Antibacterial Properties of Metallic Elements for Alloying Evaluated with Application of JIS Z 2801:2000

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Antibacterial properties of 21 metallic elements used as alloying elements (Al, Si, Ti, V, Cr, Mn, Co, Ni, Cu, Zn, Zr, Nb, Mo, Pd, Ag, Sn, Ta, W, Pb, Au, Pt) was studied. Escherichia coli (Gram-negative bacteria) and Staphylococcus aureus subsp. aureus (Gram-positive bacteria) were used as model bacteria. The film attachment method was adopted for evaluation of antibacterial activity, and Japan industrial standard Z 2801:2000 was applied as the criterion for evaluate antimicrobial abilities of samples. Silver and Cu showed strong bactericidal effects as expected, and following them Co, Ni and Al were moderately toxic. Physiological effects of metals depended on the species of bacteria. For example, Ni decreased the total viable count of E. coli to 10 cfu/mL in 4 h, while it took 24 h to decrease the total viable count of S. aureus. By judgment following to JIS Z 2801:2000, Pt and Pb were effective only for E. coli, while V and Zr were only for S. aureus. Gold was not toxic even though Au3+ had been reported as strong toxic. Also Mo showed antibacterial effects which can be resulting from decrease in pH of the bacteria suspension.

KEY WORDS: metallic elements; bacteria; bactericide; film attachment method; JIS Z 2801:2000.

1. Introduction

Most engineering metals are alloy of several elements. The objectives of alloying are mainly to improve mechanical properties, electric properties, work ability and corrosion resistance. There is a few alloys aiming to increase antibacterial properties.1–3)

Recently, various disasters induced by bacteria have been reported. In a hospital, for example, hand-washing solution storage tanks were contaminated by bacteria forming biofilms, and the contamination resulted in hospital infection.4) Koening and Pierson5) reported contamination and biofilm formation in water systems in the Space Shuttle. Contamination was also found in nuclear waste repositories.6,7) For materials scientists and engineers, corrosion influenced by microorganisms are known as MIC (Microbially Influenced Corrosion or Microbially Induced Corrosion). Sulfate-reducing bacteria, sulfur-oxidizing bacteria and manganese-bacteria are well known bacteria associated with MIC.8) Microbially influenced corrosion is also induced by biofilms which consist of not only these bacteria but also popular non-specific common bacteria. The surfaces of substrates over which biofilms formed are anaerobic and surrounding areas are aerobic; they work cathodic and anodic sites respectively, constructing differential aeration cells and inducing corrosion. Annual loss resulting from MIC in the United States only was estimated several billion USD,9,10) and the amount of the loss presumably increases. Case studies,11,12) review articles9,13–19) and a textbook20) on deterioration influenced by microorganisms can be found elsewhere.

Biofilm is a consortium of several kind of bacteria.20) Prior to bacteria adhesion, the formation of a conditioning film, which consists of organic and inorganic substances, is necessary to be formed on a target surface. After formation of the conditioning films, bacteria attach over the conditioning films and then attached bacteria proliferate, forming biofilms. Environmental factors in biofilms, such as oxygen concentration and potential, are dynamic in space and in time. Such biofilms were well formed on a surface of SUS 316 stainless steels immersed in a sample solution containing sulfate-reducing bacteria for 15 d, and pitting corrosion were formed on the surface after 40 d, whereas Ag alloyed antibacterial stainless steel reduced formation of biofilms.21) More case studies on MIC were described by Ito.22) As the corrosion form is the same as general crevice corrosion or pitting corrosion, it is difficult to conclude a particular case is MIC, and microorganisms should be considered as one of environmental factors inducing corrosion, as like temperature, chloride ions and pH.23) Also it should be worth noting that the skeleton structure of MIC sites, which is thought to be a unique finger print of MIC, can be resulted from corrosion by ferric chloride aqueous solution.24)

As sessile bacteria is more tolerant against biocide in comparison with bacteria in a planktonic form, biocide is less effective for well formed biofilms, and it is, therefore, important to prevent the formation of biofilms and conditioning films which induce bacteria adhesion. Science in cleaning biofilm formed surfaces were described in a reference25) and an example of toxic metal applications looking for such functions is antibacterial eco-plating based on
HACCP (HACCP: Hazard Analysis and Critical Control Point). 26

