. On the other hand, Ag/Cu-coated catheters were associated with a beneficial decreased of 37% in the rate of bacteremia as compared to the uncoated catheters. Thus, compared to the good activity *in vitro*, the *in vivo* antimicrobial effectiveness of the Ag/Cu-coating appeared suboptimal.

A likely explanation for the poor activity of Ag/Cu-coating in preventing catheter infection *in vivo*, as compared with their efficacy *in vitro*, is that *in vivo* catheters were situated in a flowing system, where plasma proteins and other blood components, including platelets and erythrocytes, could impair the Ag/Cu effect. Few precedents exist in favour of such explanation.

A first hypothesis is that plasma proteins are rapidly deposited on the surface of implanted catheters after insertion and might complex Ag- and Cu-ions significantly reducing their availability on the outermost layers of the catheter's surface (Slawson et al. 1990; Liau et al. 1997; Schierholz et al. 1998; Cabrera et al. 2008b; Moriya et al. 2008). The antibacterial activity of Ag and Cu nanoparticles is mediated by the release of unbound, biologically active, ions (Ag^+ and Cu^+, respectively) that interact

with the bacterial cell envelope and lead to cell membrane damage and bacterial death (Jung et al. 2008; Roe et al. 2008; Randall et al. 2013). In the case of Ag, plasma proteins and amino acids with sulfhydryl groups (such as cysteine or histidine), and some vitamins and chloride ions promptly react with Ag ions and neutralize their antimicrobial effect (Slawson et al. 1990; Liau et al. 1997; Schierholz et al. 1998). In the case of Cu, several studies have demonstrated that plasma proteins with high Cu affinity (e.g., ceruloplasmin, albumin and macroglobulins) could rapidly bind and sequester Cu after catheter implantation (Cabrera et al. 2008b; Moriya et al. 2008). Therefore, plasma proteins are able to rapidly complex Ag- and Cu-ions, and significantly reduce their availability on the catheter outer surface.

A second hypothesis is that the activity of Ag/Cu-coated catheters could have been masked by the progressive formation of a plasma protein-fibrin sheath covering the coating on the catheter surface. Catheter insertion is followed by deposition of plasma components on its surface, which is believed to act as a physiological sheath. As a paradox, however, plasma proteins such as fibrinogen, as well as platelets circulating in the blood, once deposited on the catheter surface, promoted the attachment of *S. aureus* (Herrmann et al. 1988; Vaudaux et al. 1989; Cheung and Fischetti 1990; Baumgartner and Cooper 1998).

The present experiments confirm the dynamics of protein and bacterial depositions *in vivo*. SEM studies performed on catheters explanted early (30 min) after implantation demonstrated neither sheaths nor bacteria on their surface, whereas SEM observations on catheters explanted after 48 h showed the formation of a dense fibrin sheaths with embedded bacteria. Thus, catheter colonization did probably not result from the initial intravenous bacterial challenge, since bacteria was neither observed in Ag/Cu-coated nor in uncoated catheters despite all the animals

were bacteremic. The infection was probably due to a secondary seeding by bouts of low-grade bacteremia originating from colonized organs, such as the spleen.

This is in accordance with observations by Lloyd et al., who did not observe fibrin sheath formation on intravenous catheters within the first 15 to 30 min following insertion (Lloyd et al. 1993), and with those by Mehall et al., who showed that fibrin sheaths around implanted catheter favoured bacterial attachment following bacteremia (Mehall et al. 2002). Moreover, these results also support that although catheter colonization arising from an skin entry site at the time of insertion is the most frequent route of colonization, long-term implanted IVCs infections result from breakthrough seeding from other sources via transient bacteremia (Timsit et al. 2011). This explains why clinical studies on the prevention of IVC-associated infections using catheters coated with Ag gave disappointing results (Walder et al. 2002; Galiano et al. 2008; Chen et al. 2014).

In addition, SEM studies indicated that Ag/Cu-coated catheters tended to display greater fibrin sheath relative to uncoated control catheters. One likely explanation is that the nanoparticles coating the catheters would accelerate the formation of thrombin and induce strong platelet activation upon contact with plasma, as shown *in vitro* for Ag (Stevens et al. 2009). This would facilitate the adhesion of the circulating MRSA to the catheters. After attachment, the procoagulant activity of *S. aureus* (including MRSA) would further promote fibrin and other plasma proteins deposition (Vanassche et al. 2013). All in all, this could also explain why animals with Ag/Cu-coated catheters had a significant lower rate of positive blood cultures than those with uncoated catheters. Possibly, *S. aureus* embedded deeply in such compact fibrin network had a diminished invasive ability, as a consequence of the down regulation of virulence factors expression in the thick fibrin-sheath film, as already

observed with this organism in biofilms (Resch et al. 2005), and were released less efficiently in the bloodstream.

One of the issues to be solved to optimize Ag/Cu activity is limiting plasma proteins and fibrin adsorption on the catheter surface. Recent studies aimed at reducing the protein adsorption and cell adhesion on implanted biomaterials have led to encouraging nonbiocidal antiadhesive strategies. Indeed, it has been shown that poly-ethylene-glycol (PEG)-based polymers reduce or suppress protein adsorption and platelet adhesion, and were able to limit bacterial adhesion on device surfaces (Hook et al. 2012). Moreover, a recent study with Ag-embedded PEG-coated ventricular catheters has shown an enhanced effect in preventing shunt catheter infection in rats (Hazer et al. 2012). Whether the antimicrobial effect of Ag/Cu-coated catheters could be increased by embedding these nanoparticles into PEG remains to be investigated.

In conclusion, we demonstrated the efficacy of novel developed Ag/Cu-coated catheters in preventing MRSA colonization *in vitro*. These catheters also appeared not toxic for human skin in an *ex vivo* skin irritation model. *In vivo*, in a rat model of IVC implantation, the activity of Ag/Cu-coated catheters was, however, hindered by the deposition of plasma proteins and the formation of a fibrin sheath over the surface of the catheter. The development of catheters combining the antibacterial activity of Ag/Cu with compounds limiting plasma proteins adsorption on their surface warrants further investigation.

General Conclusion and Perspectives

In this thesis, we have addressed the activity of copper-based antimicrobial surfaces, e.g., polyester and catheters, to prevent bacterial colonization, in order to potentially lower the incidence of HAIs. We used DCMS to sputter Cu on polyester and prepare copper-coated intravascular catheters. First, we presented evidence that flexible copper surfaces induce a fast antimicrobial activity against a broad range of AMR pathogens. The antimicrobial activity was accelerated when pre-exposed to light irradiation. These results support the potential of Cu-PES as a valid surface to prevent microbial infection in hospital facilities. A mechanism of action of Cu-PES not involving ROS-species is suggested. Transmission electron microscopy (TEM) analysis of organisms exposed to Cu-PES showed that contact killing is the more likely mechanism leading to bacterial death.

