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Effect of Humidity on Antibacterial Activity of Copper and Its Alloy Surfaces

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Copper and its alloys are well known to have bactericidal activity and applied to touch surface to suppress the transmission of pathogens via material surface. The touch surface is exposed to real life conditions, which are generally lower in humidity than that in laboratory testing conditions. The influence of humidity on the antibacterial activity of copper and other materials was reported as contradictory results due to different inoculation methods and contact periods. In this study, the antibacterial activity of copper (C1020) and its alloys (C7060, C7150, MONEL400, and antibacterial stainless steel) was systematically investigated by a method simulating droplet transmission under controlled humidity (60 and 35%RH) with *Escherichia coli* or *Staphylococcus aureus*. Corrosion rate of C1020 was estimated by electrochemical impedance spectroscopy under the controlled humidity. As a parameter of antibacterial activity, the contact time to reduce the viable bacteria to be 1% of the inoculated ones, $T_{0.01}$, was decided based on the test results. The all materials increased their $T_{0.01}$ with reduction in humidity, indicating the decrease in their antibacterial activity. In case of C1020, the smallest $T_{0.01}$ for *E. coli* was observed at 90% RH as 6.96 min, which increased to 67.4 min at 35%RH. At both high and low humidity, the materials with high copper contents (C1020, C7060, and C7150) had the smaller $T_{0.01}$ than those with lower copper contents. *S. aureus*, which has higher tolerance to the environment with low water activity than *E. coli*, resulted in greater increase in $T_{0.01}$ with reduction in humidity (In case of C1020, $T_{0.01}$ at 90 and 35%RH for *S. aureus* were 8.50 and 230 min, respectively). The corrosion rate of C1020 decreased with reduction in humidity, suggesting the importance of copper ion release at the surface for their antibacterial activity. [doi:10.2320/matertrans.MT-M2025099]

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1. Introduction

Emergence of antimicrobial resistance (AMR) in bacteria and other pathogens become a global threat as the review paper published in 2014 estimated about 0.7 million deaths every year due to the drug-resistant infections [1]. In 2019, the global deaths associated with bacterial AMR is estimated as 4.95 million, including those attributable to AMR bacteria of 1.27 million [2]. Rapid increase in global antibiotic use increases the AMR strains, which overtakes the pace of novel antibiotic discovery. Running out of an effective antibiotic for the infection treatment drawbacks the greatest medical advances in 20th century; successful treatment for major deadly illness such as pneumonia and tuberculosis, as well as infection control for routine surgery and childbirth. To avoid this situation, unnecessary and excessive antibiotic use should be terminated not only in the medical/health care fields but also in an agricultural field based on the concept of "one health" [1, 3]. In addition, it is important to have the means to inhibit the spread of drug-resistant pathogens without antibiotics.

Copper surface is well known to have bactericidal activity so called "contact killing" [4–7]. Though its exact mechanism is not fully understood, it can rapidly eliminate a wide range of pathogenic microorganisms such as gram negative/positive bacteria, fungi, spores, and viruses [4, 5, 8]. Therefore, the application of copper and its alloys to touch surface is considered to be one of the countermoves to suppress the spread of infective pathogens. Fields trials of copper application to touch surface in hospitals and elderly care facilities reported reductions in bioburden on the surface [9–14], however, evidence for significant decrease in healthcare associated infection (HAI) is still limited [9, 10]. The meta-analysis of 3 clinical studies revealed the introduction of copper and its alloys has limited reduction in HAI by around 25% [10]. This suggests the necessity of the improvement in the antimicrobial activity of copper and its alloys for touch surface application under real life conditions.

One of the key factors for the successful touch-surface application is the environment. Most of the laboratory antimicrobial tests use bacterial suspension to inoculate onto material surface. For example, ISO22196 indicates a 0.4 mL portion of the bacterial suspension ($2.5–10 \times 10^5$ cells/mL) contacting to the specimen surface area of 40 mm × 40 mm, which is controlled with a cover of a non-toxic polymer film [15]. Then, the inoculated specimen is kept at 35°C under the relative humidity (RH) over 90% [15], which is a fully wet condition. However, in the hospital environment, the temperature and humidity are generally 20–30°C and 40–50%RH [16] varying with weather, and pathogens are applied as droplets or by direct contact to the touch surface. For the successful reduction in HAI, the materials having a sufficient antimicrobial activity under the real-life environment of the touch surface should be employed.

Focusing on the difference between the laboratory testing environment and that in real life ones, several researchers evaluated the materials antibacterial activity at the ambient temperature and low humidity. H.T. Michels *et al.* reported that the antibacterial activity of copper and its alloys contacting 24 h with methicillin resistant *Staphylococcus aureus* (MRSA) were not influenced by the temperatures (∼35°C or ∼20°C) and relative humidity (∼90%RH, ∼20%RH, or ∼24%RH), whereas that of Ag-containing antibacterial material decreased with decrease in temperature and relative humidity [17]. J. Li *et al.* reported contradictory results; the antibacterial activity of the ordinary copper foil contacting 1 h with *Escherichia coli* increased with reduction in humidity from 90 to 30%RH whereas that of nano-structured copper foil decreased [18]. A. Mayr *et al.* reported

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that the antibacterial activity of copper alloy (C71500) contacting 4 h with *S. aureus* decreased with reduction in humidity from 50 to 35%RH [19]. The available studies are limited for different testing materials at different relative humidity with different contact periods. Furthermore, different bacterial inoculation methods may contribute to the contradictory results. C.E. Santo *et al.* tested the antibacterial activity of copper with "dry" (10^6 CFU applied by swab) and "moist" (10^9 CFU in 40 µL phosphate buffered saline) inoculation, reporting the "faster" killing in the former condition [20]. Due to the large difference in inoculated number of bacteria, it is unclear that this faster killing is due to less bacteria contacting to copper surface or to the "dry" condition. J. Jann *et al.* attempted to evaluate the material antibacterial activity with a bacterial transfer from a moist gel or a dried nylon filter [21], but the results are complicated partially due to the ambiguity in the actual transferred number of bacteria to the testing material surface.