Biofilms develop on all surfaces in contact with aqueous environment. It is hardly possible to completely remove them from surfaces, and therefore one of the effective measures to protect materials against MIC is to prevent adhesion and growth of attached bacteria. Several organic biocides have been used to control the growth of bacteria. One of disadvantages regarding organic biocides usage is the impacts of flush containing the biocide upon environment. It is, therefore, strongly desired antibacterial steels to be developed in order to decrease environmental pollution. 27

Some metals, such as Ag, is toxic to lower organisms but not for human; 28 if it is in low concentrations. Another advantages of inorganic bactericide comparing to organic ones are higher in heat stability and durability, and the less appearance of resistant bacteria.

Most metals used in industries are alloy of several elements. For example, stainless steels contain Cr, Ni, Mo, Cu etc. in order to improve corrosion resistance, strength and stiffness. There might be alloying elements which function as an antibacterial agent. Such elements can be used to develop antimicrobial steels with slight modification of current products. For example Cu or Ag alloyed antibacterial steels are already under production, 1,2 and the effects of Ag alloying was already described above. Molybdenum, which was alloyed in type 316 and 316L stainless steels in order to increase toughness, inhibits growth of biofilm on stainless steels. 29 If Mo is precipitated there surfaces by any methods, including heat treatments, surface conditioning, and/or increase Mo contents, such stainless steels can have the bactericidal function. Moreover, combination of different toxic metals can increase there bactericidal effects effectively. 30

The biofilms found in the water systems of the Space Shuttle did not result in microbiologically influenced corrosion. However, the existence of bacteria and biofilm in the water systems is a potential cause of corrosion and disaster of materials. These problems will be more serious in water systems in the International Space Station. Antimicrobial alloys are potential materials for applications such as a water system in the ISS. In order to develop antibacterial alloys, it is necessary to collect data on antibacterial properties of elements. There exists, however, few research on effects of metallic elements on bacteria carried out from a metallurgical stand point, and as mentioned by R. B. Thurman and C. P. Gerbe 31 it is difficult to compare the results of one study with those of another since the conditions under which the respective studies were conducted are seldom the same. In this study, bactericidal activity of 21 metallic elements having engineering importance were examined with application of JIS Z 2801. 32 Such data should be useful for the development of antibacterial alloys.

2. Samples and Experimental

2.1. Samples

2.1.1. Materials

The purity of materials subjected to experiments are summarized in Table 1. The values in the table are obtained from manufactures. All materials are purchased and used without further purification.

Iron was excluded in this study because surface conditions of iron, such as a passive layer, are very sensitive to environmental factors and change significantly with time; less repeatability in experimental results and therefore it is difficult to examine bactericidal properties.

A 25 mm × 25 mm sample coupon was embedded in an unsaturated polyester resin (Fig. 1). Bactericidal properties of the resin had been examined and was clarified to be negligible. A surface of the sample coupon was polished under dry condition using #2000 grade emery papers, and 70% ethyl alcohol aqueous solution was used to clean and sterilize the polished surface. The specimen was, then, dried and further sterilized under ultraviolet radiation in a clean bench for more than 20 min.

For molybdenum, we obtained only flakes in shape. To make specimens, therefore, the flakes were placed on the top of the resin so that the total amount of surface area of Mo flakes was about 625 mm², which is equal to the surface area of another samples. Except flakes were used, another procedure to prepare specimens were the same as that of another sample materials.

Poly-ethylene films (PE films) were cleaned and sterilized in the same manner as the specimens.

Table 1. Sample metals and their purity (purity in mass%).

| Element | Al | Si | Ti | V | Cr | Mn |
|---------|----|----|----|---|----|----|
| purity% | 99.9 | 99.9 | 99.5 | 99.7 | 99.9 | 99.9 |

| Element | Co | Ni | Cu | Zn | Zr | Nb |
|---------|----|----|----|----|----|----|
| purity% | 99.9 | 99.7 | 99.9 | 99.6 | 99.2 | 99.9 |

| Element | Mo | Pd | Pt | Ag | Sn | Te |
|---------|----|----|----|----|----|----|
| purity% | 99.9 | 99.9 | 99.9 | 99.9 | 99.9 | 99.9 |

| Element | W | Pt | Au | Cu |
|---------|---|----|----|----|
| purity% | 99.9 | 99.7 | 99.9 |

Fig. 1. Specimens. (a) A Cr coupon embedded in a resin, (b) Mo flakes placed on the surface of a resin.

