Antibacterial Mechanism of Copper-bearing Antibacterial Stainless Steel against E.Coli

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A preliminary study was made on the antibacterial mechanism of copper-bearing antibacterial stainless steels against E.coli through experiments of microbiology such as EDTA (ethylenediaminetetraacetic acid) complexing, DNA smearing and AFM (atomic force microscope) observation. It was measured that the antibacterial stainless steels showed excellent antibacterial functions with antibacterial rate to E.coli over 99.99%. The antibacterial rate was weak if the bacteria solution was complexed by EDTA, indicating that the copper ions play a dominant role in the antibacterial effect of the antibacterial stainless steels. The electrophoresis experiment did not show the phenomenon of DNA smearing for E.coli after contacting antibacterial stainless steels, which meant that DNA of E.coli was not obviously damaged. It was observed by AFM that the morphology of E.coli changed a lot after contacting antibacterial stainless steels, such as cell walls being seriously changed and lots of contents in the cells being leaked.

KEY WORDS: Copper; Stainless steel; E.coli; Antibacterial mechanism

1. Introduction

With sustainable improvement of the people’s living level, public awareness on safety during food and medicine processing has been rapidly raised. In this case, many products with antibacterial functions, such as sanitation fixtures, plastic products and articles of clothing, have been developed in various fields. However, antibacterial mechanisms of antibacterial products are rather complex. At present, though some research has been made on the antibacterial mechanism, few results have been acquired to reveal the process of inhibiting and killing bacteria. As to the antibacterial mechanism of metal ions, some possible antibacterial mechanisms have been put forward. For instance, bacteria are killed by metal ions through absorption in the electric field, or through a catalysis process, or through damaging the enzymatic system that affects the normal metabolism and so on.

Copper-bearing antibacterial stainless steel is a novel structural and functional material, which not only keeps the particular properties of metallic structural materials, such as high strength and toughness, conductivity, high temperature resistance, workability, but also possesses a new antibacterial function. However, there was almost no detailed work made on the antibacterial mechanism of such novel steel. Based on the previous research, this work conducted a preliminary investigation on the antibacterial mechanism of antibacterial stainless steel against E.coli by using some research methods in microbiology, and some instructive results were obtained.

2 Experimental

2.1 Materials

In this work, the antibacterial stainless steels included a copper-bearing austenitic antibacterial stainless steel (0Cr18Ni9–3.8 wt pct Cu) and a copper-bearing ferritic antibacterial stainless steel (0Cr17–1.8 wt pct Cu). The contrast steels were 0Cr18Ni9 and 0Cr17, respectively. Experimental samples were cut into slices (40 mm × 40 mm × 1 mm). Bacterium for the experiment, Escherichia coli (E.coli) ATCC25922, was supplied by a preserving center for bacterium in China.

2.2 Experimental procedures

2.2.1 Antibacterial activity test

The film attachment method was adopted for the antibacterial test, which is one of the most commonly used testing methods for antibacterial properties measurement for solid materials, quantitatively testing the antibacterial rate of the material.

Antibacterial tests were conducted with standard Gram-negative bacteria, E.coli ATCC25922. The broth for culturing was made up by dissolving 5.0 g flesh extract, 5.0 g NaCl and 10.0 g peptone into 1000 ml of distilled water and its pH was adjusted to 7.0–7.2. The culturing solution containing the bacteria was diluted to 10⁶ cfu/ml (colony forming units/ml). 0.3 ml of this solution was homogeneously added dropwise by a dispenser onto the surface of samples, which had been horizontally placed into a sterilized Petri-dish, and then covered with sterile plastic films. The bacteria on samples were incubated at 37°C for 24 h in an incubator. After the incubation, the bacterial solution on the sample surface was diluted with 15 ml of phosphate buffer solution, and 0.1 ml of the bacterial solution was added into a 9 cm diameter Petri-dish.

The antibacterial effect was recognized by the antibacterial rate, which was calculated as below:

\[
\text{Antibacterial rate (\%) = 100 \times \frac{(A - B)}{A} }
\]

where \(A\) is the number of bacteria colonies in the Petri-dish for the contrast stainless steel acting with...
**E. coli**, and *B* is the number of bacteria colonies for the antibacterial stainless steel acting with **E. coli**.