In the present study, our primary purpose is to investigate the effect of humidity on the antibacterial activity of copper and its alloys mimicking the real-life condition applied to touch surface. Test specimens were prepared with intentional storage in ambient atmosphere for long time after polishing. The antibacterial activity of test specimens was systematically investigated using *E. coli* (gram negative bacteria) and *S. aureus* (gram positive bacteria) at different contact periods upto 30 min under controlled humidity. To avoid the ambiguity, a small portion of the bacterial suspension was directly applied to each of the testing material surface, simulating the droplet transmission. Electrochemical impedance measurement was introduced to evaluate the corrosion rate of copper under relatively low humidity.

2. Experimental Procedure

2.1 Testing materials

Materials used are oxygen-free copper (C1020), three kinds of copper-nickel alloys (C7060, C7150, and MONEL400), and antibacterial stainless steel (NSSAM3, abbreviated as ABSS). Copper alloys are provided by the courtesy of Mitsubishi Materials Corporation. All samples are commercially available. Chemical compositions of these materials are shown in Table 1.

All materials were cut into 15–20 mm squares and 0.05–2 mm thick for the antibacterial assay. Alloy specimens were ground by SiC paper up to #1200 (~5 µm), followed by rinsing with ultrapure water. C1020 specimens were in form of thin plate (0.05 mm), so they were used as received. The cut and polished specimens were stored under ambient atmosphere of the laboratory (22–24°C and 40–60%RH) over 7 days, considering a real-world condition for indoor touch surface application. In prior to the antibacterial assays, the specimen surfaces were cleaned with a detergent (a mixed solution of sodium α-dodecan-1-yl-ω-(sulfonatooxy)poly-(oxyethylene) and fatty acid alkanolamide), followed by rinsing with ultrapure water and air-dry. For the antibacterial assay by the film method, the bottom surface of each specimen was covered with a thin silicone film to avoid the contact to the collecting solution of survived bacteria.

2.2 Antibacterial assay under controlled humidity

The antibacterial assay procedure was schematically shown in supplementary Fig. S1. The bacterial cell lines, *Escherichia coli* (ATCC 8739, 0483-PEC, Microbiologics, Inc.) and *Staphylococcus aureus* (ATCC 6538, 0485-PEC, Microbiologics, Inc.), were employed and prepared following the protocol supplied with the kit. Each of the testing materials was placed on the bottom of the glass dish individually. A bacteria suspension was prepared in a phosphate buffer supplied with the kit adding Tween 80 [Polyoxyethylene (20) Sorbitan Monooleate, FUJIFILM Wako Pure Chemical Corporation] to be 0.5%. This surfactant was added to improve the spreadability of the bacteria suspension on the material surface, which influences the contact area and drying time of the suspension.

A 1 µL portion of the bacteria suspension (containing $\sim 1 \times 10^5$ CFU) was spread about 10 mm square on the specimen surface using a tip of the digital pipette. As a control surface, the bacteria suspension was spread on the bottom of a glass dish in the same manner. Each specimen was dried at the ambient temperature and humidity in the biosafety cabinet for 5 min and then, incubated at 25 ± 1°C under the controlled humidity as 35–90%RH for additional 5, 10, and 30 min. For ABSS, the incubation period was set as 5, 120, and 1440 min based on our previous study [22]. The humidity in the incubator was monitored by a hygrometer (Weathecom II electronic thermos and hygrometer EX-502, EMPEX Instruments, Inc.) and was controlled with the open surface area of the water except the case of 35% RH, in which a silica gel pack was used instead of water.

After the incubation, the testing material surface was wiped with a polyester swab containing 20 µL of sterile Nutrient Broth 'Eiken' (abbreviated as NB, Eiken Chemical Co. Ltd.). The swab was applied 20 times horizontally and 20

Table 1 Chemical compositions of the testing materials (mass%).

Material	Cu	Ni	Fe	Mn	Pb	Zn	Cr	Si	C	Al	S
C1020	>99.99	—	—	—	—	—	—	—	—	—	—
C7060	Rem.	10.3	1.19	0.83	0.01	0.01	—	—	—	—	—
C7150	Rem.	30.1	0.28	0.77	0.001	0.001	—	—	—	—	—
MONEL	Rem.	64.7	0.03	1.56	—	—	—	0.34	0.004	0.001	0.001
ABSS	3.8	9.4	Rem.	1.40	—	—	18.1	0.6	—	—	—

times vertically, alternately up to 5 sets (100 times in total) covering the loaded area of the bacteria suspension. The head of the swab was cut into a 480 µL portion of NB and vortexed for 30 sec to extract collected bacteria. Then, the number of viable bacteria in the collected solution was estimated using a water-soluble tetrazolium salt (WST) [23, 24]. The WST penetrates into cells and forms a soluble formazan dye by succinate-tetrazolium reductase in the mitochondrial respiratory chain, which is only active in viable cells. Therefore, the concentration of the formazan dye correlates to the number of viable cells. A brief explanation of the procedure is as follows; a 180 µL portion of the NB with collected bacteria was poured into a well of a 96-well microplate, followed by the addition of 20 µL of the mixture of 5 mM WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, Dojindo Laboratories] and 0.2 mM 1-methoxy PMS (1-Methoxy-5-methylphenazinium methylsulfate, Dojindo Laboratories) solution. NB (without bacteria) was poured into the blank well. Then, the microplate was sealed and incubated at 35°C up to 16 h while the absorbance at 450 nm was measured every 20 min for *E. coli*. For *S. aureus*, the interval for the absorbance measurement was set as 30 min during 24 h of incubation.

