Biofilm Inhibition and Antiviral Response of Cold Sprayed and Shot Peened Copper Surfaces: Effect of Surface Morphology and Microstructure

Maryam Razavipour¹ · Mayte Gonzalez² · Naveen Singh¹ · Cagla Eren Cimenci² · Nicole Chu² · Emilio I. Alarcon^{2,3} · Julio Villafuerte⁴ · Bertrand Jodoin¹

Submitted: 1 September 2021 / in revised form: 14 December 2021 / Accepted: 19 December 2021 / Published online: 6 January 2022
© ASM International 2022

Abstract Antibacterial properties of copper against planktonic bacteria population are affected by surface microstructure and topography. However, copper interactions with bacteria in a biofilm state are less studied. This work aims at better understanding the difference in biofilm inhibition of bulk, cold-sprayed, and shot-peened copper surfaces and gaining further insights on the underlying mechanisms using optical and scanning electron microscopy to investigate the topography and microstructure of the surfaces. The biofilm inhibition ability is reported for all surfaces. Results show that the biofilm inhibition performance of cold sprayed copper, while initially better,

decreases with time and results in an almost identical performance than as-received copper after 18h incubation time. The shot-peened samples with a rough and ultrafine microstructure demonstrated an enhanced biofilm control, especially at 18 hr. The biofilm control mechanisms were explained by the diffusion rates and concentration of copper ions and the interaction between these ions and the biofilm, while surface topography plays a role in the bacteria attachment at the early planktonic state. Furthermore, the data suggest that surface topography plays a key role in antiviral activity of the materials tested, with a smooth surface being the most efficient.

This article is an invited paper selected from presentations at the 2021 International Thermal Spray Conference, ITSC2021, that was held virtually May 25-28, 2021 due to travel restrictions related to the coronavirus (COVID-19) pandemic. It has been expanded from the original presentation.

✉ Maryam Razavipour
Maryam.razavipour@uottawa.ca

¹ Cold Spray Research Laboratory, University of Ottawa, Ottawa, ON, Canada

² Division of Cardiac Surgery, BEaTS Research, University of Ottawa Heart Institute, Ottawa, Ontario, Canada

³ Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

⁴ Centerline Windsor, Ltd, Windsor, Ontario, Canada

Graphical Abstract

Keywords antiviral properties · biofilm inhibition response · cold spray · copper ion diffusion · shot peening · surface topography · surface microstructure

Introduction

The antimicrobial properties of copper surfaces have been extensively studied (Ref 1–4). It was shown that exposure to metallic copper can rapidly kill bacteria according to

several potential mechanisms (Ref 5). Many studies suggested that copper ions released from the copper surfaces cause a decline in the cell membrane integrity leading to leakage of cell nutrients causing loss of function of proteins (Ref 6). Other studies related the antibacterial activity of copper to its capacity to release ions which function as catalysts in reactions generating reactive oxygen species (Ref 7). DNA damage is also known as a secondary reason causing cell death (Ref 8). The inherent contact killing effectiveness of copper can be further improved by surface

modification processes such as cold spray (CS) or shot peening. CS is a solid-state deposition process in which the feedstock powder is accelerated at high speed towards a part to be coated. Upon impact, the powder undergoes severe plastic deformation at a temperature below their melting point and forms a coating (Ref 9). CS generally induces localized grain refinement and dynamic recrystallization of the sprayed material (Ref 10), as a result of the accumulation of dislocations during severe plastic deformation. These accumulated dislocations restructure and form subgrains that eventually turn into ultrafine grains (Ref 10, 11). The improved antibacterial properties of CS copper coating over bulk copper, at the planktonic state, have been explained by the presence of many favorable ion diffusion paths which enhance the surface copper ionic release (Ref 12–15).

The antiviral effectiveness of cold sprayed copper can be improved by using nanocrystalline copper powder (Ref 15). This was attributed to the increased grain boundaries and dislocation densities in the nanocrystalline copper coatings leading to higher ion diffusivity (Ref 15). Surface modification techniques, such as surface nanocrystallization (SNC), can be used to enhance surface ion diffusion by increasing surface dislocation densities and grain refinement. Shot peened surfaces present enhanced antibacterial properties over bulk copper (Ref 16).

Several studies have investigated the effects of surface roughness on cell attachment. It has been suggested that surface topography affects the ability of bacteria to adhere to the surface and to develop colonies. In one study, surfaces with similar surface chemistry but different surface roughness exhibited different bacteria retention abilities depending on the size of bacteria used (Ref 17). It was also reported that high surface roughness improves the antibacterial property of copper by improving its hydrophobicity (Ref 18). As opposed to many studies reporting hydrophobicity in favor of antibacterial effectiveness, others demonstrated higher bacterial adhesion on hydrophobic surfaces compared to hydrophilic surfaces (Ref 19, 20), with the mechanism still not understood. A different contact killing mechanism is considered when dealing with nanoscale surface roughness. It was proposed that the contact killing mechanism of nanotextured surfaces was mechanical rupture of cell membrane induced upon contact with the sharp nanofeatures (Ref 21, 22).

Many uncertainties are involved in identifying methodically and separately the effect and mechanisms of surface roughness on the bacterial attachment and inactivation. This makes the problem even more complicated when surface modification/treatment processes involving surface topography modifications also involve grain size modification at surfaces, creating a convoluted situation (Ref 23). Furthermore, examination of the literature (Ref

1, 2, 18, 24–28) on anti-pathogenic properties of copper surfaces reveals that almost all of these studies were focused on antibacterial properties in a planktonic state (Ref 1, 2, 18, 24–28), not as a biofilm (Ref 29–31).

Undesired biofilm represents a significant economic burden regardless of the industry or economic area and is a major threat to public health (Ref 32, 33). The development of biofilms occurs in five stages: Cell attachment, micro-colony formation, biofilm formation, biofilm growth and maturation, and eventually dispersal stage where the bacterial cells depart biofilms and can return to planktonic state (Ref 34, 35). The antibacterial properties of copper are commonly associated with the released copper ions that will cause cell damage (Ref 1, 2, 26). However, the copper interaction with biofilm is significantly different and the action of copper surfaces and copper ions will not only focus on cell interaction but will also interfere with extracellular polymeric secretions (EPS) (Ref 36). It was reported that exposure to CuO nanoparticles increased the production of loosely bound EPS on wastewater biofilms. These CuO nanoparticles altered the EPS structure and its chemical composition, affecting significantly the functional groups of proteins and polysaccharides of loosely bound EPS (Ref 29). CuO nanoparticles have been found attached to and embedded in an EPS matrix in wastewater biofilm (Ref 29). Developing antimicrobial surfaces with improved anti-biofilm and self-sanitizing performances is a critical task. This can be tailored by first understanding the mechanisms affecting the anti-biofilm response of the surfaces. Further, limited studies also reported the antiviral properties of metallic copper and copper ions (Ref 37–40).

