Biocidal and Sporicidal Efficacy of Thermal Spray Copper Alloy Coating with Varying Degrees of Roughness

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
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Abstract

The biocidal and sporicidal efficacy of copper alloy coating was compared to copper alloy sheet and stainless steel coupons. For surfaces with roughness values of 0.1 and 3.5 μm, no significant difference in the killing of *Escherischia coli*, *Staphylococcus epidermidis* and *Bacillus subtilis* was observed between copper alloy sheet and thermal spray copper alloy coupons. Scanning electron microscopic analyses revealed that the degradation of *B. subtilis* endospores and formation of nanoflowers begin within 2 hours after exposure to the copper surfaces. Flower-like nanostructures appeared in intimate contact with partially degraded endospores. Energy dispersive technology analysis revealed that nanoflowers were composed of carbon-copper-phosphate crystals. Focused ion beam sectioning revealed that the majority of nanoflowers have petal-like structures. This study indicates that thermal spray copper alloy coatings are as effective as copper alloy sheet in the destruction of endospores; thus representing a cost-effective and viable strategy for decreasing the risk of infection within hospital settings.
Acknowledgments

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To my parents

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>DPA</td>
<td>Dipicolinic Acid</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy Dispersive Spectrometer</td>
</tr>
<tr>
<td>FIB</td>
<td>Focused Ion Beam Sectioning</td>
</tr>
<tr>
<td>GIS</td>
<td>Gas Injection System</td>
</tr>
<tr>
<td>HAI</td>
<td>Hospital Acquired Infection</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>LB</td>
<td>Lurian-Bertani</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-Binding-Protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>Ra</td>
<td>Roughness value</td>
</tr>
<tr>
<td>SASP</td>
<td>Small Acid Soluble Spore</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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CHAPTER 1. BACKGROUND

In healthcare settings, despite considerable efforts to develop the protocols to control the infections, hospital acquired infections (HAIs) remain a main reason of mortality. Microbes are able to thrive on any surface. Studies have shown, some microbes are able to stay alive on stainless steel and polymer substrates for long periods of time [1, 2]. The endospores of some strains may stay dormant for many years [3], and as a result, surfaces act as microbial reservoirs. Numerous studies have focused on disinfectants that can improve surface hygiene. One of the challenges with the use of disinfectants is the lack of lasting impact, meaning that they cannot continuously reduce the level of surface contamination. In addition, some disinfectants are hazardous to human health (skin irritation and chronic cough) [4]; thus, it is important to ensure surfaces do not recontaminate quickly. Ideal surfaces should keep their antimicrobial properties permanently, reducing availability for microbial contamination.

As such, considerable research efforts are currently directed towards the development of metallic surfaces such as silver, zinc, and copper alloys, which provide antimicrobial activity and are both environment friendly and wear resistant. Numerous studies have demonstrated that alloys with greater than 70% copper content can reduce bacteria contamination within a few minutes. Furthermore, copper retains its antimicrobial properties over time [1, 5]. However, using copper alloy sheet instead of stainless steel to construct hospital furniture and equipment is expensive and a major engineering challenge. Previous studies demonstrated that thermal spraying of copper
alloy onto an organic surface leads to the killing of *E. coli* and *S. epidermidis* within 2 hours[6]. Furthermore, thermal spray copper alloy coating combines relatively low cost with good mechanical properties, and over time can be widely used to coat furniture and medical devices found within hospitals. Moreover, the thermal spray copper alloy coating makes a rough surface topography with a high surface area due to coating formation, which could increase the bacterial adhesion to the surface thereby increasing the numbers killed. While thermal spray copper alloy coating holds promise for rapidly killing microbes, its ability to kill microbial endospores has not been evaluated.

This study was focused on the biocidal efficacy of copper alloy sheet and thermal spray copper alloy coating each at two different surface topographies, and the sporicidal impact of the copper alloy sheet and thermal-sprayed copper alloy coating.
1.1 Introduction

1.1.1 Spread of microbial infections

Nosocomial Infections (NIs) are a major threat to human health in healthcare settings. HAIs are often caused by the buildup of surface contaminants—microbes and viruses—on medical equipment and furniture that [7] leads to infection rates. The spread of microbial pathogens remains a major issue in spite of stringent cleaning protocols with disinfectants. Microbes have evolved diverse survival strategies for overcoming environmental stress, toxins, and antibiotics.

One strategy used by microbes to survive is the formation of biofilms that consist of bacteria surrounded by an amorphous polysaccharide layer that makes them more resistant to disinfectants [8]. Another adaption to environmental stress is the formation of endospores that are highly resistant to common sterilization techniques (e.g. heat, radiation). Endospores can remain viable in a dormant state for centuries [3]. In contrast to biofilms, endospores bind to the surface reversibly, facilitating their spread in healthcare environments.

An initial weak reversible bond of bacteria to substrates precedes the formation of biofilms and endospores. Consequently, a great deal of effort has been spent in the development of antimicrobial coating substrates. Not only do these have biocidal efficacy but they are also refractory to the primary microbial adhesion mechanisms (biofilms and endospores) [9].

Hospital furniture and equipment are commonly made of stainless steel, due to its relatively low manufacturing cost, aesthetic appeal, strength and inertness. However, repeated contact with abrasive detergents and hard materials creates indentations and scratches that can harbor microbes and viruses. Trapping of bacteria in grooves increases their resistance to mechanical shear forces. As such, the development of antimicrobial metallic materials to manufacture furniture and equipment in healthcare centers and facilities. Antimicrobial metallic materials should have two
Chapter 1. Background

characteristics: 1) they must have a redox–active surface under ambient conditions, and 2) they must release ions that are toxic to the microbes and viruses. Antimicrobial metals include: silver, zinc, and copper. Among these, copper alloy is the most promising because of its ductility, corrosion resistance, strength, and cost-effectiveness. In addition, copper has been registered as the first antimicrobial material with the U.S. Environment Protection Agency[10].