The difference in absorbance between the sample well and the blank well was calculated and find the incubation time when this difference reaches to 0.5 ($T_{Abs0.5}$). This incubation time was utilized to estimate the number of viable bacteria in the collected solution, NVB_{col} (CFU/mL), based on the calibration curve prepared in NB as shown in Fig. S1(c). The NVB_{col} was used to calculate the survival rate of bacteria, *SRB* as following equation;

$$SRB = NVB_{col} \times 0.5/INB_{mat}$$

where INB_{mat} indicates the inoculated number of bacteria per specimen surface ($\sim 1 \times 10^5$ CFU). Then, the contact time to reduce the *SRB* to 0.01, $T_{0.01}$ was estimated using the probit regression. All the experiments were performed in triplicate.

2.3 Antibacterial assay by film method

In order to compare the antibacterial activity under a fully wet condition, antibacterial assay by a film method [22] was carried out for C7060 and MONEL using the same bacterial cell lines, *E. coli* and *S. aureus*. Briefly, a 50 µL portion of bacterial suspension ($\sim 1 \times 10^6$ CFU) in 0.9% NaCl was placed onto a specimen surface and covered by a polyethylene film of 10 mm square. Then, the specimens were incubated at 35 ± 1°C and the humidity over 90%RH up to 1440 min. After incubation, the bacteria on the specimen surface were collected into a 1 mL portion of 0.1 mM ethylenediamine-N,N,N′,N′-tetraacetic acid, disodium salt, dihydrate (EDTA-2 Na) in 0.9% NaCl solution by pipetting. The viable bacterial number was decided using 5-cyano-2,3-ditolyl-2H-tetrazolium chloride (CTC) staining kit (-Bacstain- CTC Rapid Staining Kit for Flow cytometry, BS01, Dojindo Laboratories, Kumamoto, Japan) following its instruction. All the experiments were performed at least in duplicate and in triplicate when necessary.

2.4 Electrochemical impedance analysis

In order to investigate the corrosion rate of the C1020 under the controlled humidity, electrochemical impedance measurement was performed using a pair of parallel copper plates as electrodes [25]. Figure 1 indicates the details of the specimen and equivalent circuit model used for data analysis. A commercially available 100 µm thick C1020 foil was cut into 10 mm wide with one side covered by a 50-µm-thick polyimide insulating tape. Then, the covered sides of two pieces were faced to each other and embedded into a low-viscos resin using a mold of the inner diameter of 25.4 mm. Then, the specimen surface was polished by SiC paper up to #1200 and the periphery of the resin was covered by the insulating tape with a rim of 0.5 mm in height.

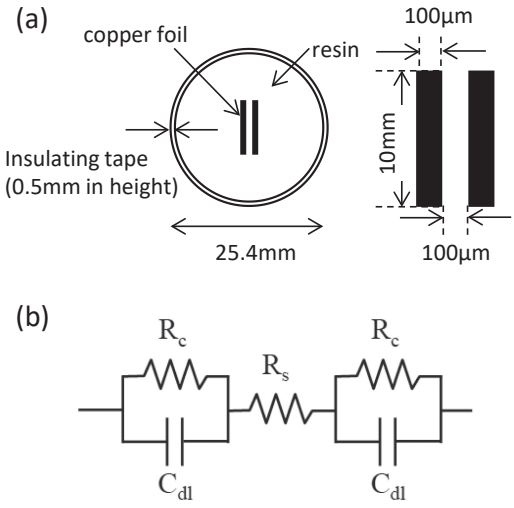

Fig. 1 Schematic explanation of the specimen (a) and equivalent circuit model (b) for the electrochemical impedance spectroscopy. R_s: the sum of the electric resistance of electrolyte and the EIS system used for the measurement. R_c: charge transfer resistance. C_{dl}: double-layer capacitance.

A 500 µL portion of an electrolyte, 0.7 M Na_2SO_4 was poured onto the specimen surface which gives the salt density of 10 mg/cm^2. Then, the specimen was placed in the incubator and electrochemical impedance spectroscopy (EIS) was performed immediately with AC amplitude of 10 mV in the frequency range of 10^{-2}–10^5 Hz using a potentiostat equipped with a frequency response analyzer (VersaSTAT4, Princeton Applied Research). This result in the electrolyte was indicated as the 100% humidity. Then, the humidity in the incubator was controlled to be 30 ± 5% RH and left overnight to dry the moisture. The EIS was performed in the next day, followed by the increase in the humidity to be 50 ± 5, 60 ± 5, 75 ± 5, and 90 ± 5% RH. EIS measurement was carried out at least 0.5 h after the humidity is stable in the designated range. The RH in the incubator was controlled and monitored in the same manner to the antibacterial test under controlled humidity.