2.1.2. Bacteria

Bacteria can be divided in two groups, i.e. Gram-positive and Gram-negative, and the major difference between them is the structure of the cell membrane: Gram-negative bacteria have an outer membrane and lipopolysaccharide which is negatively charged. Such differences in cell membrane structure can result in difference in sensitivity of bacteria to toxic materials. 33-36 Model bacteria used in this study were, therefore, Escherichia coli NBRC 3972 (E. coli) and Staphylococcus aureus subsp. aureus NBRC 12732 (S. aureus); the former is Gram-negative bacteria and the latter is Gram-positive bacteria. They were purchased from National Institute of Technology and Evaluation, Japan.
2.2. Experimental

A platinum loop of sample bacteria was suspended in nutrient broth cultivate medium (NB medium) of 200 mL and was incubated with shaking at 180 rpm at 35 ± 1°C for 18 h. After the cultivation, the suspension was diluted with adequate amount of sterilized water to obtain a sample suspension whose bacteria concentration was in the range of 1.0×10^5–1.0×10^6 cfu/mL (colony forming unit per milliliter).

The film attachment method was adopted for evaluation of the antibacterial properties of specimens. A sample suspension of 0.050 mL was dropped on the metal surface of a specimen, which was horizontally placed on a sterilized Petri dish, and the droplet was covered with a sterilized PE film to prevent the suspension drying up during following process (Fig. 2).

The specimens were placed in an incubator and bacteria were incubated for several period up to 24 h under the environment of temperature at 35 ± 1°C and relative humidity of 90%. After the incubation, the surface of the specimen and the PE film were rinsed with Soybean-Casein Digest broth with Lecithin and Polysorbate 80 (SCDLP) medium of 3.0 mL and 2.0 mL respectively to harvest bacteria exposed to the sample metal. To count the total viable count, a plate counting technique using standard agar medium (SA medium) was adapted. The total viable count was set to 10 cfu/mL in the case that the number of colony was less than 10.32)

In order to obtain reference data, a sample suspension of 0.050 mL was dropped on a sterilized Petri dish, and the droplet was covered with a sterilized PE film. Such a specimen is designated as control. For control specimens, the following processes, i.e. incubation, harvest and counting the total viable count, were the same as those for the metal specimens.

Mediums used in this study were summarized in Table 2. The nutrient broth, yeast extract and tripton were manufactured by Decton, Dickinson and Company, USA. The glucose and agar were purchased from Wako Pure Chemical Industries, Ltd., Japan, and the SCDLP was from Nihon Pharmaceutical Co., Ltd., Japan. All chemicals were used without further purification.

3. Results and Discussions

For all samples except Mo, the value of pH was in the range of 6.0 to 7.5 over an experiment. For Mo, the pH value decreased with time and reached 4.7 at 24 h after inoculation.

Experimental results for Ag, which is known by its strong bactericidal effect, are shown in Fig. 3. Total viable count, which was about 2.0×10^5 cfu/mL at the time of inoculation, decreased to 1.0×10^1 cfu/mL in less than 1 h, indicating strong bactericidal activities of Ag, as expected.

Physiological effects of metals depend on the specie of subjected bacteria. Experimental results for Ni were shown in Fig. 4 as an example showing bacteria specie dependent physiological properties of metals. In the case that E. coli was exposed to Ni, the total viable count decreased to 1.0×10^1 cfu/mL in 4 h, while it took 24 h to fall into 1.0×10^1 cfu/mL in the case of S. aureus.