1.1.2 Antimicrobial Properties of Copper

Copper ions are essential in many biological processes. Copper is a co-factor for several enzymes, including cytochrome oxidase. For these enzymes, depending on the copper redox state, copper serves as either an electron donor (Cu^{+1}) or receiver (Cu^{+2}). While, copper ions are fundamental, high concentrations are lethal and can kill microbes through oxidative stress, loss of enzymatic activity, and membrane damage. The highly reactive species produced through Fenton chemistry—a reaction in which iron catalyzes hydrogen peroxide and generates highly reactive hydroxyl radicals—can participate in lipid peroxidation and oxidation of proteins, which can lead to loss of membrane fluidity and deactivation of some enzymes [10]. Cu (I) is also able to oxidize sulfhydryl groups in cycle, of the amino acids cysteine and methionine depicted in reactions 1 and 2 shown below [10]:

\[
2Cu^{2+} + 2 RSH \rightarrow 2Cu^{+} + RSSR + 2H^+ \quad (1)
\]

\[
2Cu^{+} + 2H^+ + O_2 \rightarrow 2Cu^{2+} + H_2O_2 \quad (2)
\]

Cu (I) is able to break iron-sulfur clusters in cytoplasmic hydratases and release iron (Fe), which can cause oxidative stress [11, 12]. A previous study indicated that copper can damage DNA through the generation of hydroxyl radicals and the oxidation of DNA [13]. However, it is unlikely that this was the main killing mechanism in this study, because the killing kinetics were too fast.
— over 95% of bacteria were killed in the first 5 minute exposure to the copper alloy surfaces. Furthermore, DNA analysis of dead cells [14] shows that genotoxicity, and DNA lesions are not the underlying causes of antimicrobial activity of copper.

Copper has been used as a disinfectant for water and food since ancient times [15]. Ancient civilizations benefited from the antimicrobial properties of copper before microbes had been fully understood. They used copper to treat leg ulcers or skin problems. In recent times, in order to reduce the extent of bacteria and biofilm formation intensive efforts have focused on developing metallic copper surfaces that can kill microorganisms in a process known as contact killing, which kills microorganisms on a time scale of minutes [16, 17]. However, healthcare institutions continue to manufacture furniture and equipment using polymers or stainless steel because of the high cost of copper. An alternative cost-effective method to deposit a layer of copper on furniture and equipment to give them antimicrobial properties. Thermal spray coating technology is a material deposition strategy that is cost effective, highly adaptable and allows for rapid coating of material onto a wide range of organic and inorganic surfaces.

1.1.3 **Thermal Spray Technology**

Thermal spray technology encompasses a group of coating processes that generate an energetic hot or cold gas through a torch or gun. The torches in thermal spray are for feeding, accelerating, heating, and directing the flow of materials towards a given substrate. The high temperature and speed of particles cause significant droplet deformation on impact at a surface, producing thin layers called “splats” [18]. Several layers of splats form the coating. Thermal spray processes are defined by the source of energy, and can be classified into three groups: compressed gas expansion, combustion spraying, and electrical discharge plasma spraying. The most common types of electrical discharge plasma spraying are direct current (DC), radio frequency (RF), direct
current transferred arc plasma, and twin-wire arc. Among these, the twin-wire arc spraying technique is the most economical with lowest operating equipment costs [19].

1.1.3.1 Twin-Wire Arc Spray

Twin-wire arc spraying technology was first introduced in 1910, but it was not used commercially until the early 1960s [19]. In this technology, a high current of 280 amps and a voltage of 29 V is used to generate an electric arc between two consumable wire electrodes (feed rate of 7 m/min). The electric arc ionizes a high velocity gas that passes over the electrodes, and forms a high-temperature plasma. The plasma melts the tip of the electrodes to generate particles which are then accelerated toward the target substrate.

![Figure 1: Principle of Twin-wire arc spray](Source: Oerlikon Metco Thermal Spray Brochure (moderated image))

Some of the advantages that make twin wire arc spray effective and efficient are [6, 18, 20, 21]:

In this method, the particles start cooling immediately after forming in the arc zone; thus, less heat is transferred to the substrate, which creates an opportunity to apply the coating on a variety of different surfaces such as wood, engineered medium-density fiberboard (MDF), and polymer substrates.
• Particle size and distribution can be controlled through current, voltage, and air pressure. Increasing the applied current or wire size and decreasing the voltage, pressure, flow increases the size of the particles. The size of droplets can be changed from submicron to about 200 µm.

• The simple design of the equipment and the low cost of wires and electrical power makes this process cost effective for coating surfaces.

• Twin wire arc spray is portable, making it an ideal technology for off or on-site coating.

• It uses non-flammable gases.

• Twin wire arc spray technique generates a rough surface topography. Grooves and features of the coating distributes randomly and is characterized by different parameters: arithmetical mean deviation (Ra), maximum height of profile (Rz), maximum profile valley depth (Rv), and root mean square deviation (Rq). The surface features and grooves are usually characterized by the parameter Ra.

1.2 Objectives

The objectives of my studies were:

1) To compare the impact of surface topography on biocidal efficacy of thermal spray copper alloy coating and copper alloy sheet.

2) To investigate the effectiveness of the thermal spray copper alloy coating and copper alloy sheet in killing endospores.

The thesis starts with an overview of the influence of surface topography on microbial adhesion, biofilm formation, and bacteria killing. The results present the impact of surface topography on microbial killing. In addition, sporicidal efficacy of copper and the appearance of nanoflowers are discussed.
CHAPTER 2. LITERATURE REVIEW

Nosocomial infections (NIs) are the sixth leading cause of mortality in developed countries [22]. Surface contamination is the main contributor to the outbreak of HAIs. Various surface cleaning strategies have been used in attempt to control HAIs, but the spread of microbes remains a major issue because microbes have evolved diverse strategies in response to environmental stress and toxins. One strategy is to secrete biofilms — a group of bacteria surrounded by extracellular polymeric substance, highly resistant to hospital disinfectants. Another strategy is endospore formation — highly stable structure— resistant to heat and disinfectants.

2.1 Bacterial Adhesion and Biofilm Formation

Biofilms are involved in 80% of infections [23]. Biofilms are a community of a single or multiple bacterial species surrounded by an exopolysaccharide matrix, which can strongly adhere to biotic or abiotic surfaces. The entrapped bacteria are three log10 more resistant to antibiotics. Thus, inhibiting or reducing biofilm formation could retard the spread of contamination.