This work aims at understanding the differences in biofilm resistance of bulk copper, cold-sprayed, and shot-peened copper surfaces and identifying the major biofilm control mechanisms. The growth behavior of *P. aeruginosa* biofilm on modified surfaces was compared using stainless steel as a control, due to its prevalent use for touch surfaces. First, biofilm inhibition tests are performed on copper and two types of stainless steel samples. Then the effect of surface microstructure is presented by a comparison between polished as-received (As-R) and cold sprayed (CS) copper surfaces. Last, the effect of surface roughness on the biofilm inhibition performance of copper is presented using three-shot peened samples as a comparison. The role of surface roughness and the grain size is illustrated and mechanisms affecting the biofilm inhibition properties of the surfaces are studied by performing complementary ion measurement tests using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Ref 41). Also, the antiviral performance of the copper cold sprayed and the shot-peened samples was also investigated. Using the best biofilm inhibition performance materials, the antiviral performance of these copper samples was

compared with bulk copper and control samples using a lentivirus surrogate model of SARS CoV-2.

Material Preparation and Surface Treatment

Coating and shot peening procedures were conducted using a commercially available EP Series SST cold spray system (CenterLine (Windsor) Limited, Windsor, Ontario, Canada). De-Laval tungsten carbide and steel nozzles were used for coating and shot-peening processes, respectively. These nozzles have throat and exit diameters of 2 mm and 6.6 mm, respectively, along with a diverging section length of 120 mm.

Discs of 10 mm diameter and 4 mm thickness were machined from as-received copper round bars (grade C101) as well as as-received stainless steel round bars (grade 316). The stainless steel discs were only used as control substrates for the biofilm inhibition response tests. The shot peening and cold spray processes were performed on copper discs.

In order to remove any machining imperfections and to standardize the substrate surface properties, all discs surfaces were grounded using a 220 grit ($\sim 63 \mu\text{m}$) diamond-grinding disc prior to each experiment.

The coatings were produced using as-received electrolytic copper powder (CenterLine (Windsor) Limited,

Windsor, Ontario, Canada). Figure 1(a) illustrates the feedstock powder morphology. These powders have an irregular shape with a purity of 99.7%, as specified by the manufacturer. The powder size distribution ranges from $5 \mu\text{m}$ to $45 \mu\text{m}$, as specified by the manufacturer. Powder cross-section images are shown in Fig. 1(b). As seen, these powders are dense and no internal pores or other defects are observed. The morphology of glass beads used for the shot peening process is illustrated in Fig. 1(c). These glass beads are spherical with an average particle size distribution of $88\text{--}149 \mu\text{m}$ and Mohs hardness of 4–5, as reported by the manufacturer.

The process parameters used to perform the cold spray and shot peening treatments are listed in Table 1. Three different peening parameters were used, i.e., SP1, SP2, and SP3. SP1 sample underwent the largest peening intensity and coverage. SP2 and SP3 samples experienced similar peening conditions except for the peening scan speed. These peening parameters were selected to produce refined grain sizes with different surface roughness values.

Biofilm Inhibition Test Methodology

The biofilm inhibition response of the various copper samples was evaluated using *Pseudomonas aeruginosa* bacteria (PA14). *P. aeruginosa* is a gram-negative rod-

Fig. 1 Morphology of (a) loose copper powder, (b) powder cross-section, and (c) glass beads used in the shot peening process

Table 1 Coating and shot peening process parameters with the sample ID for each surface treatment listed

Surface Treatments		Gas Condition		Gun Traverse Speed (mm/s)	Powder Feed Rate (gr/min)	No. of Surface Scans	Surface Coverage	Particle Velocity (m/s)
		Pressure (MPa)	Temperature (°C)					
Copper Coating	CS	3.4	500	40	9.1	5	...	539±66
Shot Peening	SP1	0.7	25	20	34.8	5	10000%	208±32
	SP2	0.7	25	40	16.7	1	500%	
	SP3	0.7	25	200	16.7	1	97%

Fig. 2 Schematic of the biofilm inhibition test methodology conducted for the samples in this study

shaped pathogen with an average size of 0.5–3 µm (Ref 42). This pathogen uses several attachment organelles to adhere to surfaces and form biofilm (Ref 33, 35, 43). The schematic in Fig. 2 illustrates the biofilm inhibition response test. All experiments were carried out in a biosafety level 2 (BSL2). Experiments are divided into three stages; biofilm formation, biofilm disruption, and quantification of colonies.

PA14 bacteria were allowed to grow in LB broth in an orbital shaker, at 37 °C and aerobic conditions. The absorbance characterization was performed on the bacteria suspension using spectrometer (SpectraMax M2). Bacteria suspension was adjusted to 0.7 optical density (OD) and then diluted by a factor of 10 using an LB broth (Fig. 2, Biofilm formation step a). From this solution, 500 µL of bacteria solution with initial concentration of $\sim 10^8$ colony-forming unit/mL (CFU/mL) was used for each disc. The 5 discs from each group were placed in a sterile plate and 500 µL of the bacteria stock solution was spread over each sample. Unused wells were covered with 750 µL of sterile H₂O to preserve the humidity (Fig. 2, Biofilm formation step b and c). Then the plate was placed in a humidity incubator at 37 °C at an inclination of 45° (Fig. 2, Biofilm formation step d). The discs were incubated for 1 hr, 6 hr, and 18 hr to allow bacteria colonization and

biofilm formation. Next, the biofilm was removed, see Fig. 2, and resuspended in M63 media, plated and survival colonies counted (18 hr incubation, 10–100 µL sample plated at 37 °C).

Anti-Viral Test Methodology

To evaluate the contact killing ability of copper samples when exposed to viral pathogens, a viral photoinactivation experiment was performed using a lentivirus surrogate model of SARS-CoV-2. The schematic of the antiviral test methodology is illustrated in Fig. 3. The test procedure is divided into four steps; cell preparation, virus preparation, cell/virus combination, and qRT-PCR analysis.

Cell Preparation

L929 fibroblasts were used. These cells were cultured in cell culture media DMEM/MEM supplemented with 1% Penicillin Streptomycin (PS), and 10% Fetal Bovine Serum (FBS). Cells were trypsinated, counted, and cell density adjusted to 15,000 cells/mL in 10 mL using DMEM. 100 µL of this cell solution were seeded into 96 wells and incubated for 24 hr at 37 °C.