The obtained data were analyzed by assuming the equivalent circuit shown in Fig. 1(b) [26]. At the high frequency, capacitance components approach to zero as negligible, while at the lower frequency, capacitance components approach to infinity. Therefore, when we define the impedance at high frequency range (100 kHz) and at low frequency range (10 mHz) as Z_{high} and Z_{low}, respectively, these can be expressed as following equations;

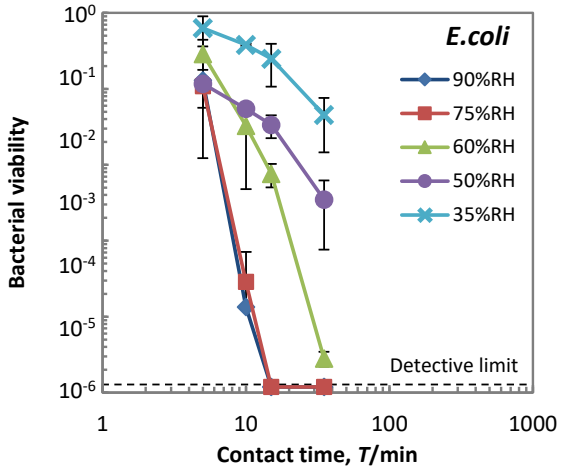

Fig. 2 The antibacterial test results of C1020 under controlled humidity using *Escherichia coli* (mean ± sd). The contact time includes the drying time (5 min) of the bacterial suspension before the sample is placed in a humidity-controlled chamber. The obtained results were statistically analyzed by the parallelism test of 2 regression lines (see supplementally Table S1). (online color)

$$Z_{high} = R_s$$

$$Z_{low} = 2R_c + R_s$$

When we consider the difference between these impedances as Z_{diff}, it can be written as follows;

$$Z_{diff} = Z_{low} - Z_{high} = 2R_c$$

Since the corrosion rate, I_{corr} is proportional to the reciprocal number of R_c as

$$I_{corr} = k/R_c$$

where k is a constant, $1/Z_{diff}$ can be employed as a corrosion parameter to monitor the change in the corrosion rate. EIS measurements were performed in triplicate.

2.5 Statistical analysis

The data obtained by antibacterial assay under controlled humidity for copper and copper alloys were statistically analyzed by parallelism test of 2 regression lines using a statistical analysis software (Kyplot 6.0, KyensLab. Inc.). The logarithm of bacteria viability of a material at a certain humidity was plotted against the logarithm of the contact time to obtain regression line, which is tested for equality of slope or vertical line separation against another regression line of another dataset such with different materials or humidity. Results of the parallelism tests were described in the supplementary Tables S1–5.

3. Results

3.1 Effect of relative humidity on antibacterial activity of copper and its alloys

The antibacterial activity of C1020 against *E. coli* was examined under the various humidity and shown in Fig. 2. At 75% RH, the *SRB* was almost equal to that of 90% RH. At lower humidity, however, the *SRB* tended to increase with reduction in humidity. For example, no bacteria survived after 15 min contact (5 min drying and 10 min incubation) at

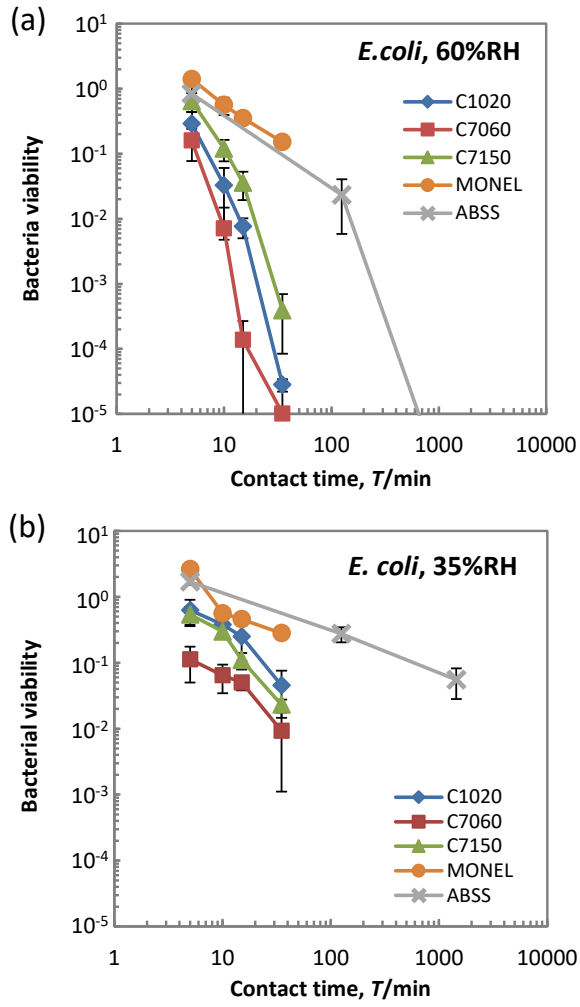

Fig. 3 The antibacterial test results of copper and its alloys under controlled humidity using *Escherichia coli* (mean ± sd). The contact time includes the drying time (5 min) of the bacterial suspension before the sample is placed in a humidity-controlled chamber. The obtained results were statistically analyzed by the parallelism test of 2 regression lines (see supplementally Tables S2 and S3). (online color)

90% and 75% RH whereas the *SRB* after 35 min contact (5 min drying and 30 min incubation) drastically increased with reduction in humidity as 60%, 50%, and 35% RH. In other words, the slope of the *SRB*–contact time curve decreased with reduction in humidity, indicating the decrease in the antibacterial activity of C1020, which agrees with the previous reports for C7150 [19] and a nanostructured copper foil [18].