The first step of biofilm formation is the adhesion of bacteria to the surface. The type of surface affects bacterial adhesion, therefore understanding the properties and topography of the surface is crucial to the inhibition of biofilm formation [24]. There are different opinions about the effect of surface properties on bacterial adhesion and biofilm formation. Some studies have shown a positive correlation between surface topography and bacterial adhesion [25, 26] whereas others have found any correlation [27, 28]. However, it was shown that surface irregularity larger than the diameter of bacteria might entrap the cells. Smooth surfaces do not favor bacterial adhesion
and biofilm formation because of lower friction and lack of shear resistance [29]. It was also shown that a small increase in surface roughness (Ra value) had a significant effect in bacterial adhesion. However, a large increase in surface roughness did not have effect on bacterial adhesion [30]. Increases in surface roughness from 0.04 µm to 0.30 µm increased bacterial adhesion while increasing surface roughness from 0.04 µm to 0.96 µm did not have a significant effect [31]. The presence of the grooves impact the cell attachment, and a great accumulation of bacteria can be seen in the bottom of the grooves because of shear forces [32]. However, groove widths have no significant impact on cell attachment [29].

Another factor that impacts bacterial adhesion is the charge of the surface, as negatively charged surfaces attract more bacteria than positively charged surfaces [24].

### 2.2 Bacterial Endospores

Bacteria are divided into two groups based upon their cell wall structures, gram-negative bacteria and gram-positive bacteria. Gram-negative bacteria do not keep the crystal violet stain that is used in the gram staining method, and they have a thin peptidoglycan cell wall between cell membrane and outer membrane—*Escherishia coli*. Gram-positive bacteria take up the crystal violet stain and become purple. They have a single lipid membrane and a thick layer of peptidoglycan, and they usually form an endospore coat under stressful conditions—*Bacillus subtilis* [33]. Gram-positive bacteria specifically *Bacillus subtilis* has been studied a lot. One of the features of *Bacilli* is their ability to form endospores. Endospores are highly resistant structures, which are formed under stressful conditions. The process in which cells make endospores was first observed about 125 years ago when anthrax disease was studied (Figure 2) [34]. Endospores can survive for hundreds to millions of years [35]. Thus, endospores have special structures and mechanisms to protect their biomolecules during dormancy. These cells are able to resist a wide
variety of stressful conditions including heat, chemicals (acids, bases, aldehyde and organic solvents, oxidizing and alkalyting agents), and radiation [36]. However, a few chemicals that can damage endospore layers and kill them. Four factors that play a main role in endospore resistance are: endospore coats, low water content of endospore core, the presence of small acid soluble proteins (SASP) of the α/β-type, and low permeability of the core—presence of dipicolinic acid DPA [37].

Endospores have unique structures (Figure 3). In Bacillus subtilis the endospore coat is the outermost layer. It is comprised a complex of more than 50 different proteins. The coat acts as the primary defense of the endospores, providing resistance to chemicals including chlorine dioxide, hypochlorite, ozone, and peroxynitrite [38-40]. These chemicals could be detoxified in the endospore coat [41]. The endospore coat also has an important role in protecting the endospore cortex from peptidoglycan-lytic enzymes [38]. The next layer after coat is called the outer layer; however, its function is not clear. There is contrasting data about its permeability against some
chemical agents [42]. The cortex and germ cell membrane are the next layers of the endospores. They are both made of peptidoglycan similar to vegetative cells, but differ in cross-linking and precise composition [43]. The cortex is degraded during endospore germination, whereas the germ cell membrane becomes the cell wall of vegetative cells after germination [44]. The inner membrane is a highly condensed layer before the endospore core. The inner membrane composition is very similar to the plasma membrane of the bacteria (*Bacillus subtilis*). However, lipid molecules in this membrane are immobile. It seems that levels of unsaturated fatty acids of inner membrane influence endospore chemical resistance, and the levels of them depend on the temperatures that the endospores were formed [45]. This layer could protect the DNA of the endospores against some of the chemicals [42].

The core contains DNA, and proteins. A high percentage of molecules within the core is similar to those present in vegetative cells. However, there are some unique molecules that play key roles in endospore resistance. The first type of molecule is dipicolinic acid (DPA), which comprises 15% of the dry weight of *Bacillus* endospores. It is synthetized in the mother cells during sporulation and excreted during the first minutes of germination. DPA is responsible for water reduction in the core, inferring wet-heat resistance [42]. The second type of molecule in the core are small, acid soluble spore proteins (SASP) of the α/β-type, which are synthetized in mother cells during sporulation. It seems, they are responsible for some resistance to chemicals, heat, and UV radiation. Therefore, to damage the endospore DNA, first α/β-type SASP must be degraded [37]. Since, most of disinfectants are water-soluble, the low water content of endospore cores could be a barrier to them.
Figure 3: Cross section of an endospore of Bacilli
Source: Joseph et al. [46] (moderated image)

As an initial weak reversible association of bacteria to substrate precedes the formation of biofilms and endospores, various surface cleaning strategies have been used to control biofilm and endospore formation. However, many of these strategies have temporary effects, they can cause environmental pollution, and be harmful to human health (phenols and chlorine that irritate asthmatic or respiratory-impaired patients) [47]. Formaldehyde and glutaraldehyde are sterilizing agents which react with the membranes of vegetative cells and endospores and kill them. However, they are carcinogenic, and highly reactive with organic and non-organic materials, which can lead to surface corrosion as well. Thus, developing sterilants that are nonreactive to nontarget materials and nonhazardous to humans is necessary.

Recent studies have focused on using surface substrates with biocidal and sporicidal properties that are refractory to the primary adhesion of microbe. The death of microbes on surfaces can significantly reduce the transmission of microbes [47]. Several studies have demonstrated the
biocidal efficacy of transition metals [48, 49]. Studies by Jessica et al (2010) showed the biocidal efficacy of transition metals is due to the production of hydroxyl radicals in Fenton reaction [50]. Therefore, these metals can be used in antimicrobial surfaces, which are not toxic to humans [51]. Silver, copper and zinc are transition metals with significant anti-microbial activity. Among the three, silver is the least cytotoxic and the most potent against bacteria, and zinc is the least effective as a bactericide. Economically, copper is more cost-effective than silver, and has strong inhibitory effects on fungi [52, 53]. In addition, copper has much greater corrosion resistance and is more suitable for general use in industrial and domestic environments [54]. Studies have suggested that surfaces containing over 60% copper can be effective in killing bacteria [10, 14].

2.3 Copper

Early records on the medical use of copper as an inorganic biocide date back to the second millennium BC when it was used to disinfect wounds and drinking water. Its therapeutic use became widespread in the 19th century, and in 1930s, it became commercially available as an antimicrobial agent for the treatment of infectious disease. With the increase in number of antibiotic-resistant bacteria within the healthcare setting, copper once again received a lot of attention for its antimicrobial properties [55]. The antimicrobial activity of copper is achieved by two steps: first, copper ions are released from the surface of a substrate, and then they adhere to the surface of the bacteria, and damage the cell membrane [23]. As an antimicrobial agent, copper can be used in the form of nanoparticles, metal surfaces and coatings [56].