Fig. 3 Graphical protocol illustrating the viral test procedure. The “+control” is the virus-only group and the “-control” is the no virus (media only) group

Virus Preparation

The lentivirus (pLL-CMV-rFLuc-T2A-GFP-Puro Lenti-Labeler™ Lentivector, LL310VA-1, SBI System Biology) was diluted in saline solution and used at a viral dosage of 10^8 International units per milliliter (IU/mL), shown in Fig. 3. Discs (two discs from each copper group) were placed in a 24 well plate. A 5 µL drop of viral solution was placed on top of each disc (Fig. 3) and incubated for 10 min at room temperature (Fig. 3, Lentivirus Preparation, step c). After 10 min, 100 µL of Hank's Balanced Salt Solution (HBSS) was added on top of each disc, which mixed with the viral solution. HBSS is a buffer used for maintaining the physiological pH range and osmotic balance of the solution. This plate was incubated for 2 hr at room temperature (Fig. 3, Lentivirus Preparation, step e).

Cell/Virus Combination

First the culture media from the 96-well plate (Fig. 3, red well plate from L929 Cell Preparation part step d) was replaced with 100 µL of fresh DMEM/MEM cell culture media to provide enough nutrients to feed cells. Then 105 µL solution from each treatment well (Fig. 3, blue well plate from Lentivirus Preparation part step e) was added to the L929 cells plate. Positive control (100 µL of DMEM/MEM culture media + 100 µL of Hank's solution with 5

µL viral solution) and negative control (100 µL of DMEM/MEM culture media + 100 µL of Hank's solution) groups were also added to the L929 cells in the 96-well plate (Fig. 3, part 3). This 96-well plate was then incubated at 37 °C for 48 hr. Then, viral RNA was extracted and quantification analysis was performed.

Quantification Analysis

Quantitative RT-PCR (qRT-PCR), shown in Fig. 3 part 4, was performed for the qPCR Lentivirus Complete Titration Kit Cat#LV900 (Applied Biological Materials Inc, Canada). Samples from the 96-well plate were collected into 0.5 mL Eppendorf tubes individually and centrifuged at 2000 g for 5 minutes to remove cells/debris. 10 µl of viral supernatant and control samples were then mixed with 10 µl of Viral Lysis Buffer and incubated at room temperature for 3 minutes. The qPCR reaction was prepared by mixing 12.5 µl fluorescent dyes (2X SYBR Green qPCR Mastermix), viral lysate or standards (2.5 µl), reagent mix (10 µl) for a total volume of 25 µl per reaction. Real-time qPCR instrument (LightCycler 480, Roche) was programmed with reverse transcription at 42 °C for 20 minutes, enzyme activation at 95 °C for 10 minutes, denaturation at 95 °C for 15 seconds, and annealing/extension at 60 °C for 1 min - repeating the last two steps for 40 cycles. Melting/Dissociation Curve Analysis was then

performed at the end of the last annealing cycle by melting the product at 95 °C. The titer of the samples was calculated using the Ct values obtained from the LightCycler® 480 Software and the Lentiviral titer calculator tool at ABM's corresponding website as suggested by the manufacturer.

Surface Characterization

Microscope observation of sample cross-section was performed to study the samples microstructure after surface treatments. The cross-sections of the samples (As-R, CS, SP1, SP2, SP3) were ground with a 220 grit (~63 µm) diamond grinding disc and diamond paste solution down to 0.05 µm and etched for 5 s in a solution of 5g FeCl₃ + 25 ml HCl + 100 ml ethanol to reveal the grain boundaries. Optical microscopy observations were performed using an optical microscope (VHX—1000, Keyence Corporation, Osaka, Japan). The surface topography of the samples was also analyzed with an optical microscope. Ten 3D images per sample were taken to calculate sample surface roughness (Ra). This was done by extracting the surface topography using VHX-2000 3D Shape Measurement Software. A Matlab code (R2018b) was developed to measure the surface roughness from the exported data points.

Detailed observation of near-surface grain structure was done using scanning electron microscope (SEM) (EVO MA-10, Carl Zeiss AG, Oberkochen, Germany) equipped with secondary (SE) and back-scattering electron (BSE) detectors. Image J software was used to perform the grain size measurements of shot-peened and cold sprayed surfaces down to 20 microns deep. Prior to the analysis, all samples were cleaned ultrasonically with ethanol and dried with air to remove any surface contamination.

Copper Ion Release

Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to measure and compare the copper ion release from the studied samples (SP1, SP2, SP3, As-R, CS). Prior to the experiment, the sidewall and base of all the discs were painted to prevent copper diffusion from these surfaces to allow comparing the copper ion diffusion of the modified surfaces only.

Copper discs from each studied group were placed in a 24-well sterile plate and covered with 500 µL of LB broth. This plate was then placed in a humidity incubator at an inclined angle of 45° and a constant temperature of 37 °C. These sample groups were incubated for 1 hr, 6 hr, or 18 hr. Next, discs were removed and the solution containing

copper ions was transferred to vials for quantification analysis.

The copper concentrations were determined for each sample using ICP-OES (Agilent Technologies Inc. 5110 ICP-OES) with the detection limit below 0.0006 ng/g or parts per billion (PPB). The weight of 0.1 ml of the solution was measured. This solution was then diluted in 1% HNO₃ solution with the dilution factor of 1:100. This diluted sample was used for the ion concentration measurement. Before each measurement, the calibration was done using the plasma calibration standard (Cu 1001 ± 5 µg/ml 4 % HNO₃). The concentration of copper in the solution is reported in µg/g.

Results and Discussion

Biofilm inhibition performance of copper is demonstrated by a comparison between bulk copper and stainless-steel samples, illustrated in Fig. 4. All sample surfaces were smooth and mirror polished prior to testing. Copper has significantly better biofilm inhibition properties when compared to stainless steel for all the incubation times.

The copper interaction with biofilm is significantly different from its interaction with bacteria cell in the planktonic state: the number of viable bacteria in the biofilm never reaches zero but rather increases. This is different from the antibacterial efficiency of copper reported using wet plating techniques studying exclusively the survival of bacteria in their planktonic state, where the number of bacteria drops as incubation time increases (Ref 2, 44, 45). As also seen in Fig. 4, there is a considerable difference in the values for copper and stainless steel after 1 hr, with this difference less significant at 18hr incubation time. This suggests that the biofilm inhibition efficiency of copper drops with time as the biofilm grows and thickens.