Finally, we demonstrated the efficacy of Cu-coated and Ag/Cu-coated catheters in preventing MRSA colonization *in vitro* and that this coating was not toxic for human skin using an *ex vivo* skin irritation model. In an *in vivo* rat model of intravascular catheter implantation, the activity of Cu-coated and Ag/Cu-coated catheters was, however, reduced and this was caused by the deposition of plasma proteins and the formation of a fibrin sheath over the surface of the catheter, which impeded the direct contact killing process.

In the immediate future, one question to be addressed is the use of Cu-sputtered flexible surfaces, not only for the prevention of infection transmission, but for the treatment of specific infections, such as its use for topical dressings in wound management. A specific example would be for the treatment of diabetic foot infections (DFI) in order to avoid its related complications, which can lead to limb amputation. Indeed, diabetes is an epidemic chronic disease that leads to lower extremity ulcers and wounds (Noor et al. 2016). Microorganisms are able to colonize

this altered environment, which can lead to damaged tissues, inflammation and finally infection. If the infection reaches deeper tissues such as the bone, this might lead to amputation (Lipsky et al. 2012). Foot ulcers are initially responsible for around 60% of lower extremity amputations. *S. aureus* is the most prevalent pathogen to cause mild infections, while moderate to severe infections are frequently polymicrobial (Omar et al. 2008). Several approaches have been defined to help control DFI: local wound management, surgical drainage and antimicrobial therapy. However, the prevalence of DFI is not restricted to one type of pathogen, the initial antimicrobial therapy is empirical, which represents a risk for patients to not be treated correctly in time (Abdulrazak et al. 2005). Moreover, even after adequate treatment, the probability that the health status of the patient worsens is around 70% (Clokie et al. 2017). Thus, there is an urgent need to find and implement new treatments. Biological dressings for the treatment of infected chronic ulcers appears to be a good approach (Abdel-Mohsen et al. 2017).

The process of skin wound healing needs to undergo different stages, such as coagulation, inflammation, proliferation of extracellular matrix proteins (ECM) and tissue remodeling (Abdel-Mohsen et al. 2017). It is known that copper is a key element in stabilizing the ECM of the skin and, the cross linkage of collagen and elastin is copper dependent (Philips et al. 2012). Furthermore, recent evidence suggests that copper contributes to accelerated wound healing, acting on the synthesis and stabilization of skin proteins (Borkow et al. 2010a; Borkow et al. 2010b). In addition, copper is unlikely to be toxic for healthcare workers or patients, as Cu is a rare sensitizer and Cu levels required for its antimicrobial activity are under the toxicity level threshold allowed for mammalian cells (Heidenau et al. 2005; Rtimi et al. 2014). This was also the case in our work when we demonstrated that the

release of Cu from DCMS-manufactured surfaces is low and that copper is nontoxic for skin. These properties of copper, in addition to its bactericidal activity against a broad number of pathogens, could make Cu-sputtered flexible surfaces excellent candidates for topical dressings in order to improve the management and healing of DFI, and maybe other wound infections, such as burn wounds.

Based on the contact killing mechanism of copper-coated surfaces, it may be of interest to look into further research related to this observation. So far, researchers have observed that Gram-negative bacteria are more sensitive to copper surfaces than Gram-positive bacteria, and the later are more sensitive than fungi (Gould et al. 2009; Grass et al. 2011). In this thesis, we have also observed that Gram-negative bacteria are killed more rapidly than Gram-positive bacteria and yeast when exposed to our Cu-PES surfaces. This suggests that the mechanism of copper killing is somehow related to the different structure of the microbial cell, which could be less permeable to copper in yeast and Gram-positive bacteria than in Gram-negative bacteria. Therefore, it would be interesting to determine the killing kinetics of microbial cells with similar genetic backgrounds, but expressing different cell wall thicknesses. For this purpose, vancomycin-intermediate *Staphylococcus aureus* would be an appropriate choice. First, because these strains are intermediately susceptible to vancomycin, which is considered the last-line antibiotic for the treatment of infections caused by antibiotic resistant Gram-positive bacteria. Second because the mechanism of resistance is related to the thickness of the cell-wall (Howden et al. 2010) and can share the genetic background with the parent strain (Shoji et al. 2011).

Finally, it would also be interesting to develop catheters that combine the antibacterial activity of Ag/Cu with compounds limiting plasma proteins adsorption on

their surface. Heparin is among the substances that could be attached to catheters in order to prevent the adhesion of plasma proteins on Ag/Cu surfaces. Heparin-bonded catheters have been shown to decrease the rate of thrombosis and positive blood cultures in a clinical trial (Pierce et al. 2000). However, the use of heparin-coated materials has major limitations, such as limiting activity over time and a risk of heparin-induced thrombocytopenia (Bannan et al. 1997). Several other studies have shown that surfaces coated with polyethylene glycol (PEG) were effective in preventing the adhesion of proteins and microbes (Chapman R. Langmuir 2000). PEG can be attached directly to the catheter or cross-linked to Ag/Cu and then the composite material grafted on the catheter (Saldarriaga Fernandez et al. 2011; Hazer et al. 2012). These approaches based on this chapter of the thesis work merit further investigation.

Annexes

Duality in the *Escherichia coli* and Methicillin-Resistant *Staphylococcus aureus* Reduction Mechanism under Actinic Light on Innovative Co-Sputtered Surfaces.
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Duality in the *Escherichia coli* and methicillin resistant *Staphylococcus aureus* reduction mechanism under actinic light on innovative co-sputtered surfaces

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ABSTRACT

The kinetics of bacterial reduction of *Staphylococcus aureus* (MRSA) on co-sputtered TiO_2/Cu-polyester (TiO_2/Cu-PES) was found to be little dependent on the applied light dose. But in the case of *Escherichia coli*, the bacterial reduction kinetics was observed to be strongly dependent on the applied light dose. The reasons for the different effect of the applied light dose on the bacterial reduction are discussed. Mechanistic considerations are suggested to account for this observation. TiO_2/Cu-PES obtained by direct current magnetron co-sputtering and the bacterial reduction features compared to PES sputtered individually by TiO_2 and Cu. This study presents the first evidence for the stabilizing effect of TiO_2 on the amounts of the Cu released during bacterial inactivation by co-sputtered surfaces compared to sequential sputtering of Ti and/or Cu on PES. The release of Cu-monitored in the ppb range by inductively coupled plasma-mass spectrometry (ICP-MS) is indicative of an oligodynamic effect leading to bacterial reduction. The bacterial reduction of MRSA ATCC 43300 on co-sputtered TiO_2/Cu led to a 5 $\log_{10}$ (99.999%) reduction within 120 min in the dark and 60 min under low intensity actinic light. Diffuse reflectance spectroscopy (DRS), transmission electron microscopy (TEM) and X-ray fluorescence (XRF) describe the TiO_2/Cu surfaces investigated in this study.