Figure 3 indicates the antibacterial activity of copper and its alloys examined at 60% and 35% RH against *E. coli*. In all tested materials, the antibacterial activity reduced at the lower humidity than at the higher. At 35% RH, no material can succeed to reduce the *SRB* less than 0.001 (99.9% reduction) within 35 min of contact (5 min drying and 30 min incubation). The order of antibacterial activity does not change from that at 60%RH; C7060 > C1020 ≥ C7150 > MONEL ~ ABSS.

In similar to *E. coli*, the antibacterial activity of copper and its alloys reduced at 35%RH for *S. aureus* as shown in Fig. 4. At the lower humidity, the reduction rate of *SRB* retarded in comparison with those at the higher humidity. The order of

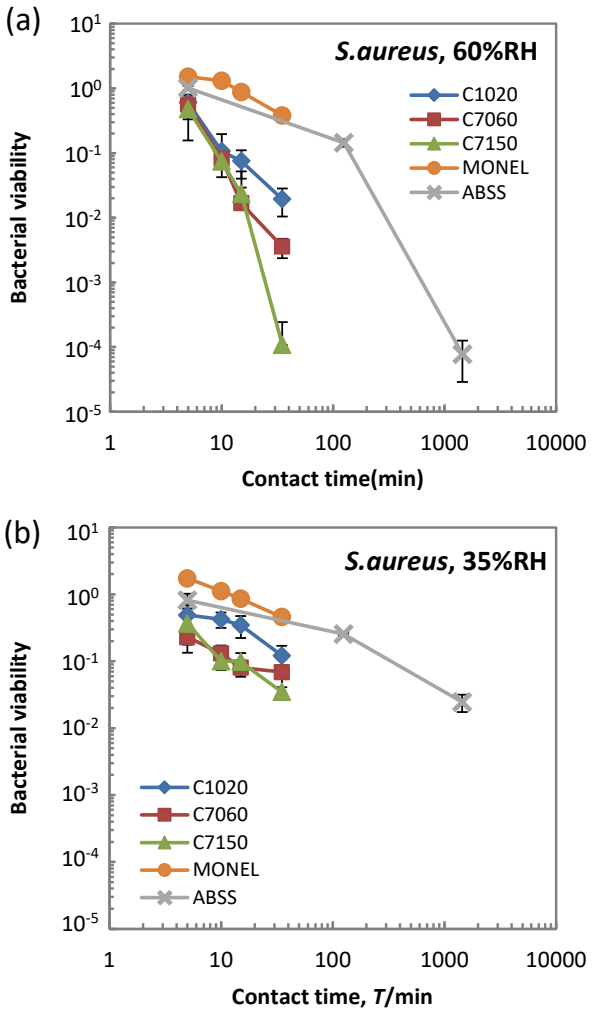

Fig. 4 The antibacterial test results of copper and its alloys under controlled humidity using *Staphylococcus aureus* (mean ± sd). The contact time includes the drying time (5 min) of the bacterial suspension before the sample is placed in a humidity-controlled chamber. The obtained results were statistically analyzed by the parallelism test of 2 regression lines (see supplementally Tables S4 and S5). (online color)

antibacterial activity was as follows; C7150 ~ C7060 > C1020 > ABSS ~ MONEL.

As the parameter of the antibacterial activity level, the time to reduce the *SRB* to 0.01 ($T_{0.01}$, min) was estimated by probit regression and shown in Table 2 together with the $T_{0.01}$ values examined by the film method referring to JIS Z2801:2012 (ISO 22196). The bacterial survival ratio–contact time curves for C7060, C7150, and MONEL obtained by the film method was displayed in supplementary Fig. S2. Table 2 also contains the $T_{0.01}$ values of MONEL and ABSS against *E. coli* at 90% RH; their survival ratio-contact time curves were displayed in supplementary Fig. S3. The $T_{0.01}$ increased with reduction in humidity for all the materials tested and bacteria used. In the film method, bacterial suspension will not dry out because of the polyethylene cover film, therefore it can be referred as the result in a fully wet condition. Except ABSS, the $T_{0.01}$ at 60%RH are close to those by the film method, but the $T_{0.01}$ at 35%RH is larger than those at 60%RH or by the film method. These data suggest that the lower humidity such as 35%RH drastically reduces the antibacterial activity of copper and its alloys.

Figure 5 plots $T_{0.01}$ against the Cu content in the alloy composition. For *E. coli*, C7060 has the smallest $T_{0.01}$ both at 60% and 30% RH, and $T_{0.01}$ increased with decrease in Cu content. For *S. aureus*, C7150 has the smallest $T_{0.01}$ both at 60% and 35% RH, and $T_{0.01}$ increased with decrease and increase in Cu content. These data indicate the dependance of $T_{0.01}$ on Cu content in the alloy composition. Cu-Ni alloy with Ni content up to 30% has superior antibacterial activity in similar or even better level of pure copper (C1020). Cu-Ni alloys with Ni content over 30% decrease its antibacterial activity with decrease in Cu content. This trend suggests the dependance of the antibacterial activity of Cu-Ni alloy in the Cu content, which agrees with the general tendency of Cu alloys; the higher Cu content gives the higher antibacterial activity [22].

Table 2 Time to reduce the viable bacteria to 1/100 of the inoculated ones on the material surface ($T_{0.01}$/min).

Material	Cu content (mass%)	Ni content (mass%)	*Escherichia coli*				*Staphylococcus aureus*		
			RH(%)			Film*	RH(%)		Film*
			35	60	90		35	60	
C1020	100	–	67.4	13.6	6.96	9.33**	230	32.4	9.86**
C7060	90	10	36.8	9.64	–	21.5	169	21.3	22.3
C7150	70	30	45.2	19.7	–	26.2	53.6	16.9	28.5
MONEL	30	65	697	35.6	27.5	89.1	396	114	90.8
ABSS	4	10	1650	180	136	1490**	3080	390	2030**

*"Film" indicates the results obtained by the film method (under fully wet condition).