The advanced use of nanoparticles (NP) is a novel approach being implemented to fight and prevent diseases. Among all nanoparticles with antimicrobial properties, metallic nanoparticles have attracted global attention because of their high chemical activity due to their large surface to volume ratios that allows them to interact with microbial membranes [57]. Nanoparticles also can
move across cell membranes faster than microparticles, and can be coated on the surfaces of medical devices and equipment [58]. However, extensive use of NP will lead to their accumulation in the environment, especially in landfills and water effluents. Furthermore, it could affect the populations of microbes that have positive effect on the environment (microbes have essential role in cycling) [59].

Copper surfaces in laboratory settings and hospital trials have showed a great killing efficiency against a wide range of microbes. Microbes that make direct contact with copper die rapidly [60]. Although the biocidal efficacy of copper is well known. Healthcare industries continue to manufacture furniture and devices using stainless steel, and polymers due to the prohibitive cost of using copper. As a result, techniques that coat a surface with copper rather than manufacturing equipment out of copper have gained a great deal of attention recently. One such technique is thermal spray copper technology.

### 2.4 Thermal Spray

There are different metal spray techniques that can be used to deposit copper alloy coating on surfaces including flame spray, plasma spray, high velocity oxygen fuel (HVOF), and twin-wire arc spray. The flame spray process generates a coating with high porosity, high oxide levels, and it is difficult to coat inner surfaces. Plasma spray is expensive and the plasma spray gun deteriorates rapidly. High velocity oxygen fuel (HVOF) generates a high-density coating with great corrosion resistance, but gives high heat, thus extra cooling of the substrate is necessary. Twin-wire arc has the highest deposition rate, is cost effective, easily coats inner surfaces, and uses low heating making it accessible for thermally-sensitive substrates [19]. A common feature of all of these techniques is the lenticular grain structure of the coating, resulting from the rapid
solidification of small droplets of copper alloys. Thermal spray produces rough surface topography that impacts bacterial adhesion and subsequently, biofilm formation.
CHAPTER 3. MATERIALS AND METHODS

3.1 Preparation of Bacteria

Bacteria used in this study were a Gram-negative bacterial strain (*Escherishia coli*), and two
Gram-positive bacteria (*Staphylococcus epidermidis* and *Bacillus subtilis*). Bacteria were grown
on Luria-Bertani (LB) medium at 37 °C overnight. A single colony of each was suspended in 5
mL of LB and left in a rotatory shaker at 37 °C for 7-8 hours. spectrophotometery was used to
monitor bacteria growth at optical density 600nm (OD$_{600}$). Cells in the middle of log phase
(OD$_{600}$=0.5) were used for adjusting to approximately 7 × 10$^7$ bacterial cells/mL by dilution in LB
broth.

3.2 Copper Alloy and Stainless Steel Coupon Preparation

A twin-wire arc spray with a high velocity gun (Sulzer Metco, Westbury, NY, US) was
utilized for coating grit blasted stainless steel coupons. The feedstock wire was CuNiZn with
composition shown in Table 1. The coating thickness was 400 µm, and all the coupons were 2.5
cm in diameter. The copper alloy sheets that were used, had the same copper content and similar
concentration for the other alloys. Copper alloy sheets were prepared using C75200 copper alloy
registered as antimicrobial by Environmental Protection Agency (US). This type of alloy has better
corrosion resistance compared to other copper alloys. In addition, C75200 copper alloy does not
tarnish after contact with skin, and looks like stainless steel. Uncoated stainless steel coupons were
used as controls in all experiments.
Chapter 3. Materials and methods

The surface of coupons was polished with a grinding and polishing apparatus (EcoMet 300, Buehler, USA). The apparatus was served with silicon carbide paper of different abrasive particle sizes. The surface roughness was measured with a stylus profiler (Alpha-Step D-120, KLA-Tencor, and Milpitas, CA).

The twin wire arc spray technique used in this study generates a dense coating with roughness value (Ra) of 20-30 µm that is not convenient to use for the surfaces of furniture and equipment in hospitals. Therefore, the surface of coupons were polished to two roughness levels; common touch surface Ra=3.5 µm, and mirror like finish Ra=0.1 µm.

To disinfect the coupons, coupons were vortexed in 1% Sparkleen, followed by washing with de-ionized water for three times then sterilized with 70% ethanol and placed in sterile petri dishes prior to use.

3.3 Inoculation of Copper Alloy and Stainless Steel Coupons with Bacteria

A single colony of each bacterial strain was inoculated in 5 ml of sterile LB broth and was kept on a rotatory shaker at 37°C for 24 h. To quantify cell survival on coupon surfaces, cell concentration in suspension was measured by Optical Density at 600 nm (OD600) and series of dilutions were prepared. To assess the biocidal activity of coupons, 25 µl aliquots of culture broth were spread over the surface of copper alloy, and stainless steel coupons and incubated for 5 min at room temperature. To detach bacteria, coupons were soaked individually in 10 ml phosphate-buffered saline (PBS), and vortexed for 1 min. Bacterial survival was assessed by plating 100 µl of bacterial suspension on LB agar and incubated at 37°C overnight. Surviving Bacteria were calculated from the colony forming units (CFU).
3.4 Sporulation, Germination and Inoculation of Spores onto Copper Alloy and Stainless Steel Coupons

The strain used in this study was *B. subtilis*. The bacteria were grown on LB agar at 37°C, harvested after 6-7 days of growth and purified by heating at 100 °C. Spores were removed by centrifugation, washed repeatedly with water [61], then pelleted, resuspended in 0.05%NaCl. For coupon inoculation, coupons were inoculated with endospores at room temperature for 2 h, 1, 3, or 7 days. The coupons were rinsed with water. In all series of experiments in order to make sure that all endospores were deactivated and killed through the exposure to copper, they were germinated before culturing [62]. The endospores used for germination were heat activated in distilled water at 70 °C for 30 min and cooled on ice for 15 min, Spores were activated in MOPS medium supplemented with 10 Mm L-asparagine, 10 mM glucose, 1 mM fructose, and 1 Mm potassium chloride [63] and then aliquots of 100 µl of suspension cultured on LB agar at 37°C overnight. CFU on the agar used to determine the sporicide effectiveness of copper coupons and exposure time.