1. Introduction

Nanoparticulate films preparation able to kill pathogenic bacteria avoiding the formation of biofilms is a topic of increasing attention. Infections caused by antibiotic-resistant bacteria and leading to healthcare associated infections (HCAIs) with higher cost treatments [1–4]. Also, these nosocomial infections caused by antibiotic resistant bacteria are becoming more frequent [5]. Recently, some laboratories [6–9], have reported antibacterial Ag, Cu and TiO_2 coatings on glass and polymer films depositing the metal/oxides by chemical vapour deposition (CVD) and sputtering techniques. Direct current (DC) magnetron sputtering as used in this work allows the deposition of metal/metal oxides at relatively low temperatures not exceeding 120–130 °C which allow the sputtering on textiles and polymers presenting a low thermal resistance. Advances in bactericidal surfaces have become a focus of attention over the last five years. Studies on the photo-activated TiO_2 and Cu–TiO_2 mediated bacterial reduction have been reported [10–12]. There is a need for active, uniform and adhesive surfaces to decrease more effectively environmental risks associated with HAI.

Recent studies [13,14] have reported the preparation of Cu and TiO_2/Cu films by sol–gel methods inducing significant bacterial inactivation under UV–vis light. Nevertheless, the sol–gel preparations deposited films are not mechanically stable, nor reproducible, present low uniformity and little adhesion since they can be wiped off by a cloth or thumb [15]. This moved us to work on the preparation of sputtered antibacterial films to overcome the shortcomings of colloidal loaded films and motivated the present study.

Reduction of *Escherichia coli* on Cu-sputtered polyester (Cu-PES) [16,17] and reduction of MRSA on Cu-PES has recently been reported by our laboratory [18] and other sources [19]. This

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study is a continuation of our previous work on TiO_2/Cu surfaces leading to *E. coli* [17–19] reduction, but extending the previous studies to show: (a) the bacterial reduction on innovative TiO_2/Cu co-sputtered PES, (b) the differential effect of the applied actinic/visible light (400–700 nm) on the bacterial reduction kinetics of *E. coli* and MRSA and suggest the rationale for this, (c) the stabilizing effect of TiO_2 on the Cu release from the co-sputtered surfaces during bacterial reduction compared to Cu sputtered individually, (d) by inductively coupled plasma mass spectrometry (ICPMS) account for the Cu-oligodynamic effect leading to the observed bacterial reduction and (e) the effect of a co-sputtered catalyst TiO_2/Cu-PES on the bacterial reduction kinetics of Gram+ and Gram− bacteria, as well as the effect of actinic/visible intensity on the bacterial reduction on these two bacterial probes.

2. Experimental

2.1. Catalyst preparation

The PES used corresponds to the EMPA test cloth sample No 407. It is a polyester Dacron polyethylene terephthalate, type 54 spun, 130 microns thick, plain weave ISO 105-F04. Direct current magnetron sputtering deposition of Ti and Cu was carried out in a CMS-18 Vacuum system from Kurt Lesker Ltd. evacuated to 5.8×10^{-3} mbar by a turbo-molecular pump. The Cu as well as the Ti target were 2 inches in diameter, 99.99% pure from K. Lesker Ltd., UK. Co-sputtering was carried out at 300 mA and 350 V. The Ti and Cu targets used for the co-sputtering were positioned at 10 cm of the PES substrate. The nominal calibration of the film thickness was carried out on the Si-wafers and the film thickness was determined with a profilometer (Alphastep500, TENCOR).

2.2. Catalyst characterization

2.2.1. Surface loading

The X-ray fluorescence (XRF) determination of the Ti and Cu on PES was evaluated in a PANalytical PW2400 spectrometer.

2.2.2. Surface release during bacterial inactivation

A Finnigan™ ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) was used to determine the Ti and Cu release during the bactericidal cycles with a resolution of 1.2×10^5 cps/ppb and detection limit of 0.2 ng/L. A washing solution of the TiO_2/Cu sample were digested with nitric acid 69% (1:1 $HNO_3 + H_2O$) to remove the organics in the solution and to guarantee that there were no remaining ions adhered to the flacon wall. The samples droplets are introduced to the ICP-MS trough a peristaltic pump to the nebulizer chamber at ~7700 °C allowing the evaporation and ionization of the elements in the sample. The Cu and Ti found in the nebulizer droplets were subsequently quantified by mass spectrometry.

2.2.3. Diffuse reflectance spectroscopy (DRS)

Diffuse reflectance spectroscopy (DRS) was carried out in a Perkin Elmer Lambda 900 UV–vis-NIR spectrometer within the wavelength range of 200–800 nm. The rough UV–vis reflectance data cannot be used directly to assess the absorption of the TiO_2/Cu/CuO-PES samples because of the large scattering contribution of the PES fabric to the DRS spectra. Normally a weak dependence is assumed for the scattering coefficient S on the wavelength. The spectra were plotted in Kubelka-Munk (KM) units.

2.2.4. High-resolution transmission electron microscopy (HRTEM) and stereomicroscopy

The transmission electron microscopy (TEM) required the PES fabrics embedding in epoxy resin (Embed 812) and cross-sectioned with an ultra-microtome (Ultracut E) up to a thin 2.25: Fluorescence stereomicroscopy and X-ray-photoelectron spectroscopy (XPS):

The fluorescence stereomicroscopy was carried out on samples inoculated with 10^8 CFU of *E. coli* and incubated for 2 h in a humidification chamber. This method uses a fluorochrome-based staining procedure from Filmtracer™ LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen). The kit contains a combination of the SYTO 9® green fluorescent nucleic acid stain and propidium iodide fluoro-chromes for the staining of live and dead cells, respectively. The sample fluorescence was monitored in a fluorescence stereomicroscope (Leica MZ16 FA, Leica Microsystems GmbH Wetzlar, Germany) and the images were processed using the LAS v.1.7.0 build 1240 software from Leica Microsystems CMS GmbH. Adhesion of bacteria to the sputtered PES was allowed for 2 min before washing the sample with sterile Milli-Q water to remove non-adherent bacteria.

2.2.5. X-ray photoelectron spectroscopy of samples

The XPS of the samples was carried out in an AXIS NOVA unit (Kratos Analytical, Manchester, UK) equipped with monochromatic AlK$_\alpha$ ($h\nu$ = 1486.6 eV) anode and the deconvolution software CasaXPS-Vision 2. The carbon C1s line with position at 284.6 eV was used as a reference to correct the charging effects. The surface atomic concentration of some elements was determined from the peak areas and known sensitivity factors [20,21]. Spectrum background was subtracted using the program of the Kratos unit. The etching depth induced by the Ar-ions was referenced to the etching of Ta of 15 atomic layers per minute equivalent to ~3 nm/min.

2.3. Catalytic tests

2.3.1. Bacterial reduction and irradiation sources

Unsputtered and sputtered PES samples were sterilized in 70% ethanol and dried overnight at room temperature (RT). Cultures for the preparation of the MRSA ATCC 43300 and *E. coli* K12 inoculum were prepared by picking a single bacteria colony from the agar plate and incubated in tryptic soy broth at 37 °C overnight. Overnight cultures of the microorganisms were washed two times in 0.9% NaCl and lately diluted to the selected concentration.