**Values from [22].

$T_{0.01}$ of C1020 at 50%RH, 75%RH for *E.coli* and at 90%RH for *S.aureus* are 23.8, 7.05 and 8.50 min, respectively.

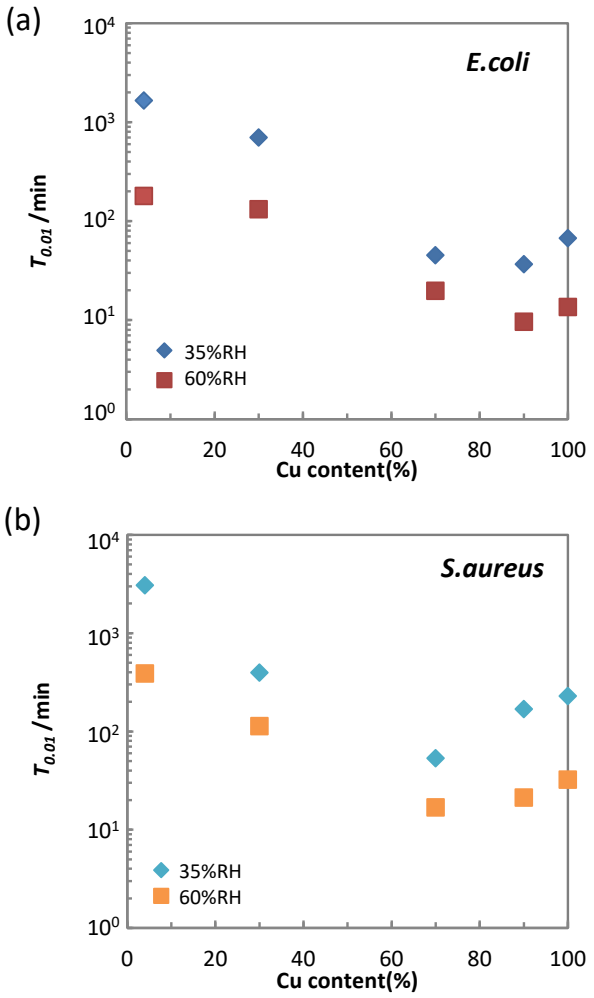

Fig. 5 Correlation of the antibacterial activity and copper content in the alloy composition. (online color)

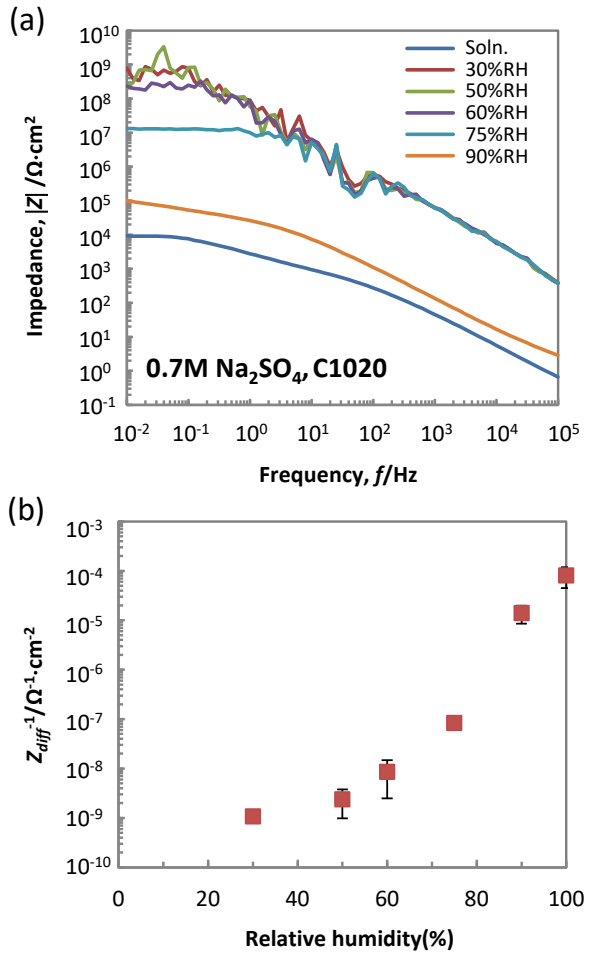

Fig. 6 Typical results of electrochemical impedance spectroscopy of C1020 under controlled humidity (a), and the average $1/Z_{diff}$ plotted against relative humidity (b), n = 3, mean ± sd. (online color)

3.2 Effect of relative humidity on corrosion of copper

The examples of the electrochemical impedance spectra of C1020 under various humidity were shown in Fig. 6. It clearly shows the increase in impedance with reduction in humidity. In order to evaluate the change in corrosion rates, the reciprocal of the difference in impedances at low (10 mHz) and high (100 kHz) frequencies, $1/Z_{diff}$ was calculated and plotted against humidity in Fig. 6(b). It indicates the decrease in the corrosion rates with reduction in humidity, which agrees with the results of other metals evaluated by a similar manner [27].