Contact killing efficiency of metallic copper is affected by many parameters and this makes the comparison between studies challenging. Contact killing properties of copper are significantly affected by the bacteria type and morphology (Ref 46), temperature and humidity (Ref 24, 27), and even the test medium composition (Ref 2, 26, 28, 47). Nevertheless, in all cases, the inherent antibacterial advantages of copper were well demonstrated.

Figure 5 compares the surface topography and microstructure of the samples used in the current study. Cross-section images of shot-peened samples SP1, SP2, and SP3 are shown in Fig. 5(a,b,c), respectively. Fig. 5(d,e) illustrates the cross-section images of As-R and CS samples, respectively. The high magnification images of the highlighted zone in the CS sample are shown in Fig. 5(f). The SP1 sample experienced the most intense shot peening process among all shot peened samples. It presents the

Fig. 4 Comparison between biofilm inhibition ability of bulk copper (As-R) with stainless steel substrates grade 316 (SS 316). The figure shows viable *Pseudomonas aeruginosa* bacteria in the biofilm formed on copper and stainless steel samples after 1, 6, and 18 hr

incubation time at 37 °C. Sample surfaces were polished and their surface roughness is nearly identical. P values calculated from t-students test vs. bulk copper (As-R)

roughest surface topography with the deepest affected zone highlighted in yellow, where the grain size was evaluated at $0.3 \pm 0.14 \mu\text{m}$. The cross-section of the SP2 sample presents a reduced depth zone of refined microstructure compared to SP1. Also, the surface topography comparison between SP1 and SP2 reveals that SP2 surface is smoother than SP1. The SP3 sample surface is the least rough among the peened samples. Shot peening parameter in SP3 resulted in less surface impingement and hence a non-uniform surface microstructure. The regions with detectable refined microstructure were highlighted in yellow. As seen, some parts of the surface barely experienced any microstructure modification. The As-R sample is characterized by coarse grains, with the coarsest surface grain sizes among all the studied samples. The cross-section of CS sample is shown with the copper coating and the substrate parts identified. The copper coating has a fine microstructure with an average grain size evaluated at $1.73 \pm 0.70 \mu\text{m}$ attributed to the fine microstructure of the feedstock powder used for the coating process and recrystallization caused by excessive plastic deformation. The magnified image of the highlighted region (blue box) in Fig. 5(e) is shown in Fig. 5(f). The powder boundary (black region) and the fine grains inside the deformed powders are observed.

Surface roughness comparison (Ra) for all samples is shown in Fig. 5(g). Both As-R and CS were mirror-polished hence have nearly identical surface roughness. For shot peened samples, the surface roughness differences are noticeable with the SP1 that has the largest Ra with the value of $9.59 \pm 2.7 \mu\text{m}$ and for the SP2 and SP3 the Ra values are $4.89 \pm 0.6 \mu\text{m}$ and $3.3 \pm 0.26 \mu\text{m}$, respectively.

Figure 6(a,c,e) compares the biofilm inhibition performance of modified surfaces (shot-peened and cold sprayed sample) with the bulk copper (As-R) sample at 1, 6, and 18

hr of incubation. The student's t-test was used to compare the antibacterial effect between the groups. The comparison is made between samples SP1, SP2, SP3, CS, and bulk copper (As-R). The p-values are reported in the figure. In all statistical tests, the significance level was considered as 0.05.

The results of copper ion diffusion, measured for these samples at the three incubation times, are also shown in Fig. 6(b,d,f). The p-value comparison demonstrates that the significance level between samples SP1, SP2, SP3, CS, and bulk copper (As-R) is all lower than 10^{-4} . The copper ion diffusion rate (CIDR) at 1, 6, and 18 hr was approximated by dividing the instantaneous copper ions concentration at these times by the time of measurement in hr and is reported in Table 2. These results reveal that the diffusion rate increases with time for all surfaces with the exception of CS which increases at 6 hr and then decreases. It also reveals that the diffusion rate for SP1 is much larger than for the other surfaces and that $\text{CIDR}_{\text{SP1}} > \text{CIDR}_{\text{SP2}} > \text{CIDR}_{\text{SP3}} > \text{CIDR}_{\text{CS}} > \text{CIDR}_{\text{As-R}}$ at all times except for $\text{CIDR}_{\text{CS}} < \text{CIDR}_{\text{As-R}}$ at 18 hr.

As shown in Fig. 6, when applied to a surface by cold spraying, copper exhibits better biofilm inhibition ability when compared to its bulk version (As-R) only for the 1 hr incubation time. The initial improved biofilm inhibition performance of CS copper compared to the as-received copper (As-R) is associated with enhanced ion diffusion and is explained by the effect of finer surface microstructure and increased dislocation densities typically found in cold spray coatings (Ref 10, 11).

Figure 7(a,b) compares the SEM cross-section images of As-R and CS samples. Fine-grain size is the characteristic of CS sample. It was shown that atomic diffusion in crystalline structure can be accelerated by the presence of defects such as dislocations, grain boundaries, and twinning

Fig. 5 Optical microscope etched cross-section images of the various copper samples. Shot peened samples (a) SP1, (b) SP2, (c) SP3, and surface polished samples of (d) As-R (bulk copper), and (e) CS. High magnification not-etched SEM image of the highlighted region in

figure (e) is shown in figure (f). Shot-peened affected zones were marked with yellow dashed lines. Surface roughness, R_a , comparison of the samples is shown in figure (g)

(Ref 13, 48). The improved antibacterial properties of CS copper coating over bulk copper, at the planktonic state, is explained by the presence of many favorable ion diffusion paths which enhance the surface copper ionic release (Ref 12-15).

As opposed to 1 hr incubation time, the enhanced inhibition ability of CS vs. bulk copper (As-R) decreases as the incubation time increases, as seen in Fig. 6(c,e). As presented, no significant differences are observed for CS and As-R for 6 and 18 hr incubation time with a p-value of 0.48. This seems to be independent of the ion release results for the As-R and CS samples. Despite the higher copper ion release from CS sample after 6 hr incubation time, illustrated in Fig. 6(d), the biofilm inhibition response of both CS and As-R are almost identical.

The reduction of ion diffusion rate for the case of CS after 18 hr is possibly explained by continuous microstructural evolution of CS samples after the deposition process (Ref 49). High elastic energy is stored in materials due to extreme plastic deformation during high-velocity impact. This stored energy triggers a continuous static recrystallization process that can take place over the course of weeks at room temperature (Ref 49).