E. coli K12 strains was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) ATCC23716, Braunschweig, Germany, to test the antibacterial activity of the co-sputtered samples. The PES fabrics were sterilized by autoclaving at 121 °C for 2 h. The 20 μL culture aliquots with an initial concentration of ~10^6 CFU mL^{-1} in NaCl/KCl (pH 7) were placed on coated and uncoated (control) PES fabric. Samples were then placed on Petri dishes provided with a lid to prevent evaporation. After each determination, the fabric was transferred into a sterile 2 mL Eppendorf tube containing 1 mL autoclaved NaCl/KCl saline solution. This solution was subsequently mixed thoroughly using a Vortex for 3 min. Serial dilutions were made in NaCl/KCl solution. A 100 μL aliquot was pipetted onto a nutrient agar plate and then spread over the surface of the plate using standard plate method. Agar plates were incubated lid down, at 37 °C for 24 h before colonies were counted. Three independent assays were done for each sputtered sample during the course of this study. To verify that no re-growth of *E. coli* occurs after the first bacterial inactivation cycle, the TiO_2/Cu-film was incubated for 24 h at 37 °C. Then, the bacterial suspension of 100 μL was deposited on three Petri dishes to obtain replicates. The samples were incubated at 37 °C for 24 h. No bacterial re-growth was observed for these samples.

For MRSA testing, PES samples (4 cm^2) were inoculated with 20 μl of cell culture and incubated for 30, 60 and 120 min in a humidified chamber at room temperature (RT) in the dark or under light. The temperature inside the light box was maintained

(20–23 °C). Bacterial loss of viability on TiO_2/Cu-PES samples was then evaluated by direct agar transfer plate (incubated at 37 °C).

2.3.2. Irradiation sources

The irradiation of the *E. coli* and MRSA bacteria on the co-sputtered samples was carried by a Philips Master TLD-18 W/865 actinic lamps as used in hospital facilities with an emission between 400 and 720 nm with different light doses between 1.01 mW/cm^2 and 4.65 mW/cm^2.

3. Results and discussion

3.1. Surface characterization of sputtered TiO_2/Cu-PES

Co-sputtering Ti and Cu for 3 min in Ar-O_2 atmosphere led to a coating thickness of ~135 nm (equivalent to ~700 atomic layers). The Cu, TiO_2 and TiO_2/Cu content were determined by X-ray fluorescence (XRF) and are reported in Table 1. The amounts of Cu and TiO_2 are seen to increase with sputtering time as expected. It is important to note that TiO_2 sputtered for ~2 min has an equivalent to the amount of TiO_2 co-sputtering TiO_2/Cu for 3 min. CuO sputtered for ~1 min deposited Cu equivalent to a Cu/TiO_2 film co-sputtered for 3 min.

The diffuse reflectance spectra (DRS) show an increase in the optical absorption as a function of the co-sputtering time in Kubelka-Munk units up to 3 min as shown in Fig. 1. The wide spectral range for Cu(I)/Cu(II)-species between 200 and 800 nm extending allows a considerable absorption of the actinic light between 400 and 700. The optical absorption between 500 and 600 nm is due to the inter-band transition of Cu(I) and the absorption between 600 and 720 nm is attributed to the exciton band and the Cu(II) *d–d* transition fluorescence stereomicroscopy [17,21].

Fig. 2 shows the transmission electron microscopy (TEM) of TiO_2/Cu co-sputtered for 3 min on PES. The more dense Cu-clusters presented diameters between 16 and 20 nm while the TiO_2 clusters revealed smaller sizes between 5 and 10 nm. The TiO_2/Cu coating of 120–160 nm thick (500–800 atomic layers) presented a close contact between the TiO_2 and Cu-nanoparticles.

3.2. Evaluation of the E. coli reduction and effect of the applied light dose

Fig. 3 shows the bacterial reduction by TiO_2/Cu-PES under actinic light irradiation and in dark as a function of TiO_2/Cu co-sputtering times. PES by itself in the dark and actinic light does not reduce *E. coli*. But in the dark, *E. coli* bacterial reduction proceeds within 120 min in the dark on TiO_2/Cu-PES. The mechanism of TiO_2/Cu mediated *E. coli* inactivation under light irradiation has been reported in detail and for this reason it will not be addressed in the present study [13,14,16–18]. Reduction in the dark as shown in Fig. 3, trace 4 proceeds through a mechanism involving the

Table 1
Cu, TiO_2 and TiO_2/Cu determined by X-ray fluorescence (XRF) as a function of the DC-sputtering time.

Sample	%wt Cu/wt PES	%wt CuO/wt PES	%wt Ti/wt PES	%wt TiO_2/wt PES
Cu (5 min)	0.19	0.24	–	–
Cu (3 min)	0.16	0.20	–	–
Cu (1 min)	0.05	0.07	–	–
TiO_2 (5 min)	–	–	0.13	0.20
TiO_2 (3 min)	–	–	0.12	0.14
TiO_2 (1 min)	–	–	0.09	0.10
TiO_2/Cu (5 min)	0.09	011	011	0.17
TiO_2/Cu (3 min)	0.06	0.07	0.10	0.14
TiO_2/Cu (1 min)	0.02	0.04	0.02	0.03

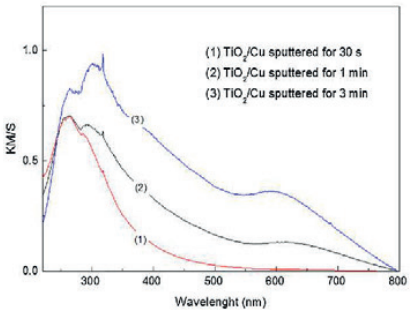

Fig. 1. Diffuse reflectance spectroscopy (DRS) of co-sputtered TiO_2/Cu on PES for the times: (1) 30 s, (2) 1 min and (3) 3 min.

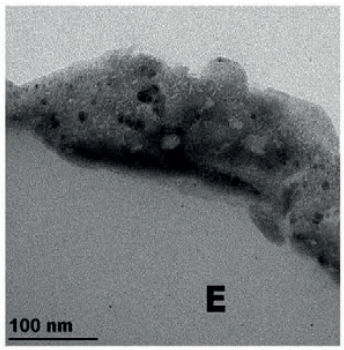

Fig. 2. Transmission electron microscopy (TEM) of TiO_2/Cu co-sputtered for 3 min on PES. E stands for the epoxide that is required to embed the sample during the preparation of the TEM image.