The major object of this study is to evaluate antibacterial activity of metals with application of JIS Z 2081. In JIS Z 2801, the value of antimicrobial activity is defined as the following equation:

\[
R = \log \left( \frac{B}{A} \right) - \log \left( \frac{C}{A} \right) = \log \left( \frac{B}{C} \right) \tag{1}
\]

Table 2. Compositions of mediums and buffer.

| Constituents          | pH      |
|----------------------|---------|
| Nutrient Broth (NB)  | 8.00 g  |
| Nutrient broth       | 1000 ml |
| Distilled water      | 8.04 ± 0.2 |
| SCDLP                | 38.0 g  |
| SCDLP                 | 1000 ml |
| Distilled water      | 7.04 ± 0.2 |
| Standard Agar (SA)   | 1.25 g  |
| Yeast extract        | 7.12 ± 0.1 |
| Tryptone              | 2.50 g  |
| Glucose               | 0.50 g  |
| Agar                  | 7.50 g  |
| Distilled water      | 5000 ml |

SCDLP stands for Soybean-Casein Digest broth with Lecithin and Polysorbate 80.
where 

\[ A: \text{the average value of the number of active bacteria exposed to a control immediately after inoculation} \]

\[ B: \text{the average value of the number of active bacteria exposed to the control for 24 h} \]

\[ C: \text{the average value of the number of active bacteria exposed to a specimen for 24 h} \]

and the antibacterial activity of the specimen is positive if the \( R \) value is equal to or larger than 2.0, i.e. the specimen decreased the number of active bacteria more than two factors of 10 compared to the control in 24 h. The results are summarized in Table 3 and shown in Fig. 5. From the experimental results, the samples can be classified into four groups depending on their antibacterial properties:

+ Al, Co, Ni, Cu, Zn, Mo, Pd, Ag and W
+ only for \( E. \ coli \): Pt and Pb
+ only for \( S. \ aureus \): V and Zr
+ −: Si, Ti, Cr, Mn, Nb, Sn, Ta and Au

The range of pH in which \( E. \ coli \) can initiate growing is from 4.4 to 9.0, and that for \( S. \ aureus \) is from 4.0 to 9.8. For all samples except Mo, the value of pH was in the range of 6.0 to 7.5 over an experiment: such an environment was normal for these bacteria and, therefore, the change in pH value during experiments was not major cause of the decrease in the total viable count. For Mo, the pH value decreased with time and reached 4.7 at 24 h after inoculation. As such an acid environment is harmful for both \( E. \ coli \) and \( S. \ aureus \), the antibacterial activity of Mo might be resulting from the decrease in pH of microorganism suspensions.

In JIS Z 2801, the bactericidal activity is measured by the \( R \) value, which is measured at 24 h after inoculation, and the bactericidal quickness of the samples is, therefore, not taken into account in Fig. 5. For example, the total viable count of \( S. \ aureus \) exposed to Ag decreased to 10 cfu/mL in less than one hour, while it took almost 24 h when Ni was subjected to the experiments. The \( R \) values of these two samples were however the same, because only the total viable count at 24 h after inoculation were used to calculation and their references, i.e. controls, were the same. In order to compare biocidal efficiency of the samples, we calculated the death rate constant \( k \) by fitting following equation to experimental results.

\[
\frac{dn(t)}{dt} = k \cdot n(t) \quad \text{................................(2)}
\]

where \( n(t) \) is the total viable count at time \( t \) (\( t \) in hour). Experimental results used for evaluation of the \( k \) values were obtained from the time of inoculation to the time at which the total viable count decreased to 10 cfu/mL.

It should be mentioned that we don’t insist on the first order kinetic of the bactericidal activity of solid metals by using the equation. The major mechanism of bactericidal activity of metallic elements is resulting from chemical attacks of metal ions or metal ions in complex forms and must be rather complicated processes. In our experiment, the origin of metal ions is the sample plates. As dissolve processes of metallic ions from solid metals are highly complex processes, including formation of a passivation layer and they are affected by environmental factors, it is hardly possible to take into consideration the change in
cation concentration distribution in the microorganisms suspension resulting from such dissolve processes. We therefore used the simple equation and the purpose of using such a simple equation is only to make the comparison.

The value of the death rate constant $k$ for the samples are presented in Table 4 and shown in Figs. 6 and 7. The $k$ values for Ag and Cu were significantly higher than those values of other samples, indicating strong biocidal activities of these metals. Following them, Co, Ni and Al were moderately toxic, and Mo, Pd, Zn, W and Pb were weak toxic. Most of these elements have been used as alloying elements and, hence, they are potential alloying elements for antibacterial alloys.