The lower copper ion diffusion for CS compared to SP1 is attributed to surface microstructural differences between these two samples. As presented in Fig. 7(b,c), SP1 sample has finer surface grains compared to the CS sample. The intensive plastic deformation in SP1 sample resulted in this finer microstructure, with higher dislocation densities and hence larger ion diffusion ability. It should be noted that the induced increased surface area of shot-peened samples

Fig. 6 *Pseudomonas aeruginosa* bacteria survival in the form of microcolonies and biofilm reported in “CFU/mL” at (a) 1 hr, (c) 6 hr, and (e) 18 hr incubation time. The copper ion released from the samples reported in “µg/g” is shown at (b) 1 hr, (d) 6 hr, and (f) 18 hr

Table 2 Copper ion diffusion rate (CIDR) approximated at 1, 6, and 18 hr

	Diffusion Rate (µg/g.hr)		
	1 hr	6 hr	18 hr
SP1	73.7	107.7	507.4
SP2	43.1	58.0	149.9
SP3	38.4	44.6	43.7
CS	25.8	37.9	12.6
As-R	14.5	18.7	33.2

were calculated and only 5% increased area was observed for the SP1 sample. This increased surface was even smaller for the SP2 and SP3 samples with the value lower than for 2 %. These limited area increases cannot explain

incubation time. Standard error of the mean value is shown. P-values were evaluated by t-test comparison between SP1, SP2, SP3, and CS vs. As-R

the highly increased ion diffusion from the shot-peened samples. Therefore it is concluded that it is rather the refined microstructure and dislocation density that play a role in the high copper ion release. Shot peening induces severe plastic deformation that results in refined microstructure and high dislocation density at the surface and near-surface regions (Ref 50). As seen in Fig. 6(b,d,f), copper ion concentrations of shot-peened samples are consistently higher than As-R sample, for all the incubation times with this difference even more significant for SP1 sample at 18 hr. Also, as presented in Table 2, the copper diffusion rate is much higher for SP1 compared to As-R at all incubation times. Consequently, as shown in Fig. 6, an improved biofilm inhibition performance of shot-peened

Fig. 7 Microstructure comparison for (a) As-R (bulk copper) and (b) CS samples, and (c) SP1. The SEM image shows the channeling contrast due to differently oriented grains. The grain boundary in the As-R sample is highlighted with the dashed line

samples over As-R sample is achieved with the observed improvement to be the most significant for SP1 at 18 hr of incubation time. For this sample, the biofilm inhibition response and the amount of copper ion concentration are directly correlated.

As presented in Fig. 6, the biofilm inhibition response of CS samples was similar to that of the SP1, SP2 SP3, and bulk copper samples for 6 hr incubation times, as p-values are all similar, despite lower copper ion diffusion of bulk copper at all incubation times. At 18 hr incubation time, the biofilm resistance performance of SP1 overcame that of CS and As-R.

The schematic in Fig. 8 illustrates an approximate model for bacteria attachment in planktonic state and biofilm formation in SP1, CS, and As-R samples at different incubation times considering the differences in surface topography and copper ion concentration. It illustrates that SP1 and CS initially presented better biofilm inhibition response compared with As-R sample. It also schematically explains the similar biofilm inhibition performance of CS and SP1 samples at 6 hr incubation time despite the lower copper ion release in CS. As already seen in Fig. 5(a), rough surface topography was induced by the peening process for the SP1 sample. This micro surface roughness plays a role in bacteria adhesion as well as the expected nano-roughness created by the peening process that was not

captured by the imaging resolution. Bacteria use different attachment mechanisms to increase the number of surface attachment points. Rod-shaped bacteria like *P. aeruginosa* have a large linear surface and this increases the area of contact. Besides, bacteria can create appendages, like flagella, to increase the area of contact and promote attachment. These flagella also help in the motility of bacteria to overcome hydrodynamic and surface repulsive forces and to maintain adherence (Ref 35, 46, 51). The combination of micro and nano roughness contributes to higher bacterial adhesion and increases the number of cell-to-surface contacts at the initial planktonic state for the SP1 sample. This explains the similar biofilm inhibition of polished CS surface and the rough SP1 sample for 6 hr incubation time despite their noticeable copper ion diffusion differences. However, the trend changes at 18 hr of incubation time as the whole biofilm formation process is dynamic. The enhanced biofilm inhibition characteristics and contact killing properties of the SP1 sample at 18 hr are explained by the increased copper ions concentration as a result of the larger copper diffusion ability from the finer grains.

As explained, SP1 is prone to bacteria attachment in the initial planktonic state due to its surface topography (Fig. 8, 1 hr incubation time). However, for this sample, due to its finer microstructure and dislocation density, the ion

Fig. 8 Schematic comparing the biofilm control proposed mechanism of SP1, CS, and As-R samples. SP1 shot-peened sample with rough surface topography and very fine surface microstructure. CS sample, with smooth surface and fine microstructure. The As-R sample with

coarse grain and smooth surface. The copper ion concentration in $SP1 > CS > As-R$ at all incubation times with the exception at 18 hr incubation in which ion diffusion in $CS < As-R$

diffusion is significantly larger than for the CS sample. At 1 and 6 hr incubation time, the concentration of copper ion was not enough to kill a large number of bacteria in a planktonic state and prevent biofilm formation. As time evolves, copper ions travel over longer distances and this leads to progressively higher copper ions release from the surface (Fig. 8, at 18 hr incubation time). As discussed and illustrated in Fig. 6(f), for 18 hr, the ion concentration of the SP1 sample is sufficiently high to disrupt biofilm

structural integrity and decelerate any further bacteria proliferation and additional bacteria colonization. As observed in Fig. 6(e), a significant reduction of biofilm production is a characteristic of the SP1 sample. The destructive interaction of copper nanoparticles with biofilm was also reported in previous studies (Ref 31). It was shown that copper nanoparticles can effectively disperse the biofilm architecture by loosening the bacteria microcolonies.

The SP2 sample follows a similar trend with similar mechanism as the SP1 sample. The SP2 sample is smoother and has a less refined microstructure than SP1. The smoother surfaces provide fewer bacteria attachment points while the less refined microstructures lead to less ion diffusion than SP1. Eventually, with time, when ion diffusion increases, it regulates the biofilm development behavior resulting in improved biofilm inhibition properties. However, no statistically significant biofilm inhibition improvement was noticed for SP3 sample.