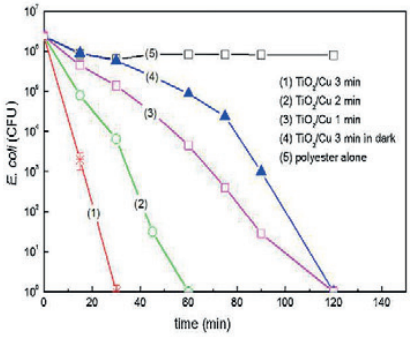

Fig. 3. *E. coli* inactivation on TiO_2/Cu co-sputtered for different times on PES as indicated in the traces: (1) 3 min, (2) 2 min, (3) 1 min, (4) co-sputtered TiO_2/Cu for 3 min in the dark and (5) PES-alone. The bacterial reduction under light irradiation used a lamp Philips Master-18 W/865 (4.65 mW/cm^2).

reaction of O_2 (air) with the Cu/Cu-ions. It will be discussed below in the XPS section and to suggest a possible reaction mechanism

Fig. 3, trace 1 shows a complete bacterial reduction under visible light irradiation within 30 min for TiO_2/Cu samples co-sputtered for 3 min. In this case, sufficient amount of TiO_2 and Cu was coated on the PES leading to a number of exposed catalytic sites inducing the fastest *E. coli* load reduction (see Table 1). Co-sputtering for 1 and 2 min (Fig. 3, traces 2 and 3) did not attain the necessary TiO_2 and

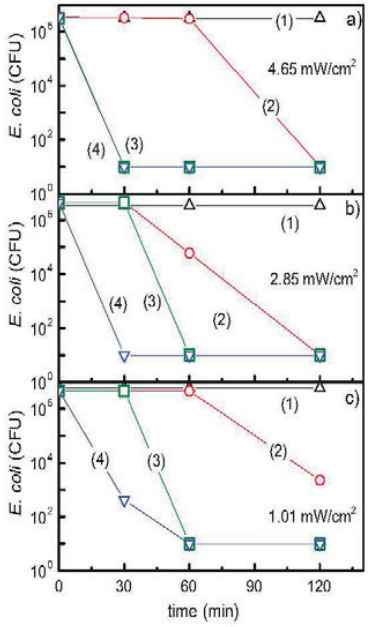

Fig. 4. *E. coli* reduction under Philips Master-18 W/865 (4.65 mW/cm^2) light irradiation: (1) PES alone (2) TiO_2 sputtered on PES for 2 min, (3) TiO_2/Cu-PES sputtered 3 min and (4) Cu sputtered on polyester for 1 min.

Cu sites to induce fast bactericidal activity. Fig. 3, trace 4 shows the complete bacterial reduction in the dark, a more important results than bacterial reduction induced by light due to the continuous nature of the dark bacterial reduction process. The effect of Cu on bacteria has been associated with the ingestion of copper leading to adverse reactions with transport-proteins of enzymes regulating the respiratory chain [16,17,23–26].

Fig. 4 show that TiO_2/Cu-PES surfaces damage the *E. coli* cell wall more than the TiO_2 surface under light and in the dark due to the added effect of cytotoxic Cu. This suggests that *E. coli* damage is located at the cell wall [27] in contact with the bactericide TiO_2/Cu-PES surface in Fig. 4. TiO_2 and Cu on PES proceeded with a similar trend in the bacterial reduction kinetics in Fig. 4. Increased Cu-loading on PES led to faster bacterial inactivation kinetics, but this effect was not observed in the case of TiO_2 (Fig. 4).

Fig. 4 also presents the effect of the actinic light intensity on the *E. coli* reduction mediated by TiO_2/Cu-PES surface at different light intensities. At the highest light dose of 4.65 mW/cm^2 the PES by itself does not lead to *E. coli* reduction (Fig. 4a, trace 1). TiO_2 sputtered samples for 2 min are seen to inactivate *E. coli* within 120 min (Fig. 4a, trace 2). Fig. 4a, trace 4 indicate that Cu sputtered for 1 min with the same surface content of Cu found in TiO_2/Cu co-sputtered for 3 min reduced *E. coli* as TiO_2/Cu-samples co-sputtered for 3 min. A similar trend was observed for the *E. coli* bacterial reduction applying intensities of 2.85 mW/cm^2 (Fig. 4b) and 1.01 mW/cm^2 (Fig. 4c). Under light irradiation TiO_2/Cu is a p-type semiconductor with band gap of 1.7 eV, a flat-band potential of −0.3 V vs SCE (pH 7) and a valence band of +1.4 V SCE [22].

Fig. 4a shows that the bacterial reduction effect shown by TiO_2 (trace 2) is far below the bacterial reduction induced by Cu (trace 4). TiO_2 is acting as a semiconductor generating highly oxidative species under light irradiation. But Cu induces toxicity in the sputtered films that more effectively inactivate bacteria. The interfacial charge transfer (IFCT) [17] from CuO to TiO_2 inhibits the O_2 reduction by Cu_2O/CuO since cb electrons are injected into the TiO_2 decreasing the amount of electrons available to reduce O_2 by Cu_2O/CuO that subsequently would lead more effectively to HO_2° and OH°.

The effect of the light dose in Fig. 4 shows that the density of active sites on the TiO_2/Cu-PES surface leading to *E. coli* reduction is higher than the photon/cm^2 reaching the TiO_2/Cu-PES surface. A higher light dose led to light saturation on the photocatalyst accelerating bacterial reduction (Fig. 4). Cu/CuO/NPs-ions and other heavy metals have been reported to denature bacteria proteins by binding to reactive groups resulting in their precipitation and inactivation through an oligodynamic effect [28,29]. This last effect will be accounted in the section below providing the data for the Cu released in ppb quantities during bacterial reduction as monitored by ICP-MS.

3.3. Repetitive bacterial reduction and Ti and Cu-release during disinfection: mechanistic implications

Repetitive bactericidal cycles of *E. coli* were carried out by TiO_2/Cu-PES under light irradiation and showed a stable repetitive performance (data not shown). The time necessary for the complete bacterial reduction up to the 5th cycle was about 40 min and increased by a small amount during the last repetitive recycling. After each cycle, the co-sputtered samples were washed thoroughly with sterilized MQ-water, vortexed for 3 min and dried. No bacteria remained on the TiO_2/Cu-PES sample. This observation moved us to assess by ICP-MS, the Cu- and Ti-release from the TiO_2/Cu-PES during bacterial reduction. The metal released during the bacterial reduction cycles when sputtering separately Ti (2 min) and Cu (1 min) on PES is shown in Table 2. Table 2 shows the Cu, Ti release at the end of the bacterial reduction when both metals were co-sputtered for 3 min. The release of Ti and Cu decreases to 2 and 4 ppb/cm^2, respectively after the fifth bacterial reduction cycle. Table 2 shows that the Cu and TiO_2 released during the cycles leading to complete reduction on samples co-sputtered for 3 min are significantly below the Cu and TiO_2 amounts released from samples loaded independently with similar amounts of Cu and TiO_2 on PES. A slower release of Cu allows a longer operational lifetime for the TiO_2/Cu-PES samples inducing bacterial reduction and this is important for any practical application of these films.

The copper toxicity toward mammalian cells have been reported with a median of lower effective concentration at 50% e.g. (L(E)C50 of 25 mg/L for mammalian cells [23–26]. The low release Cu of 4 < 25 ppb/cm^2 shown in Table 2 is well below the Cu-cytocompatible level [23]. Cu-levels of 4 ppb/cm^2 inducing bacterial reduction provide disinfection through an effective

Table 2
Metal-ions released during bacterial loss of viability cycles when sputtering TiO_2 or Cu on PES compared to ions release in co-sputtered TiO_2/Cu-PES.

		First cycle	Third cycle	Fifth cycle
TiO_2-PES (2 min)	Ti (ppb/cm^2)	14	11	8
Cu-PES (1 min)	Cu (ppb/cm^2)	11	5	9
Co-sputtered TiO_2/Cu-PES (3 min)	Ti (ppb/cm^2)	7	3	2
	Cu (ppb/cm^2)	6	4	4

Table 3
Surface atomic percentages determined by XPS of TiO_2/Cu-PES co-sputtered for 3 min as detected by XPS.