3.5 Energy Dispersive Spectrometer (EDS), and Surface Topography Analysis

To evaluate molecular composition of the coupon surfaces, endospores, and nanoflowers, EDS (Quantax 70 Bruker Nano GmbH, Berlin, Germany) was used. The 3D surface images were captured with a Hitachi 3000 scanning electron microscope (SEM) equipped with a 3D-image Viewer (Denshi Kougaky Kenkyusyo Co.). All 3D surface images were obtained by merging four SEM images taken at different angles using 3D Image Viewer. EDS microanalysis provides identification of all elements, and results are reported as atomic percent and weight percent.
3.6 Contact Angle Measurement

Surface topography influences hydrophobicity and hydrophilicity of the surface, and combinations of these factors affect bacterial adhesion and mobility [64]. To compare wettability of thermal spray copper alloy coating with copper alloy sheet, the contact angle was measured on left, and right side of the drop, using imageJ software from National Institute of Health (USA).

3.7 Scanning Electron Microscopy (SEM)

Inoculated coupons were fixed using 3% glutaraldehyde in PBS overnight at 4°C. Samples were washed three times with PBS and stained with osmium tetroxide (OsO4)—to sputter coat and create a high electron scattering rate—for 45 min. The samples were then washed with PBS three times and dehydrated in an ethanol (ETOH) series of increasing concentration 50, 70, 80, 90, and 2X 100%. Coupons were then critical point dried by a series of ETOH:hexamethyldisilazane (HMDS) at ratios of 3:1, 1:1, 1:3, 0:1, and 0:1 for 10 min each. In the final 100% HMDS, the samples were left covered in the fume hood overnight. After drying, samples were mounted on stubs and sputter coated with gold-palladium using Balzers Sputter Coated SCD 050 (Capovani Brothers Inc., NY, USA), and visualized using scanning electron microscope SU3500 (Hitachi Ltd, Tokyo, Japan).

3.8 Focused Ion Beam (FIB) Sectioning

The SEM samples were used for FIB sectioning—samples were sputter-coated with gold-palladium. The conductivity of the samples was achieved by addition of two spots of silver paste onto the sample holder. Samples were air-dried before transferring into FIB specimen chamber. Tungsten gas was injected to the area of interest via the needle of the gas injection system (GIS). After deposition of the tungsten gas, nanoflowers were cross-sectioned at an acceleration voltage of 2kV and an ion beam current of 53 µA.
3.9 Statistical Analysis

The Scientific 2D graphic and statistics software GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) was used to calculate significant differences among results. The two way and one way ANOVA with Post-hoc “Tukey's test” were used for multiple sample comparisons.
4.1 Results

Previous studies have demonstrated that microbes are killed within a few minutes of exposure to surfaces containing greater than 60% copper [12]. Thus, copper content of the copper alloy sheet and thermal spray copper alloy coupons used in this study was above 60% (Table 1). The 316 stainless steel coupons (with ~ 60-70% iron, 18% chromium and 13% nickel content by mass) were used as controls.

<table>
<thead>
<tr>
<th>Table 1: Thermal spray copper alloy and copper alloy sheet compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Elements wt.%</strong></td>
</tr>
<tr>
<td>Copper</td>
</tr>
<tr>
<td>Nickel</td>
</tr>
<tr>
<td>Zinc</td>
</tr>
<tr>
<td>Oxygen</td>
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</tbody>
</table>

Sprayed coatings always have a certain level of surface void, which may affect the total surface area, although the level of roughness is the same. Thus, the total surface area that the bacteria were exposed on both the copper alloy sheet, and thermal spray copper alloy coating was measured (Table 2).

<table>
<thead>
<tr>
<th>Table 2: Total surface area on copper alloy sheet, and thermal spray copper alloy coating.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coupon</strong></td>
</tr>
<tr>
<td>Surface roughness</td>
</tr>
<tr>
<td>Total surface area (µm²)</td>
</tr>
</tbody>
</table>

Several reports have evaluated the influence of surface roughness on bacterial adhesion, colonization, biofilm formation and consequently bacterial death. To evaluate the impact of
topography on biocidal efficacy of copper surfaces in this study, bacteria were exposed on copper coupons with different roughnesses for 5 min. Plate count method was used to quantify the number of viable bacteria (Figure 4).
Figure 4: Bacterial survival on copper alloy sheet and thermal spray copper alloy coating for two Ra values.

(a) *E. coli*, (b) *S. epidermidis* and (c) *B. subtilis*. No statistical difference is observed between Ra = 0.1 µm and Ra = 3.5 µm (n=3) (P<0.0001).
Despite a 35-fold difference in Ra values, copper alloy sheet and thermal spray copper alloy coating, were equally effective in killing Gram-negative, *E. coli*, and Gram-positive, *S. epidermidis* and *B. subtilis*. Greater than 95% of the vegetative Gram-negative and Gram-positive bacteria were killed within 5 minutes. Since variations in surface roughness have been shown to promote adhesion of a large variety of bacterial species, the surface topography of the substrates were visualized by SEM equipped with 3D-image viewer (Figure 5).

![3D-Images of surface topography](image)

Figure 5: 3D-Images of surface topography
Sheet metal a₁ (Ra=0.1 μm), a₂ (Ra=3.5 μm), Thermal spray copper alloy coating b₁ (Ra=0.1 μm), b₂ (Ra=3.5 μm).

3D-images of surfaces indicate that, although large variations of surfaces can have similar Ra values (Figure 5), the grooves can have different shapes (V-shaped or flat), and features are dispersed randomly. Furthermore, Figure 5 shows that surfaces with an Ra value of 0.1 μm were composed of parallel grooves, whereas the grooves and the scratches on the rougher surfaces (Ra = 3.5 μm) were wider and oriented in random directions. For further studies, the morphology
and orientation of *E. coli* on the coupons were visualized by SEM. The cells on stainless steel did not show any significant changes and maintained their rod-shapes. While on copper surfaces, the cells lost their regular shapes and aggregated loosely or elongated. Furthermore, the surfaces with Ra value of 3.5 μm exhibited higher fraction of the cells adhered on scratches in different orientations.

![SEM image of adhesion pattern](image)

**Figure 6:** SEM image of adhesion pattern
(a) Thermal spray copper alloy surface with Ra = 3.5 random orientation of bacteria on scratches, (b) Bacteria elongation.