The copper samples with the best biofilm control abilities for 1 hr incubation, i.e., CS and SP1, and 18 hr incubation, SP1, were selected to assess their antiviral effects as well. The bulk copper (As-R) and stainless steel control substrate were also included in the test as references for comparison purposes. The qRT-PCR test results for these samples are reported in Fig. 9. The values are presented in relative expression meaning that the antiviral response values were normalized to the kit's standard virus concentration value and reported in percentage. As seen, both the control sample and the virus only show similar values meaning that the control sample has no antiviral properties. SP1 sample demonstrates a slightly lower value than the control sample, with values of 69.5% and 52.4% for control and SP1, respectively. No detectable viruses were observed for As-R and CS samples. This suggests that these two samples have effective virus deactivation properties over a short contact time. It is evident that surface topography contribution in virus adhesion is dominant. Even in the presence of a reduced concentration of copper ion, the antiviral response of a flat and smooth surface is immediate while for the time frame considered it is not the case for the rougher SP1 surface despite its ability to deliver more copper ions at the surface.

Fig. 9 qRT-PCR test results for control, SP1, As-R, and CS samples. The test results were normalized to the kit's standard virus concentration value. Kit's standard is the calibration kit used for the test. Virus only is the virus solution added to the assay without any treatment and the media only group is the media with no virus. Media only group is the control to ensure there is no virus contamination in the media

Conclusion

Biofilm inhibition and the antiviral response of bulk copper, copper cold sprayed, and shot-peened samples were investigated. It was found that the biofilm inhibition ability of copper surfaces is different from their interaction with bacteria in the planktonic state: the number of bacteria count in the biofilm always increases with time. The as-received bulk copper sample is the least effective sample in preventing biofilm formation at all incubation times. Biofilm inhibition effectiveness of cold sprayed copper, while initially better, decreased with time and resulted in almost identical performance than as-received bulk copper. Shot peened samples demonstrated the best biofilm inhibition properties at 18 hr incubation, which was attributed to significantly higher copper ion concentration and its interaction with the biofilm. In summary, the surface topography, including both nano and micro surface texture, can facilitate the adhesion of bacteria at the planktonic state. However, if the surface modification process is accompanied by induced grain refinement and increase of dislocation densities, it results in an increase in the copper ion release rate. The latter takes over the initial adherence enhancement property of the surface and results in improved biofilm inhibition properties by interfering with the biofilm structural integrity and reducing the biofilm formation. The antiviral properties of SP1 and CS sample were compared to the As-R and stainless steel samples. It was shown that it is rather the surface topography that plays the main role in antiviral properties than ion diffusion. A smooth surface is the best antiviral choice even when exposed to low copper ion concentration.

Acknowledgment The authors would like to thank Dr. Peter King for providing us with useful suggestions on performing copper ion diffusion tests. We would also like to acknowledge the work performed by Dr. Nimal De Silva and Ms. Smitarani Mohanty at the Geochemistry Laboratory (copper ion measurement test). Figure 2, 3, and 8 in this work were created using the BioRender platform.

References

1. G. Grass, C. Rensing and M. Solioz, Metallic Copper as an Antimicrobial Surface, *Appl. Environ. Microbiol.*, 2011, **77**(5), p 1541–1547. <https://doi.org/10.1128/AEM.02766-10>
2. C. Molteni, H.K. Abicht and M. Solioz, Killing of Bacteria by Copper Surfaces Involves Dissolved Copper, *Appl. Environ. Microbiol.*, 2010, **76**(12), p 4099–4101. <https://doi.org/10.1128/AEM.00424-10>
3. C. Espírito Santo, E.W. Lam, C.G. Elowsky, D. Quaranta, D.W. Domaille, C.J. Chang and G. Grass, Bacterial Killing by Dry Metallic Copper Surfaces, *Appl. Environ. Microbiol.*, 2011, **77**(3), p 794–802. <https://doi.org/10.1128/AEM.01599-10>
4. S. Mehtar, I. Wiid and S.D. Todorov, The Antimicrobial Activity of Copper and Copper Alloys against Nosocomial Pathogens and Mycobacterium Tuberculosis Isolated from Healthcare Facilities