The effects of the outer membrane and the lipopolysaccharide of Gram-negative bacteria on bactericidal activity of metal ions are not clear. Gram-negative bacteria are said to be more resistant to bactericide than Gram-positive bacteria are, but such a tendency has a lot of exceptions depending on groups of bacteria and types of bactericide.39) For example, there exists experimental results indicating that the outer membrane defends bacteria against toxic materials and Gram-negative bacteria are, therefore, more tolerant to bactericidal catechin than Gram-positive bacteria are,26 whereas the negatively charged lipopolysaccharide, which exists outside of the outer membrane, attracts catechine–copper(II) complexes,33 indicating the possibility that the Gram-negative bacteria may be less tolerant to metal ions compared with Gram-positive bacteria. In our experimental results (Figs. 6 and 7), using solid metals as bactericide, only a little and non-systematic differences were found in the bactericidal activity of metal ions between *E. coli* (Gram-negative) and *S. aureus* (Gram-positive), and our data is not enough to advance discussion.

The minimal inhibitory concentration of Au$^{3+}$ was about the same as Ag$^{+}$,38 indicating Au$^{3+}$ was rather toxic. In our experimental results, on the other hand, the bactericidal activity of solid Au was far less than that of Ag. Gold, which is the most noble metals on the earth, hardly dissolves in water, and hence antimicrobial activity was not observed in our experiment.

Miyano *et al.*40 examined antibacterial properties of solid metals (Zn, Ni, Pb, Sn, Ti, Co, Zr, Mo and Cu) against *E. coli* and *S. aureus*, employing the same protocol as that used in this study. Their experimental results are similar to our results, but some exceptions. In the Ref. 40), Ti and Zr were classified as antibacterial substances against *E. coli*. Contrary to the results in the reference, Ti and Zr were not classified as antibacterial substances against *E. coli* in this study. Yet, the $R$ values for Ti and Zr against *E. coli* obtained in this study were 1.91 and 1.26 respectively, close to 2.0 indicating these substances were weakly toxic. The difference between the reference and our results are, therefore, not significant.

Also in the Ref. 40), Pb showed strong bactericidal activity against *S. aureus*, and the value of pH of the sample suspension increased to 12 in 24 h. The authors of the reference attributed the strong bactericidal activity of Pb to the increase in pH value. In our experimental results, on the other hand, Pb showed weak antibacterial activity against *S. aureus*, and the range of pH value was from 7.0 to 8.0 over experiments, which is normal environment for *S. aureus*. These results indicate that antibacterial property of metal substances is affected by the value of pH, or metal substances bias there antibacterial properties by changing the pH value of there suspensions.

**4. Conclusions**

Bactericidal effects of solid metals were examined with application of JIS Z 2801 and the results are as the following:

- +: Al, Co, Ni, Cu, Zn, Mo, Pd, Ag and W
- **only for E. coli**: Pt and Pb
- **only for S. aureus**: V and Zr
- −: Si, Ti Cr, Mn, Nb, Sn, Ta and Au

Silver and Cu were strong biocide and Al, Co, Ni were moderately toxic. Following them are Mo, Pd, Zn, W and Pb. The antimicrobial effects of Mo might be archived by reducing pH.

![Table 4. Death rate constants $k$ of metals against *E. coli* and *S. aureus*.](image)

| Element | Al | Si | Ti | V |
|---------|----|----|----|---|
| *E. coli* | 1.23 | -0.25 | 0.08 | -0.06 |
| *S. aureus* | 1.19 | 0.02 | 0.13 | 0.38 |
| Cr | Mn | Co | Ni | Cu | Zn |
| -0.04 | 0.04 | 2.81 | 2.77 | 13.57 | 0.53 |
| 0.08 | 0.14 | 2.49 | 0.54 | 12.51 | 0.53 |
| Zr | Nb | Mo | Pd | Ag | Sn |
| 0.01 | -0.08 | 0.64 | 1.18 | 11.91 | -0.04 |
| 0.38 | -0.09 | 0.59 | 0.34 | 11.66 | 0.13 |
| Ta | W | Pt | Au | Pb |
| -0.12 | 0.31 | 0.17 | -0.07 | 0.38 |
| 0.15 | 0.30 | 0.11 | 0.09 | 0.15 |

![Fig. 6. Death rate constants for *E. coli*. Elements not tested are hashed.](image)

![Fig. 7. Death rate constants for *S. aureus*. Elements not tested are hashed.](image)
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