In addition, contact angle measurements showed that copper alloy sheet and thermal spray copper alloy coating had similar contact angles, which corresponded to the same free surface energy. For example, the average equilibrium contact angles for the copper alloy sheet, and thermal spray copper alloy were Θ = 94.4 °, and Θ = 91.1°, respectively (Figure 7).
Figure 7: Water drop in equilibrium on polished metal and coating surfaces, $\theta_s = 94.4^\circ$ and $\theta_c = 91.1^\circ$
4.2 Discussion

Previous reports have indicated a positive correlation between Ra values and biocidal efficacy of copper surfaces [6, 65]. Our study indicates that Ra values of 0.1 and 3.5 μm had no significant impact on the biocidal activity of copper alloy sheet and thermal spray copper alloy coating on *E. coli*, *S. epidermidis* and *B. subtilis*. The discrepancy observed in the reported values for bacteria kill may be due to the variables associated with surface and bacterial cell characteristics.

A commonly proposed explanation of the impact of surface roughness on bacterial adhesion is that rougher surfaces provide more surface area for attachment. Bacteria can become entrapped in grooves when surface topographies are wide and deep. Grooves also provide shear force protection so bacteria can accumulate in them in greater numbers. The attachment of bacteria in and on surface features increase the direct contact area and bacteria kill [29]. Moreover, reports show the binding strength of bacteria oriented longitudinally inside “V”-shaped grooves is stronger than bacteria oriented perpendicularly to the grooves. However, on rougher surfaces with wider scratches, a higher percent of bacteria oriented other than parallel [66]. Thus, shapes, profiles, and orientations of the features have impacts on bacterial adhesion, and biocidal efficacy of copper surfaces [24, 67].

Aside from the effect of surface topography on bacterial adhesion, there is evidence that indicates bacterial adhesion is mediated by factors that include morphology, size, and presence of pili or flagella, cell surface hydrophobicity [68].

Bacteria vary in shape—spherical (cocci), rod (baccilus), spiral (spirilla), comma (vibrios), corkscrew (spirochaetes)—and size—from 0.2-2.0 μm in diameter and less than 1 μm to over tens of micron in length [69]. The similar effect of surface roughness on bacteria kill in this study might be due to the similar cell diameters of the species used: *E. coli* ~ 2.0 μm long and 0.25–1.0 μm in
diameter; Staphylococcus epidermidis ~ 0.5–1.25 μm in diameter; and Bacillus subtilis ~ 2.0 μm long and 0.25–1.0 μm in diameter.

An alternative explanation according to the 3D-images of the surfaces (Figure 5) might be the non-uniform distribution of features that inhibited the parallel alignment of a high proportion of bacteria on the surfaces with Ra values of 3.5 μm. It is reported that maximum adhesion and subsequent bacteria kill occurred at an Ra value close to the diameter of the bacterial cells based on the entrapment hypothesis [24]. Furthermore, the increase of surface air content due to the presence of microstructures leads to higher surface contact angle and lower bacterial adhesion on the samples with rougher surface [70, 71] (Ra=3.5 μm).

Bacteria are relatively rigid due to external peptidoglycan, which is thicker in gram-positive than gram-negative bacteria. In gram-negative bacteria, the presence of an extra lipopolysaccharide layer also helps them to maintain their shapes and hinder their interaction with surfaces that have an Ra value smaller than their diameter [72].

Bacteria also have some proteinaceous features such as pili and flagella, which have an important role in adhesion [73]. However, E. coli, S. epidermidis and B. subtilis used in this study did not have flagella or pili.

Cell surface hydrophobicity is another factor that has an important role in the attachment to the surface. This attachment is usually considered as one of the strongest non-covalent interactions in biological systems. Several studies have been performed on the effect of the hydrophobic outermost layer of the bacteria in contact with solid surfaces and their adhesion [74, 75].

As shown in my results, surface topography also has an effect on bacterial morphology, depending on the species. Some species became elongated while others became smaller [72] (
Figure 6). A change in morphology is one of a bacteria’s responses to an environmental stress, and the maximum changes could be seen when they were exposed to sub-inhibitory concentrations of copper. Metal ions such as Ca$^{+2}$ can maintain the cell envelope (lipopolysaccharide) through binding with penicillin-binding-proteins (PBPs)—located in the cell envelope. Exposing the bacteria to copper surfaces force out Ca$^{+2}$ and deactivates PBPs to cause morphological changes.

### 4.3 Conclusion

The results obtained in this study indicated that changes in Ra values of 0.1 and 3.5 μm had no significant impact on the biocidal activity of copper allow sheet and thermal sprayed copper alloy coating on *E. coli*, *S. epidermidis* and *B. subtilils*. However, this study indicates that thermal spray copper alloy coatings are as effective as copper alloy sheet in the destruction of vegetative cells within 5 minutes. Moreover, since the technology is cost-effective and easy to operate it. It can be applied for a clinical setting.

### 4.4 Recommendations for Future Work

Several studies have focused on the biocidal activities of alloys with different copper contents. However, more research is required on the impact of different bacterial morphologies and sensory appendages, such as flagella, on their adhesion and colonization on copper alloy surfaces. Live imaging of cells using high-speed atomic force microscopy would provide direct and real-time valuable insight at the single-cell level into the adhesion of different bacterial species to surfaces with different topographies and their impacts on biocidal activity.
CHAPTER 5. SPORICIDAL ACTIVITY OF COPPER ALLOY SHEET AND THERMAL SPRAY COPPER ALLOY

5.1 Result

Bacterial endospores present one the most serious challenges in the healthcare services, since some of them are able to survive for a long period. Endospore formation is a process that is usually induced by nutrient reduction and unfavorable survival environment [76]. Endospores (Figure 3) are resistant to a variety of treatments including chemical, heat and radiation. The factors involved in endospore resistance include the multilayer endospore coat, the dehydrated cytoplasm, and the presence of some proteins that protect the endospore’s DNA [77]. There have been extensive research over the last decade to develop disinfectants that are not hazardous to humans or to the environment [78], but can kill endospores. The effectiveness of copper ions in killing microbes has been well established. However, there is little information on deactivation of bacterial endospores with copper ions. Since endospore formation can be induced by adverse conditions such as nutrient deficiency, endospore-forming bacteria, *B. subtilis*, were inoculated on LB plates and incubated at 37°C for one week. Schaeffer-Fulton staining of colonies showed greater than 85 – 90% of bacteria changed to endospores (Figure 8).
Chapter 5. Sporicidal Activity of copper alloy sheet and thermal spray copper alloy

Figure 8: Schaeffer-Fulton staining of *B. subtilis* before (a) and after (b) sporulation

To kill the residual bacteria before adding to coupons, endospore suspension in saline was heated at 100°C for 10 minutes. Then it was centrifuged at 1530 revolution per minute (rpm) for 2 min at 4°C, washed, and re-suspended in water. Copper alloy coupons, and stainless steel were inoculated with 20 µl of the suspension for 2 hours. Despite the preincubated of the endospore suspension, a small amount of viable bacteria were noticed after 2-hour exposure of endospores to stainless steel at room temperature (Figure 10). However, exposure of preincubated endospore suspension to copper alloy sheet and thermal spray copper alloy coupons indicated the complete killing of residual vegetative cells (Figure 9).
Figure 9: Spore germination before using germination solution
(a) Stainless steel, (b) control sample, (c) copper alloy sheet, (d) thermal spray copper alloy.