- in the Western Cape: An in-Vitro Study, *J. Hosp. Infect.*, 2008, **68**(1), p 45–51. <https://doi.org/10.1016/j.jhin.2007.10.009>
5. M. Vincent, R.E. Duval, P. Hartemann and M. Engels-Deutsch, Contact Killing and Antimicrobial Properties of Copper, *J. Appl. Microbiol.*, 2018, **124**(5), p 1032–1046. <https://doi.org/10.1111/jam.13681>
 6. C.E. Santo, D. Quaranta and G. Grass, Antimicrobial Metallic Copper Surfaces Kill Staphylococcus Haemolyticus via Membrane Damage, *Microbiologopen*, 2012, **1**(1), p 46–52. <https://doi.org/10.1002/mbo3.2>
 7. C. Espírito Santo, N. Taudte, D.H. Nies and G. Grass, Contribution of Copper Ion Resistance to Survival of Escherichia Coli on Metallic Copper Surfaces, *Appl Environ Microbiol.*, 2008, **74**(4), p 977–986. <https://doi.org/10.1128/AEM.01938-07>
 8. S.L. Warnes, S.M. Green, H.T. Michels and C.W. Keevil, Biotic Efficacy of Copper Alloys against Pathogenic Enterococci Involves Degradation of Genomic and Plasmid DNAs, *Appl. Environ. Microbiol.*, 2010, **76**(16), p 5390–5401. <https://doi.org/10.1128/AEM.03050-09>
 9. A. Papyrin, V. Kosarev, S. Klinkov, A. Alkhimov, and V.M. Fomin, “Cold Spray Technology,” Elsevier, 2006.
 10. Y. Zou, W. Qin, E. Irissou, J.-G. Legoux, S. Yue and J.A. Szpunar, Dynamic Recrystallization in the Particle/Particle Interfacial Region of Cold-Sprayed Nickel Coating: Electron Backscatter Diffraction Characterization, *Scripta Mater.*, 2009, **61**(9), p 899–902. <https://doi.org/10.1016/j.scriptamat.2009.07.020>
 11. A. Sabard and T. Hussain, Bonding Mechanisms in Cold Spray Deposition of Gas Atomised and Solution Heat-Treated Al 6061 Powder by EBSD, *[cond-mat]*, 2018, <http://arxiv.org/abs/1811.08694>. Accessed 28 July 2021
 12. B.C. Sousa, K.L. Sundberg, M.A. Gleason and D.L. Cote, Understanding the Antipathogenic Performance of Nanostructured and Conventional Copper Cold Spray Material Consolidations and Coated Surfaces, *Crystals*, 2020, **10**(6), p 504. <https://doi.org/10.3390/cryst10060504>
 13. K. Sundberg, Y. Wang, B. Mishra, A. Carl, R. Grimm, A. Te, L. Lozeau, R. Sisson and D. Cote, The Effect of Corrosion on Conventional and Nanomaterial Copper Cold Spray Surfaces for Antimicrobial Applications, *Biomed. J. Scient. Tech. Res.*, 2019, **22**(3), p 16753–16763.
 14. V.K. Champagne and D.J. Helfrich, A Demonstration of the Antimicrobial Effectiveness of Various Copper Surfaces, *J. Biol. Eng.*, 2013, **7**(1), p 1–7. <https://doi.org/10.1186/1754-1611-7-8>
 15. K. Sundberg and V. Champagne, Effectiveness of Nanomaterial Copper Cold Spray Surfaces on Inactivation of Influenza A Virus, *J Biotechnol Biomater*, 2015 <https://doi.org/10.4172/2155-952X.1000205>
 16. Y. Sun, Z. Zhang, Y. Qin, X. Xu and S. Yang, The Effect of Rotation Accelerated Shot Peening on Mechanical Property and Antimicrobial Activity of Pure Copper, *Surf. Coat. Technol.*, 2020, **384**, 125319. <https://doi.org/10.1016/j.surfcoat.2019.125319>
 17. K.A. Whitehead, J. Colligon and J. Verran, Retention of Microbial Cells in Substratum Surface Features of Micrometer and Sub-Micrometer Dimensions, *Colloids Surf., B*, 2005, **41**(2), p 129–138. <https://doi.org/10.1016/j.colsurfb.2004.11.010>
 18. O. Sharifahmadian, H.R. Salimijazi, M.H. Fathi, J. Mostaghimi and L. Pershin, Relationship between Surface Properties and Antibacterial Behavior of Wire Arc Spray Copper Coatings, *Surf. Coat. Technol.*, 2013, **233**, p 74–79. <https://doi.org/10.1016/j.surfcoat.2013.01.060>
 19. E. Maikranz, C. Spengler, N. Thewes, A. Thewes, F. Nolle, P. Jung, M. Bischoff, L. Santen and K. Jacobs, Different Binding Mechanisms of Staphylococcus Aureus to Hydrophobic and Hydrophilic Surfaces, *Nanoscale*, 2020, **12**(37), p 19267–19275. <https://doi.org/10.1039/D0NR03134H>
 20. M.C.M. van Loosdrecht, W. Norde, J. Lyklema and A.J.B. Zehnder, Hydrophobic and Electrostatic Parameters in Bacterial Adhesion, *Aquatic Sci.*, 1990, **52**(1), p 103–114. <https://doi.org/10.1007/BF00878244>
 21. A. Tripathy, P. Sen, B. Su and W.H. Briscoe, Natural and Bioinspired Nanostructured Bactericidal Surfaces, *Adv. Coll. Interface. Sci.*, 2017, **248**, p 85–104. <https://doi.org/10.1016/j.cis.2017.07.030>
 22. A. Jaggesar, H. Shahali, A. Mathew and P.K.D.V. Yarlagadda, Bio-Mimicking Nano and Micro-Structured Surface Fabrication for Antibacterial Properties in Medical Implants, *J. Nanobiotechnol.*, 2017, **15**(1), p 64. <https://doi.org/10.1186/s12951-017-0306-1>
 23. S. Bagherifard, D.J. Hickey, A.C. de Luca, V.N. Malheiro, A.E. Markaki, M. Guagliano and T.J. Webster, The Influence of Nanostructured Features on Bacterial Adhesion and Bone Cell Functions on Severely Shot Peened 316L Stainless Steel, *Biomaterials*, 2015, **73**, p 185–197. <https://doi.org/10.1016/j.biomaterials.2015.09.019>
 24. J. Elguindi, J. Wagner and C. Rensing, Genes Involved in Copper Resistance Influence Survival of Pseudomonas Aeruginosa on Copper Surfaces, *J. Appl. Microbiol.*, 2009, **106**(5), p 1448–1455. <https://doi.org/10.1111/j.1365-2672.2009.04148.x>
 25. D.W. Müller, S. Lößlein, E. Terriac, K. Brix, K. Siems, R. Moeller, R. Kautenburger and F. Mücklich, Increasing Antibacterial Efficiency of Cu Surfaces by Targeted Surface Functionalization via Ultrashort Pulsed Direct Laser Interference Patterning, *Adv. Mater. Interfaces*, 2021, **8**(5), p 2001656. <https://doi.org/10.1002/admi.202001656>
 26. M. Rosenberg, H. Vija, A. Kahru, C.W. Keevil and A. Ivask, Rapid in Situ Assessment of Cu-Ion Mediated Effects and Antibacterial Efficacy of Copper Surfaces, *Scient. Reports*, 2018, **8**(1), p 8172. <https://doi.org/10.1038/s41598-018-26391-8>
 27. H. Michels, J. Noyce and C. Keevil, Effects of Temperature and Humidity on the Efficacy of Methicillin-Resistant Staphylococcus Aureus Challenged Antimicrobial Materials Containing Silver and Copper, *Lett Appl Microbiol.*, 2009, **49**(2), p 191–195. <https://doi.org/10.1111/j.1472-765X.2009.02637.x>
 28. M. Hans, A. Erbe, S. Mathews, Y. Chen, M. Solioz and F. Mücklich, Role of Copper Oxides in Contact Killing of Bacteria, *Langmuir*, 2013, **29**(52), p 16160–16166. <https://doi.org/10.1021/la404091z>
 29. L. Miao, C. Wang, J. Hou, P. Wang, Y. Ao, Y. Li, Y. Yao, B. Lv, Y. Yang, G. You, Y. Xu and Q. Gu, Response of Wastewater Biofilm to CuO Nanoparticle Exposure in Terms of Extracellular Polymeric Substances and Microbial Community Structure, *Sci. Total Environ.*, 2017, **579**, p 588–597. <https://doi.org/10.1016/j.scitotenv.2016.11.056>
 30. I.B. Gomes, L.C. Simões and M. Simões, Influence of Surface Copper Content on Stenotrophomonas Maltophilia Biofilm Control Using Chlorine and Mechanical Stress, *Biofouling*, 2020, **36**(1), p 1–13. <https://doi.org/10.1080/08927014.2019.1708334>
 31. N. Chari, L. Felix, M. Davoodbasha, A. Sulaiman Ali and T. Nooruddin, In Vitro and in Vivo Antibiofilm Effect of Copper Nanoparticles against Aquaculture Pathogens, *Biocatalysis. Agric. Biotechnol.*, 2017, **10**, p 336–341. <https://doi.org/10.1016/j.bcab.2017.04.013>
 32. R. Srinivasan, S. Santhakumari, P. Poonguzhali, M. Geetha, M. Dyavaiah and L. Xiangmin, Bacterial Biofilm Inhibition: A Focused Review on Recent Therapeutic Strategies for Combating the Biofilm Mediated Infections, *Front. Microbiol.*, 2021 <https://doi.org/10.3389/fmicb.2021.676458>
 33. T. Rasamiravaka, Q. Labtani, P. Duez and M. El Jaziri, The Formation of Biofilms by Pseudomonas Aeruginosa: A Review of