	C	O	N	Cu	Ti
Before bacterial loss of viability	31.8	22.1	0.7	19.3	26.1
After bacterial loss of viability	39.0	19.4	0.9	16.6	24.1

oligodynamic [17]. The Cu-ions have been reported to bind S, N and COO^- and other electron donor negative groups of the bacteria cell wall or entering the bacteria cytoplasm. Copper in the blood exists in two forms: bound to ceruloplasmin (85–95%), and the rest "free Cu" loosely bound to small molecules, protein, lipids and DNA.

The small amounts of toxic Cu NP's below 25 ppb/cm^2 released by the Cu-polyester allow for a higher cytocompatibility compared to a similar Ag-concentration as reported for mammalian cells. Cu is a metabolizable agent compared to Ag remaining in the body after ingestion increasing the Ag-serum levels [23–27].

3.4. Ar etching of TiO_2/Cu-PES film and Ti, Cu depth profile determined by XPS

By XPS the depth of Cu deposition on PES was investigated as well as the state of oxidation of the Ti- and Cu-species during bacterial reduction. The surface atomic percentage composition of C, O, N, Cu, and Ti is shown in Table 3 at time zero and after 30 min bacterial reduction of *E. coli* as shown in Fig. 3, trace 1. Table 3 shows a small increase in the C-content due to bacterial decomposition residues besides the C-absorbed from the atmosphere during the bacterial reduction. A small decrease in the Cu and Ti-surface concentration after 30 min bacterial reduction was observed and was triggered by the increase of C on the topmost layers described in the preceding paragraph.

Fig. 5a presents the Cu doublet at time zero and Fig. 5b presents the changes in the XPS spectra after bacterial reduction. This doublet is attributed to the presence of the Cu^{2+}-oxidation state (Cu^{2+}) prevalent in the Cu-sputtered sample exposed to air [13,16]. The Cu/Cu_2O with BE 932.2 eV at time zero remains stable after bacteria reduction at 933.1 eV. The CuO deconvoluted peak at time zero shifts to 943.2 eV after bacterial reduction as shown in Fig. 5b. A shift of >0.2 eV is indicative of a change in the oxidation state in the XPS specie [20,21]. Redox catalysis seems to take place during the disinfection process. The redox chemistry is associated with the production of highly oxidative radicals for composites of TiO_2 and Cu-composites [13–15].

The Cu oxidation state at time zero was monitored to be 75.5% for Cu/Cu_2O and 24.3% for CuO. A significant change in the oxidation state was observed after bacterial reduction rendering Cu/Cu_2O 14.1% and 85.7% for CuO. The Ti^{3+}/Ti^{4+} surface electron traps have been reported to enhance the O_2 chemisorption in the presence of Cu in TiO_2/Cu-more markedly than in the case of TiO_2 alone [17].

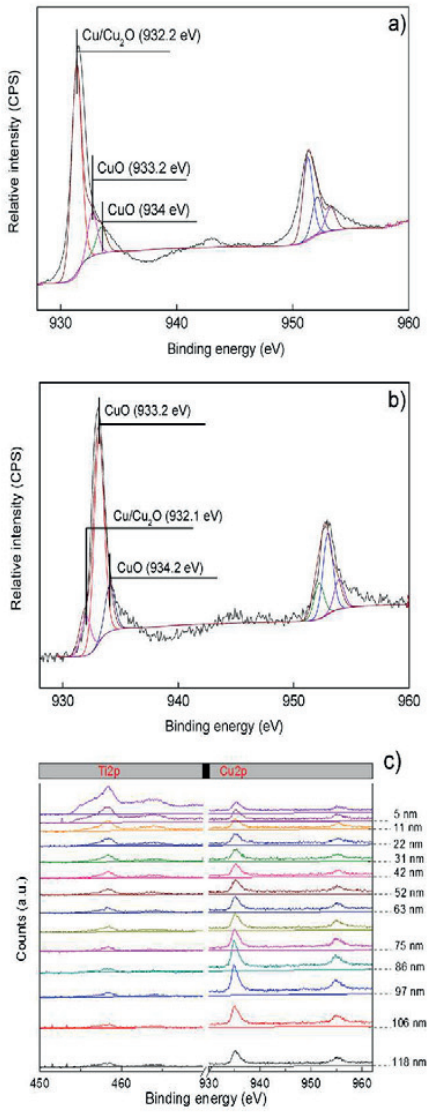

Fig. 5. (a) XPS deconvolution of the Cu2p peak of co-sputtered TiO_2/Cu PES 3 min samples (a) before bacterial reduction of *E. coli*. (b) XPS deconvolution of the Cu2p peak of co-sputtered TiO_2/Cu PES 3 min samples bacterial inactivation of *E. coli* under a Philips Master lamp irradiation18 W/865 (4.65 mW/cm^2). (c) XPS of the TiO_2/Cu-PES top-most layers for a sample co-sputtered for 3 min and etched by Ar^+-ions (5 keV) up to a depth of 118 nm.

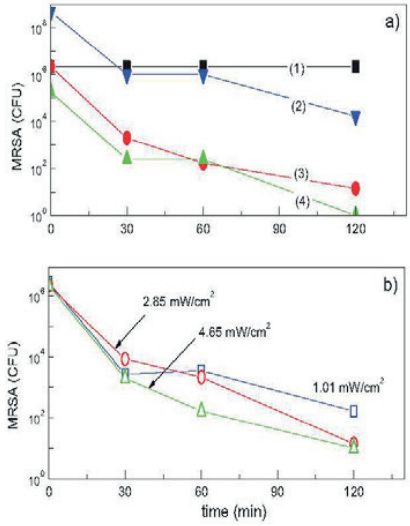

Fig. 6. (a) Effect of initial bacterial concentration of MRSA loss of viability on: (1) PES alone (2) co-sputtered TiO_2/Cu-PES for 3 min tested in the dark, 10^8 CFU/ml, (3) co-sputtered TiO_2/Cu-PES for 3 min tested in the dark, 10^6 CFU/ml, (4) co-sputtered TiO_2/Cu-PES for 3 min tested in the dark, 10^5 CFU/ml. (b) Effect of light intensity irradiation on MRSA bacterial reduction on co-sputtered TiO_2/Cu-PES for 3 min under: 1.01 mW/cm^2, 2.85 mW/cm^2 and 4.65 mW/cm^2.

Production of reactive oxygen species (ROS) by the Cu-clusters on the PES leads to bacterial inactivation due to the interaction of the Cu-clusters with *E. coli* in the dark

$$Cu^{+} + O_2^{\circ-} \leftrightarrow Cu^{\circ} + O_2 \quad (1)$$

$$Cu^{\circ} + O_2 \leftrightarrow Cu^{+} + O_2 \quad (2)$$

The CuO (Cu^{2+}) is reduced

$$Cu^{2+} + e^{-}_{cb} \leftrightarrow Cu^{+} \quad (3)$$

Or by a two electron transfer from Cu^{2+} leading to Cu° atoms

$$Cu^{2+} + 2e^{-} \leftrightarrow Cu^{\circ} \quad (4)$$

The Cu-atoms then coalesce to Cu° nanoparticles settling in the Cu-network of the PES with $E_{redox} = -0.34$ V vs. NHE [30].