To assess the viability of endospores after exposure to copper alloy sheet, and thermal spray copper alloy coupons, endospores were removed from the coupons, the suspension was heat activated (30 min at 70°C) and grown in rich media (germination buffer) [79]. Viability of the endospores was determined 12 hours later by colony counting (Figure 11). Heat activation and addition of germination buffer to endospore suspension resulted an approximately 6-fold increase in the number of colonies. The results demonstrated that endospores could rescue from dormancy following or during exposure to stainless steel (Figure 10). However, exposure of the endospores to germination buffer before plating on LB agar demonstrated an 85 – 90 % decrease in endospore survival (Figure 10).
Figure 10: Comparisons of *B. subtilis* endospore killing effectiveness of stainless steel, copper alloy sheet and thermal spray copper alloy

(a) Before germination (b) after germination for surface with Ra = 3.5 μm (N = 3, P < 0.0001).
Figure 11: Spore germination after using germination solution
(a) Stainless steel, (b) control sample, (c) copper alloy sheet, (d) thermal spray copper alloy.
5.2 Nanoflower Generation by Endospores

As endospores can remain in contact with hospital surfaces for long periods of time, it was decided to determine the impact of time exposure on the structural integrity of the endospores. SEM analyses indicated that exposure of endospores to stainless steel with roughness value of 3.5 µm for 2 hours to a week had no significant impact on their morphology (Figure 12a1–a4). However, some morphology changes were detected in a subset of the endospores that were exposed to copper alloy sheet with Ra value of 3.5 µm for 2 hours (Figure 12b1). Flower-like nanostructures became visible by the day 1 (Figure 12b2) and the number of them increased by the day 3 (Figure 12b3), by the day 7, almost all the endospores were degraded and nanostructures with two different morphologies were observed (Figure 12b4). A subset of the nanostructures had a petal-like surface appearance, while others were mainly composed of densely packed filamentous aggregates. Similar to the copper alloy sheet results, a subset of the endospores showed degradation after a 2-hour exposure to thermal spray copper alloy coating coupons with Ra value of 3.5 µm (Figure 12b3). However, amorphous aggregates and petal-like nanostructures became visible after 2-hour exposure to the thermal spray copper alloy coating coupons and after 7 days, almost all the endospores changed into petal-like nanoflowers (Figure 12c4). However, in contrast to copper alloy sheet, the presence of amorphous aggregates and petal-like nanostructures on thermal spray copper alloy coating coupons after 2-hour indicative of a more severe impact of endospore integrity. The flower-like nanostructures appeared to increase in size from a 2-hour to 3-day exposure (Figure 12c1-4). For both the copper alloy sheet, and thermal spray copper alloy coating, intact endospores were still observed until the day 3. In contrast to copper alloy sheet, no intact endospores were detected on thermal spray copper alloy coating coupons by the day 7. Instead, coupons were covered with numerous near uniform size nanoflowers.
Figure 12: B. subtilis endospores disassembly and transformation residual material into nanoflowers.

Endospores deposited on stainless steel (a1-4), copper alloy metal sheet (b1-4) and thermal spray copper coating (c1-4) and incubate for 2 hours (row1), 1 day (row 2), 3 days (row 3), and 7 days (row 4). Endospores deposited on stainless steel (a1-4), copper alloy metal sheet (b1-4) and thermal spray copper coating (c1-4) and incubate for 2 hours (row1), 1 day (row 2), 3 days (row 3), and 7 days (row 4).
5.3 Atomic Analysis of Endospores and Nanoflowers

Energy dispersive spectroscopy (EDS) was used to characterize the elemental composition of the flower-like nanostructures and endospores. EDS analysis revealed that endospores incubated on stainless steel had 14% carbon, 2.9% oxygen, 3.6% phosphorous and only trace levels of copper (0.2%). EDS analysis of nanoflowers on copper alloy sheet showed 48.2% copper, 26.2% oxygen with a 3.8% increase in phosphorous compared to intact endospores. Furthermore, the nanoflowers formed on copper alloy sheet contained approximately 50% less carbon than intact endospores. EDS profiling of nanoflowers on thermal spray copper alloy coating presented a similar elemental composition as those on copper alloy sheet; 40.2% copper, 20.6% oxygen, and 8.7% phosphorous, while the carbon content of nanoflowers on thermal spray copper alloy was similar to intact endospores (16.45%). As can be seen inhere was no significant difference in the carbon content of surfaces adjacent to the nanoflowers on copper alloy sheet and thermal spray copper alloy coating coupons. The surfaces of copper alloy sheet and thermal spray copper alloy coating coupons also had approximately 6.5-7.5% carbon. Both surfaces had a greater than 60% copper, with low levels of oxygen and phosphorous. Collectively, the data are consistent with other studies, indicating nanoflowers are hybrid organic-inorganic nanostructures.
Figure 13: EDS compositional analysis of endospores, nanoflowers and surfaces adjacent to them.