The plot of the $T_{0.01}$ of C1020 against $1/Z_{diff}$ is shown in Fig. 7. When the $1/Z_{diff}$ was larger than 10^{-7} ($/\Omega\cdot cm^2$), which corresponded to the humidity over 75%RH, the $T_{0.01}$ stayed in a similar level (~10 min). However, when the $1/Z_{diff}$ was smaller than 10^{-7} ($/\Omega\cdot cm^2$), which corresponded to the humidity under 75%RH, the $T_{0.01}$ increased, indicating the decrease in its antibacterial activity. This trend suggests the importance of the corrosion rate of the copper on its antibacterial activity in middle to low humidity environment.

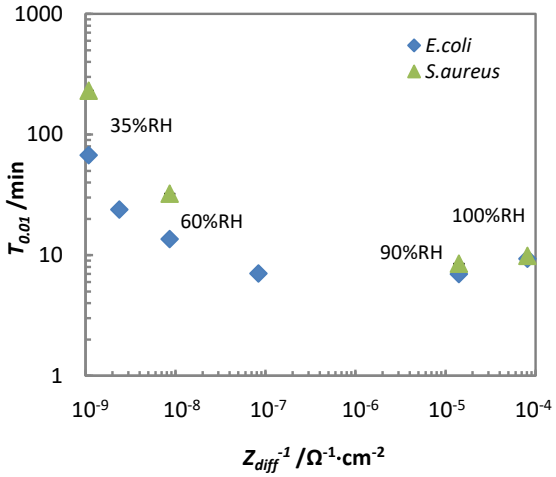

Fig. 7 Correlation of antibacterial activity ($T_{0.01}$) and corrosion parameter ($1/Z_{diff}$) of C1020 under controlled humidity. (online color)

4. Discussion

The effect of a water content in media on the bacterial growth had been studied and reviewed by microbiologists. A water activity (a_w) is a parameter to indicate the ratio of free water in the total water content of food or cultural media. The reduction in a_w influences bacterial growth via increase in lag phase and decrease in growth rate. The maximum growth rate of bacteria is generally observed between a_w 0.990~0.995 [28]. The minimum a_w for growth depends on the type

of bacteria; 0.950 for the gram-negative bacteria *E. coli* whereas a relatively low value of 0.860 for the gram-positive bacteria *S. aureus* [28]. Based on these data, the reduction in humidity is expected to assist the antibacterial activity of chemicals/materials, more effectively for *E. coli* than *S. aureus*, since low humidity itself suppresses the bacterial growth.

In the present study, the bacteria suspension was applied as a 1 μL portion spreading over about 1 cm^2 of the specimen surface with 5 min of drying time at an ambient temperature and humidity (~24°C and 45–50%RH), simulating the droplet transmission of pathogens. After the drying time, the specimens were incubated under controlled humidity for a certain period of time before the quantification of survived bacteria. As summarized in Table 2, $T_{0.01}$, the time to reduce the viable bacteria to 1/100 of the inoculated ones (which is equivalent to $\log_{10}$ reduction of 2) increased with reduction in humidity. In detail, $T_{0.01}$ of C1020 for *E. coli* decreased from 9.33 to 6.96 min with reduction in humidity from a fully wet condition (film) to 90%RH, but it increased with further reduction in humidity to 35%RH. The same trend was also observed for *S. aureus*; $T_{0.01}$ of C1020 decreased from 9.86 to 8.50 min from "wet" to 90%RH, and then, increased to 230 min at 35%RH. C7060 and C7150 had the similar trend for both bacteria; the minimum $T_{0.01}$ values were observed at 60%RH. This first phase of decrease in $T_{0.01}$ with reduction in humidity may be attributed to the inhibition of bacterial growth due to the decrease in available water, as described in the previous paragraph. However, further reduction in humidity resulted in the increase in $T_{0.01}$, as a second phase. This change cannot be explained simply by the effect of a_w on bacterial growth; it may be attributed to the change in material surface by the reduction in humidity.

For the bactericidal activity of copper and its alloys known as "contact killing", their corrosion and ion release play an important role [4, 29]. Accumulation of copper inside the bacteria contacted to copper was also confirmed [20]. If the humidity influences the corrosion reaction of copper, i.e. ion release, it should result in change of copper bactericidal activity. However, it is hard to measure the corrosion rate or ion release of the copper contacting to a small amount of bacterial suspension. As described above, the 1 μL portion of the bacteria suspension was spread over 1 cm^2 of the specimen surface, which is calculated to form 10 nm thin layer on the specimen surface. In the present study, the corrosion rate of copper under various humidity was evaluated by the electrochemical impedance measurement with a thin electrolyte layer. This method is often employed for the evaluation of atmospheric corrosion in steel and other metals [25, 26]. As shown in Fig. 6, a corrosion rate parameter, $1/Z_{diff}$ of C1020 decreased with reduction in humidity from 100% (in 0.7 M Na_2SO_4 before drying) to 35%RH. Figure 7 summarized the correlation between the antibacterial activity ($T_{0.01}$) and corrosion rate ($1/Z_{diff}$) of C1020. When the $1/Z_{diff}$ was over 10^{-7} ($\Omega^{-1}\cdot cm^{-2}$), there are a little change in $T_{0.01}$ as ~3 min, which corresponds to the first phase mentioned earlier. In the humidity range less than 75%RH, however, $T_{0.01}$ increased more clearly with decrease in $1/Z_{diff}$, which corresponds to the second phase. The first phase can be described as the range where the corrosion rate of C1020 is high enough to have efficient bactericidal action against bacteria, resulting in less difference in $T_{0.01}$, or even in slight decrease of it from the "wet" condition to 90%RH probably due to the a_w effect on the bacterial growth. In the second phase, the corrosion rate of C1020 is lower than the required level to have the efficient antibacterial activity, resulting in the increase in $T_{0.01}$ with decrease in the corrosion rate. This suggests the decrease in the corrosion rate of C1020 has a greater effect on the bacterial growth than a_w, the latter only inhibits bacterial growth but not bactericidal. Nevertheless, the a_w still has visible influence in the second phase; the $T_{0.01}$ of C1020 showed more remarkable increase for *S. aureus* than for *E. coli*, where the former has the relatively low minimum a_w for growth, indicating its relatively high tolerance for low humidity environment. The Fig. 8 schematically illustrates the influence of the humidity on antibacterial activity of copper surface. At high humidity, the copper releases enough ions to kill the bacteria on its surface, but at low humidity, the amount of released ions decreased, resulting in reduction of its antibacterial activity. However, the thinner electrolyte layer formed on copper surfaces at low humidity limits available free water for bacterial growth. Therefore, the survival ratio of the bacteria on copper surface is the combination of the damage by released copper ions and low a_w. In other words, the bacteria having higher tolerance for low humidity environment result in more survival on copper surface at low humidity.