Fig. 5c presents the deconvolution of the Ti and Cu, of TiO_2/Cu-PES samples co-sputtered for 3 min as a function of penetration depth of the Ar-ions. By following the decrease of the Ti2p doublet in Fig. 5 it is readily seen that the Ti penetration into the PES reaches ~118 nm (or ~600 atomic layers). The penetration depth of Cu in Fig. 5 on PES goes beyond 118 nm, since the Cu2p doublet is still present when the etching by Ar-ions has attained 118 nm. The etching of TiO_2/Cu-PES layers essentially takes off mostly the PES C-layers.

3.5. MRSA reduction as a function of initial CFU concentration and the effect of applied light intensity

Fig. 6a shows the MRSA reduction as a function of the bacterial loading of the sample on: (1) PES alone (2) co-sputtered TiO_2/Cu-PES for 3 min in the dark with 10^8 CFU/ml, (3) co-sputtered TiO_2/Cu-PES for 3 min in the dark with 10^6 CFU/ml, (4) co-sputtered TiO_2/Cu-PES for 3 min in the dark with 10^5 CFU/ml. After initial optimization the co-sputtered TiO_2/Cu-PES (3 min) samples presented an MRSA bacterial reduction above the activity presented by co-sputtered TiO_2/Cu-PES samples sputtered for 1 and 2 min and are therefore the results presented in Fig. 6. The bacterial reduction of MRSA in the dark is an important observation indicating the potential use of this catalyst in health facilities without any necessity of using photo-energy to activate the disinfection process.

Fig. 6b shows the effect of light intensity on MRSA bacterial reduction by a TiO_2/Cu-PES sample co-sputtered for 3 min applying light intensities of 1.01 mW/cm^2, 2.85 mW/cm^2 and 4.65 mW/cm^2. Fig. 6b shows that only a marginal increase in the MRSA bacterial

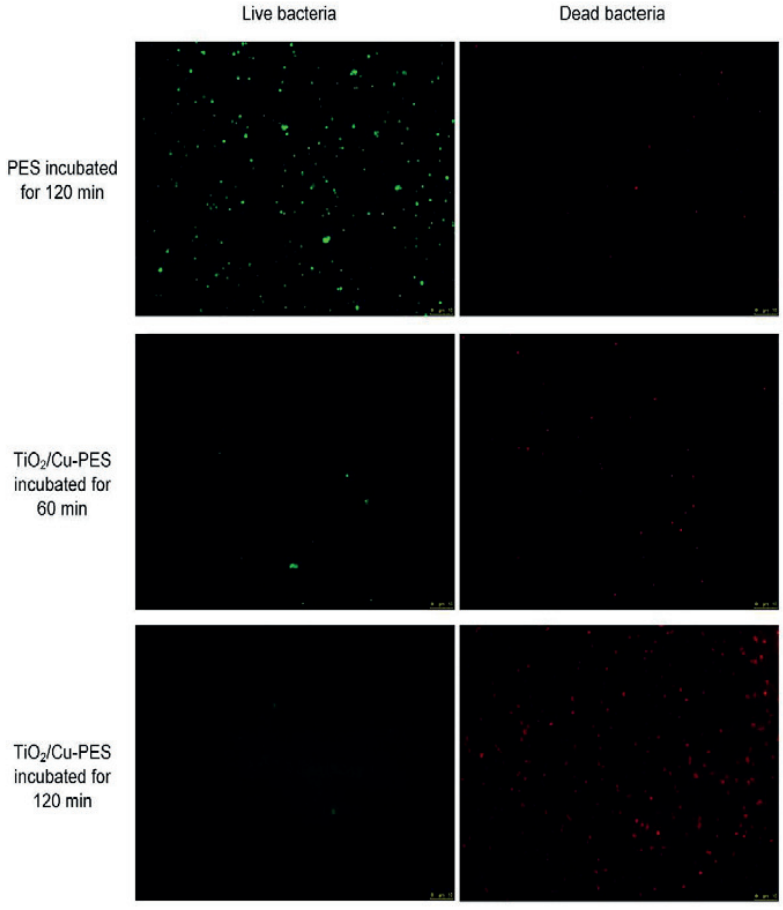

Fig. 7. Stereomicroscopy images of live and dead *E. coli* on TiO_2/Cu-PES showing the loss of bacterial viability within 120 min.

reduction was observed with an increase in light intensity. We suggest that the MRSA reduction is predominantly controlled by the Cu/Cu-ions inherent toxicity and not by the reactive oxygen species (ROS) induced by light irradiation of the TiO_2/Cu-PES sample.

In the case of *E. coli* bacterial reduction under light shown in Fig. 4a, the TiO_2/Cu-PES samples sputtered for 3 min (trace 3) reduced by a factor of two the *E. coli* reduction time when the light intensity was increased from 1.01 to 4.65 mW/cm^2. An accelerated reduction was observed for *E. coli*, but this was not the case for MRSA. The Gram-negative *E. coli* has a thinner peptoglycan cell wall compared to the Gram-positive MRSA and containing additional bilayers with a high structural complexity [11,12]. The significant difference in wall thickness/wall microstructure between MRSA and *E. coli* lead to the different interaction for *E. coli* and MRSA with TiO_2/Cu-PES surface under light irradiation. MRSA is a Gram-positive bacteria presenting a cell-wall ~40–80 nm thick with a peptoglycan content >50%, a lipid content of <3% and no lipo-polysaccharide content. *E. coli* is a Gram-negative bacteria presenting a cell wall thickness of ~10 nm with a peptoglycan content of 10–20%, a lipid content of <58% and a lipo-polysaccharide content of 13%.

Kühn et al. [31] reported recently a fast bacterial reduction of *E. coli* and *P. aeruginosa* compared to *Staphyloccocus aureus* presenting a thicker cell wall. Another important difference for the interaction of both types of bacteria with CuNP's is the strong electrostatic interaction of Cu NP's-positive-ions with the negative lipopolysaccharide (LPS) outer layer of *E. coli*. This is not the case for the interaction between MRSA and the Cu NP's positive-ions since both surfaces present similar charges. Cu-NP's absorption has been reported on the teichoic acid and the peptoglycan outer MRSA cell wall [32]. For *E. coli* and MRSA the bacterial reduction has been reported to occur on several metal and oxide agents [29,33,34].

3.6. E. coli *staining and viability on* TiO_2*/Cu-PES samples*

Fig. 7 shows the live and dead *E. coli* bacteria by using the dye fluorochrome that enters the cell and stains the DNA only if the cell wall membranes are damaged since under this condition they show an abnormal high permeability [35]. To test the time at which *E. coli* cells suffer destabilization/damage of the cell wall leading ultimately to cell dead (red dots indicate membrane damage), the cells were incubated on PES in the dark and also incubated for 60 and 120 min on TiO_2/Cu-PES. The *E. coli* cells kept alive (green dots) on PES alone up to 120 min of incubation. But for cell incubated for 60 and 120 min on TiO_2/Cu-PES, the loss of viability becomes faster as the incubation time increases leading to membrane damage and cell death as shown by the red dots in Fig. 7 [36].