Area subjected analyses are highlighted by a circle insets within the rectangles. Stainless steel; endospores (a1) and adjacent region (a2). Representative copper alloy sheet nanoflower (b1) and adjacent region (b2). Representative thermal spray copper alloy coating; nanoflower (c1) and adjacent region (c2) (n=3) (Mean ± SEM).
5.4 Cross-Sectional Analysis of Nanoflowers

The generation of Nanoflowers has three steps, in the first step the initial binding of the amine groups of the proteins to copper ions trigger the formation of copper phosphate crystals [80]. Repeated nucleation-crystal formation generates petal-like structures. Despite the distinct surface topographies, focused ion beam sectioning (FIB) of mature tungsten-coated nanoflowers reveal strikingly similar inner anisotropic growth profiles (Figure 14a3 and b3). The cores of all the mature nanoflowers appeared composed a dense network of anastomosing morphologically irregular petal-like structures of variable sizes, orientation and separation. For the nanoflowers with a petal-like surface appearance (Figure 14b1), the dense inner cores transitioned into large plate-like networks. In contrast, needle-like projections radiated towards the surface from the petal-like cores (Figure 14a3), consistent with the needle-like surface topography of the nanoflowers (Figure 14a1).
(a1) Filament-like nanostructure on copper alloy sheet; SEM surface view with 5000 – fold magnification, (a2) a nanoflower with a tungsten layer, (a3) and section view. (a1) Flower-like nanostructure on thermal spray copper alloy coating, SEM surface view with 5000 – fold magnification, (b2) a nanoflower with a tungsten layer, (b3) and section view.

Figure 14: FIB sectioned of nanoflower-like structures with different surface topographies.

5.5 Discussion

Weaver et al reported that copper alloys with greater than 70% copper content lead to the complete killing of *C. difficile* endospores within 24-48 hours [81]. SEM data in this study indicate that a subset of *B. subtilis* endospores remain intact on copper alloy sheet and thermal spray copper alloy coating up to three days of incubation. However, endospores with aberrant surface morphologies and nanoflower-like structures were evident after only a 2-hour exposure. Remnants of degraded endospores were visible after 7 days on the copper alloy sheets, but not on the thermal spray copper alloy coatings. Moreover, the nanoflowers appeared to have reached their maximum size on the thermal spray copper coatings after 7 days, while more intermediate size nanoflowers were visible on the copper alloy sheet coupons. The differences in the rate of endospore killing may have been due to differences in copper content between the copper alloy sheet (65%) and
thermal spray copper coating (69%). Thus, alloys with copper contents higher than 60% exhibit sporicidal activity within a few hours of exposure.

This finding indicated that copper alloy surfaces are effective at killing endospores. However, an extended time exposure is necessary. This time extension may be due to the structure of endospores.

Endospores consist of a core with a copy of bacteria’s DNA, embedded in a chelating complex of dipicolinic acid (DPA) and divalent cations such as Ca$^{+2}$ and Mg$^{+2}$. The core is surrounded by a lipid membrane and a thick proteinaceous coat (peptidoglycan). Although, there are striking differences in structures between vegetative cells and endospores, the reports showed that, the functional groups such carboxylate, phosphate and amino groups in the surface of Gram-positive bacteria and their endospores are almost the same [34, 82]. The studies also indicated that, the negative charge of the endospore coat is a barrier against germination, and that neutralizing the negative charge with positively charged ions such as calcium promotes endospore germination [83]. It is therefore likely copper in copper surfaces reacts with negatively charged coat ions such as phosphate ions and initiates a series of reactions, leading to killing of the endospores.

Sporicidal efficacy of copper began after penetrating into the endospore coat and changing the permeability of the coat. Fenton-like reaction mediated by the interaction of copper ions with dissolved oxygen could generate hydrogen peroxide and reactive oxygen species that could trigger the destruction of the protective endospore coat and internal structures (reactions 3, 4, and 5) [78].

$$2Cu^+ + 2O_2 \rightarrow 2Cu^{2+} + 2O_2^- \quad 3$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad 4$$
\[ H_2O_2 + O_2^- \rightarrow O_2 + OH^- + OH^+ \]

Low water content of the endospore cores, and high concentration of small acid soluble proteins (SASP) reduced the production or migration of free radicals [84].

The observation of nanoflowers following the exposure of endospores to the copper surfaces evaluated the effective killing of *B. subtilis* endospores by copper alloys. Based on experimental results and previous reports, nanoflowers are hybrid organic-inorganic nanostructures, comprising three components: copper ions, phosphate ions and proteins. Studies have also proved that by a similar mechanism (in vitro), copper ions react with phosphate groups of the endospore coat to form copper phosphate complexes. Formation of hybrid phosphate-copper complexes is the first step of flower-like nanostructures formation. In the next step, amino groups of proteins triggered the formation of copper phosphate crystals. Repeated cycles of nucleation lead to the formation of petal-like organic-copper phosphate structures [80]. It is likely that proteins of nanoflowers derived during endospore degradation.

Previous studies also indicated that Cl⁻ ions have a critical role in the formation of copper phosphate crystals [85]. In this study, the endospores were washed with NaCl suspension, which may be a likely source of Cl⁻ ions. Alternatively, the Cl⁻ ions could also have originated from the degraded endospores.
5.6 Conclusion

This study indicates that degradation of *B. subtilis* endospore begins within 2 hours after exposure to the copper alloy sheet and copper alloy coating with Ra = 3.5 μm. By day seven, only nanostructures are visible on the copper alloy coating, whereas extensively degraded endospores and nanostructures are visible on the copper alloy sheet surfaces. This indicates that thermal spray copper alloy coatings are as effective as copper alloy sheet in the destruction of endospores within hours and superior after one week. Therefore, the thermal spray coating of hospital surfaces and equipment with copper alloys holds promise as a rapid and cost-effective infection control strategy.

5.7 Recommendations for Future work

The molecular basis of how endospores are killed by copper ions is poorly understood. Data from a recent study indicates that copper ions are internalized, generating hydroxyl radicals through a modified Fenton Reaction. Since hydroxyl radicals could result in the destruction of endospores, measuring reactive species within endospores could provide evidence that destruction of endospores is from the inside out. Fluorescent probes such as coppersensor 1 (CS1) or Coppersensor 3 (CS3) [86, 87] can indicate the copper ion diffusion through endospore coat to generate hydroxyl radicals. Since the inner cores of endospores have low water content, the diffusion of copper ions is expected to be slow. Thus, endospores could be hydrated for few days before measuring copper ion levels [78]. Studies could also be performed to identify compounds that accelerate the internalization of copper ions. For example KCl. Negatively charged reagents can increase the diffusion of copper ions through the endospore coat to satisfy the electroneutrality and osmotic pressure [78]. Furthermore, fluorescent dyes can be used to assess the viability of the endospores. Two fluorescence -based viability assays are CTC/DAPI respiratory activity assessment, and the Live/Dead BacLight kit. These assays provide some information on
respiratory potential and integrity of the endospore coats. These assays are more time-consuming and accurate compared with plate cultures [87].
References