- the Natural and Synthetic Compounds Interfering with Control Mechanisms, *Biomed Res Int*, 2015, **2015**, 759348. <https://doi.org/10.1155/2015/759348>
34. K. Kierek-Pearson and E. Karatan, Biofilm Development in Bacteria, *Adv. Appl. Microbiol.*, 2005 [https://doi.org/10.1016/S0065-2164\(05\)57003-5](https://doi.org/10.1016/S0065-2164(05)57003-5)
 35. M. Kostakioti, M. Hadjifrangiskou and S.J. Hultgren, Bacterial Biofilms: Development, Dispersal, and Therapeutic Strategies in the Dawn of the Postantibiotic Era, *Cold Spring Harb Perspect Med*, 2013, **3**(4), p a010306. <https://doi.org/10.1101/cshperspect.a010306>
 36. I.B. Gomes, M. Simões and L.C. Simões, Copper Surfaces in Biofilm Control, *Nanomaterials (Basel)*, 2020, **10**(12), p E2491. <https://doi.org/10.3390/nano10122491>
 37. J.O. Noyce, H. Michels and C.W. Keevil, Inactivation of Influenza A Virus on Copper versus Stainless Steel Surfaces, *Appl. Environ. Microbiol.*, 2007, **73**(8), p 2748–2750. <https://doi.org/10.1128/AEM.01139-06>
 38. T. Ishida, Antiviral Activities of Cu²⁺ Ions in Viral Prevention, Replication, RNA Degradation, and for Antiviral Efficacies of Lytic Virus, ROS-Mediated Virus, Copper Chelation, *undefined*, 2018, <https://www.semanticscholar.org/paper/Antiviral-Activities-of-Cu2%2B-Ions-in-Viral-RNA-and-Ishida/4ac10816ee5d408f2cf590d34aa82a65dee19a13>. Accessed 13 August 2021
 39. J.-L. Sagripanti and M.M. Lightfoote, Cupric and Ferric Ions Inactivate HIV, *AIDS Res. Human Retroviruses*, 1996, **12**(4), p 333–336. <https://doi.org/10.1089/aid.1996.12.333>
 40. G. Borkow, S.S. Zhou, T. Page and J. Gabbay, A Novel Anti-Influenza Copper Oxide Containing Respiratory Face Mask, *PLOS ONE*, 2010, **5**(6), p e11295. <https://doi.org/10.1371/journal.pone.0011295>
 41. S.C. Wilschefske and M.R. Baxter, Inductively Coupled Plasma Mass Spectrometry: Introduction to Analytical Aspects, *Clin. Biochem. Rev.*, 2019, **40**(3), p 115–133. <https://doi.org/10.33176/AACB-19-00024>
 42. B.H. Iglewski, *Pseudomonas*, *Medical Microbiology*, S. Baron, Ed., 4th ed., (Galveston (TX)), University of Texas Medical Branch at Galveston, 1996, <http://www.ncbi.nlm.nih.gov/books/NBK8326/>. Accessed 4 August 2021
 43. T.F. Mah and G.A. O'Toole, Mechanisms of Biofilm Resistance to Antimicrobial Agents, *Trends Microbiol.*, 2001, **9**(1), p 34–39. [https://doi.org/10.1016/S0966-842X\(00\)01913-2](https://doi.org/10.1016/S0966-842X(00)01913-2)
 44. J.O. Noyce, H. Michels and C.W. Keevil, Potential Use of Copper Surfaces to Reduce Survival of Epidemic Meticillin-Resistant Staphylococcus Aureus in the Healthcare Environment, *J. Hosp. Infect.*, 2006, **63**(3), p 289–297. <https://doi.org/10.1016/j.jhin.2005.12.008>
 45. B.C. Sousa, C.J. Massar, M.A. Gleason and D.L. Cote, On the Emergence of Antibacterial and Antiviral Copper Cold Spray Coatings, *J. Biol. Eng.*, 2021, **15**(1), p 8. <https://doi.org/10.1186/s13036-021-00256-7>
 46. K.D. Young, The Selective Value of Bacterial Shape, *Microbiol. Mol. Biol. Rev.*, 2006, **70**(3), p 660–703. <https://doi.org/10.1128/MMBR.00001-06>
 47. A. Käkinen, O. Bondarenko, A. Ivask and A. Kahru, The Effect of Composition of Different Ecotoxicological Test Media on Free and Bioavailable Copper from CuSO₄ and CuO Nanoparticles: Comparative Evidence from a Cu-Selective Electrode and a Cu-Biosensor, *Sensors*, 2011, **11**(11), p 10502–10521. <https://doi.org/10.3390/s111110502>
 48. M. Legros, G. Dehm, E. Arzt and T.J. Balk, Observation of Giant Diffusivity Along Dislocation Cores, *Science*, 2008, **319**(5870), p 1646–1649. <https://doi.org/10.1126/science.1151771>
 49. R. Thevamaran, O. Lawal, S. Yazdi, S.-J. Jeon, J.-H. Lee and E.L. Thomas, Dynamic Creation and Evolution of Gradient Nanostructure in Single-Crystal Metallic Microcubes, *Science*, 2016, **354**(6310), p 312–316. <https://doi.org/10.1126/science.aag1768>
 50. K. Li, Y. He, C. Fang, H. Ma, J. Kim, H. Lee, J. Song, C.-W. Yang, J.-H. Lee and K. Shin, Surface Nanocrystallization of Pure Cu Induced by Ultrasonic Shot Peening, *J. Nanosci. Nanotechnol.*, 2014, **14**(12), p 9637–9643. <https://doi.org/10.1166/jnn.2014.10176>
 51. H.H.M. Rijnaarts, W. Norde, E.J. Bouwer, J. Lyklema and A.J.B. Zehnder, Reversibility and Mechanism of Bacterial Adhesion, *Colloids Surf., B*, 1995, **4**(1), p 5–22. [https://doi.org/10.1016/0927-7765\(94\)01146-V](https://doi.org/10.1016/0927-7765(94)01146-V)

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.