4. Conclusions

The trend presented in visible light induced bacterial extent and reduction kinetics of *E. coli* and MRSA on TiO_2/Cu-PES are shown to be different. Possible reasons are suggested/discussed to account this observation. There seems to be a significant stabilizing effect of the TiO_2/Cu-PES on the release of Cu during bacterial reduction compared to Cu alone as monitored by ICP-MS in the ppb range. This suggests an oligodynamic effect responsible for the bacterial reduction of *E. coli* and MRSA. This effect is beneficial for a longer operational lifetime during repetitive bacterial reduction processes.

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Zhang, L., Dillert, R., Bahnemann, D. and Vormoor, M. (2012) Photo-induced hydrophilicity and self-cleaning: models and reality. *Energy & Environmental Science* **5**, 7491-7507.

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Zimlichman, E., Henderson, D., Tamir, O. and et al. (2013) Health care–associated infections: A meta-analysis of costs and financial impact on the us health care system. *JAMA* **173**, 2039-2046.

Curriculum Vitae

Poster and Presentations

- Ballo M. K. S., Rtimi S., Kiwi J., Pulgarin C., Moreillon P., Entenza J.M., Bizzini A., *In Vitro* and *in Vivo* Protective Activity of Novel Metal Nanoparticle-based Catheter Coating Against Methicillin-Resistant *Staphylococcus aureus* infection; 12th annual assembly of the D-Day. 29th March 2016. Lausanne, Switzerland.

- Ballo M. K. S., Rtimi S., Kiwi J., Pulgarin C., Moreillon P., Entenza J.M., Bizzini A., *In Vitro* and *in Vivo* Protective Activity of Novel Metal Nanoparticle-based Catheter Coating Against Methicillin-Resistant *Staphylococcus aureus* infection; Abstract B-082. 55th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC). 17 to 21 September 2015. San Diego, USA.

- Ballo M. K. S., Rtimi S., Kiwi J., Pulgarin C., Moreillon P., Entenza J.M., Bizzini A., *In vitro* activity of innovative metal combination coated catheters against methicillin-resistant *Staphylococcus aureus*Abstract P018. 73nd Annual Assembly of the Swiss Society for Microbiology. 27-29 May, 2015. Lugano, Switzerland.

- Ballo M.K.S., Rtimi S., Entenza J.M., Kiwi J., Pulgarin C., Bizzini A., *Activity of copper-sputtered surfaces against multidrug-resistant Staphylococcus aureus and Staphylococcus epidermidis. Sy*mpostaph: Des infections staphylococciques à la biologie du microorganisme. Lyon,October 2014

- Ballo M.K.S., Rtimi S., Entenza J.M., Kiwi J., Pulgarin C., Bizzini A., *Activity of copper-sputtered surfaces against multidrug-resistant pathogens.* Abstract P097. 72nd annual assembly of the Swiss Society for Microbiology (SSM). Fribourg, June 2014

Invited Seminars

- Invited speaker in the Electronic Microscopy Facility (EMF) jubilee symposium 27th October 2015

- I gave an annual presentation during the “Friday seminar Program” at the Département de Microbiologie Fondamentale (DMF). Within the DMF, every Friday, a seminar where guests and also members (Ph.D. student and Post-doc) of the DMF can present their current work, is organized. 8th May 2016

Teaching

- Teaching at “Practical courses of Microbiology of prokaryotes” during spring semester (2015/2016) at UNIL.
  Responsables: Pr. J. R. van der MER, Dr C. Keel, Dr J.M. Entenza

- Lecture: “Alternative approach to fight against nosocomial infections”. Given during the course “Résistance Bactérienne aux Antibiotiques”
  Responsable: Dr J.M. Entenza

Awards

- Winner of the MT 180 (Ma thèse en 180 secondes) of IDEUM (IMUL, DMF and EPFL Unconventional Meeting) 1st out 11 participants; 28th June 2016

Myriam K. S. BALLO

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Passionate by science I am looking for a new position to advance my career in clinical microbiology. I gained excellent technical and communication skills in working multidisciplinary teams. My spirit of initiative and my ambition appreciated during all my previous professional experiences will be valuable assets for this post.

EDUCATION

2013-2017 **PhD in clinical microbiology**
Swiss Federal Institute of Technology Lausanne (CH)
Evaluation of antimicrobial surfaces for the prevention of nosocomial infections

3 publications in international peer reviewed journals
Participation in international conferences

Teaching:
- Practical courses on microbiology of prokaryotes
- Lecturing on Bacterial resistance to antibiotics

Certification of Animal Testing from the Swiss Federation of Cantonal Veterinary Surgeons

2008-2012 **Master and Bachelor in Health Engineering**
University of Montpellier (FR)
Mastering the specificities of the medical product throughout its lifecycle in the company

Clinical trials: Knowledge of drug discovery process
Biotechnology: Monoclonal antibody production

SOFT SKILLS

Chair of the student office:
- Obtaining of sponsors for sports events: ski/kayak
- Budget management

Languages:
- French: native
- English: fluent
- Spanish: intermediate

Computer skills:
- Microsoft office; Image J; End Note; Adobe illustrator; Graphpad Prism

INTERESTS

Volunteering:

- Nouvelle Aventure Junior: Counselor in summer camp
- Caribana Festival: Drinks and food supplying sector

Travels

Belgium; Spain; United-Kingdom; Italy; Mali; Tunisia; India; United-states; Cuba

PROFESSIONAL EXPERIENCES

2012-12 months **Research assistant**
Sanofi (FR)
Preclinical study: Evaluation of antifungals agents against candidiasis caused by *C. glabrata* in a murine model

2011-5 months **Research assistant**
University of Manchester (UK)
Monitoring the stress response of bacteria induced by drying process

2010-2 months **Research assistant**
University of Brighton (UK)
Assessment of bacteriophage based therapies for the prevention of infection

2009-2 months **Production operator**
Naho cosmetic (FR)
Production of natural cosmetics in accordance with the Health and Safety legislation (EcoCert)

2008-2 months **Research technician**
National Center for Scientific Research (FR)
Visualization of *Toxoplasma gondii* membrane

TECHNICAL SKILLS

Presentation of results in lab-meeting
Performed data, analysis and written reports
Good Laboratory Practice/Biosafety laboratory (level 2)
Good Manufacturing Practice
Preparation of culture media, reagents for bacterial growth
Establishment of biofilm model
Isolation and identification of microorganisms
Evaluation of antimicrobial resistance
Conducting bacteriophages plaque assay
In vivo experiments: Intraperitoneal and intravenous injection; anesthesia; implantation of medical device
Fluorescence Microscopy
Transmission electron microscopy
Anaerobic chamber
Multifunctional microplate filter-based reader
Protein extraction
Immunodetection