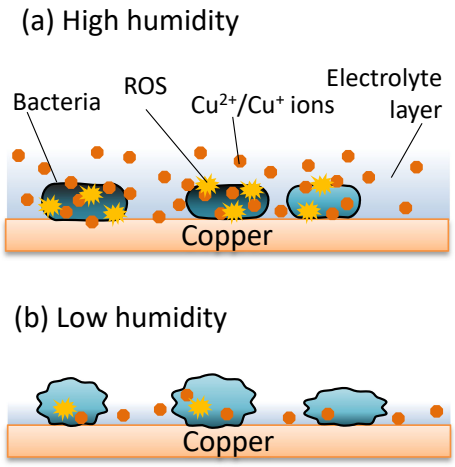

Fig. 8 Schematic explanation of the bacterial damage on copper surface at (a) high and (b) low humidity. ROS: reactive oxygen species. At high humidity, the corrosion rate of copper is high, releasing many ions giving the damages on bacteria. At low humidity, the corrosion rate of the copper is low, but low availability of water suppresses the bacterial growth. (online color)

As is discussed by other researchers, the inoculation methods (and their environmental conditions) are also influential for evaluating the copper antibacterial activity on low humidity environment. Several researchers performed aerosol inoculation to mimic the airborne transmission. McDonald *et al.* reported that the inoculation by nebulization takes longer time as 30 min, giving some damages on bacterial cells during nebulization [30]. At the end of nebulization period, the number of bacteria on copper surface reduces to 1/10 of that on stainless steel surface [30]. Even

so, the reduction rate in the survived bacteria on copper surface was much less by nebulization than by a droplet application with a 1 μL portion of bacteria suspension under the incubation condition of 20°C and 40%RH [30]. This may suggest that the nebulization allowed less water contact to the copper surface, resulting in less corrosion and ion release. Interestingly, the difference on antibacterial activity between the nebulization and a droplet application was almost negligible for stainless steel [30], that also suggests the importance of copper ion release under low humidity condition.

Even by the nebulization method, the lower humidity during the incubation after nebulization reduced antibacterial activity of copper and its alloys [16], which agrees with the results of the present study, indicating the criticalness of corrosion on the antibacterial activity of the copper. As described in Table 2, the influence of the humidity on antibacterial activity ($T_{0.01}$) depends on the types of alloys. In Fig. 5, $T_{0.01}$ was plotted against the copper content in the testing materials. For *E. coli*, the values of $T_{0.01}$ had the minimum peak at copper content of 90% (C7060) whereas those for *S. aureus* had the minimum peak at 70% (C7150). After the minimum peaks, the decrease in copper content increased $T_{0.01}$ for both bacteria. This suggests the dependance of antibacterial activity on the copper content in the alloy especially in the lower range, agreeing with the previous study [22]. This is another evidence showing the predominant effect of released copper ions on the antibacterial activity of copper and its alloys. For the application of copper and its alloys to high touch surface in low humidity environment, it is important to select the one having the reasonably high corrosion rate for its antibacterial activity. It also suggests that the humidity control near the material surface may efficiently improve the antibacterial activity of copper and its alloys for touch surface application.

In the present study, test specimens were prepared with long exposure to ambient environment after polishing to mimic the real-life conditions as indoor touch surface. This condition predicts the formation of relatively thick oxide layer on copper surface, composed of Cu_2O and CuO [31]. Further exposure to the droplet of bacterial suspension and low or high humidity environment may influence the composition of the oxide layer. For further elucidation of the mechanism for the influence of humidity on copper antibacterial activity, it is mandatory to perform surface analysis of the copper surface to clarify the change in oxide layer composition, which can be planned as our future study.

5. Conclusion

The effect of humidity on the antibacterial activity of copper and its alloys was systematically investigated by the inoculation method simulating the droplet transmission. The study found that the antibacterial activity of copper increased by the slight reduction in humidity, and thereafter, it decreased with further reduction in humidity. The retardation in copper corrosion in low humidity environment is a predominant cause of the decrease in its antibacterial activity than the reduction in water activity, a_w. *S. aureus*, which has a lower minimum a_w for growth than *E. coli*, has relatively high survival ratio on the copper surface at low humidity environment than *E. coli*.

All the tested materials, including copper containing ABSS decreased their antibacterial activity with reduction in humidity. Even in low humidity environment, the alloys with higher copper contents had the higher antibacterial activity than those with low copper contents. For successful application to high-touch surface, copper alloys with high copper content with reasonably high corrosion rate in low humidity environment should be selected.

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