2.2.2 EDTA complexing test EDTA, *i.e.*, ethylenediaminetetraacetic acid, has the effect on complexing metal cations, especially on the bivalent cations, usually used for complexing titration of bivalent cations. Enlightened from this function, by addition of proper amount of EDTA into the bacterial solution before covering the sterile plastic films, the antibacterial rate should decline, if antibacterial effect is due to the action of copper ions dissolved from the steel, which should loss the role of killing bacteria after complexing by EDTA.

In the experiment of film attachment mentioned above, 1 mmol/L EDTA was added into the 10⁶ cfu/ml **E. coli** solution, and then 0.3 ml of intermixture was homogeneously dripped on the samples, incubating at 37°C for 24 h in the incubator. After the incubation, the bacteria solution on the sample surface was diluted with 15 ml of phosphate buffer solution, and gradually diluted to 10² cfu/ml in certain gradience. Finally, the number of colonies was figured out.

2.2.3 DNA smearing test If the DNA, which is the vital substance for bacteria, cannot be restored after smearing, bacteria will die. According to some correlative data reports, antibacterial samples can act with protein of the cell[15], leading to the DNA smearing effect, thus resulting in death of bacteria. In order to prove whether the antibacterial stainless steel has such mechanism of killing bacteria, the DNA smearing test was designed as follows.

Firstly, some **E. coli** solution was gotten by centrifugation at low temperature (8–10°C, 12000 r/min, 15 min), and then the clear solution was discarded and 580 µl TE buffer (10 mmol/L tris, 1 mmol/L EDTA) was added into the sediment, which was beaten repeatedly and made into suspending. Secondly, 30 µl of 10% SDS was mixed with 3 µl of 20 mg/ml protein enzyme, preserved at 37°C in a water boiler for 1 h, and then 100 µl of 5 mol/L NaCl and 80 µl of CTAB/NaCl were added, which was well mixed and preserved at 60°C for 10 min. Thirdly, the same volume chloroform and isopentanol were added, which was separated by centrifugation at speed of 6000 r/min for 5 min. Then the supernatant liquid was taken into another new tube, and the step was repeated again. Subsequently, 0.6 diploid isopropyl alcohol was added and they were dispersed for 2 min by centrifugation (10000 r/min, 2 min). Then 1 ml of 70% ice ethanol (8000 r/min, 2 min) was added, and the clear solution was discarded, deposited and cooled. Finally, 30 µl TE buffer was added in the compound.

In the electrophoresis experiment, TBE buffer (890 mmol/L tris, 89 mmol/L boric acid, 2 mmol/L EDTA, pH8.0) was prepared at first, and then 4 µl treated samples was mixed with 2 µl buffer (every sample was repeated once) in the dotting hole. At the same time, 4 µl marker was dripped in as the standard molecule. Finally, in the electrophoresis for 3 h at a voltage of 80 V (DYY-6B Electrophoresis), pictures could be observed and taken in gel imaging present (BIO-RAD).

2.2.4 Atomic force microscope observation The observation of **E. coli** under atomic force microscope (AFM) was conducted on both copper-bearing ferritic antibacterial stainless steel and the contrast steel acted with the bacteria in order to see the morphology change of bacteria on the surfaces of both steels. The samples were prepared by surface grinding on different SiC sand papers until the 2000th grade on a pre-milling machine, and then surface polishing with different diamond polishing pastes (2.5, 1.5 and 0.5 µm). The film attachment method was still adopted by the bacteria incubation on the surface of samples. The observation was carried out on a Nanoscope (R) III Bioscope AFM (Digital Instruments, Veeco Metrology Group CA, USA).

3. Results

3.1 Antibacterial rates

Table 1 shows the antibacterial rate of the copper-bearing antibacterial stainless steels. The results reveal that certain amount of addition of Cu in stainless steels provides excellent antibacterial properties for the steels. Figure 1 presents the breeding status of **E. coli** after actions with antibacterial stainless steels and the contrast stainless steel. It can be clearly seen that after 24 h cultivation of **E. coli** contacting different steels, there was almost no bacteria in the dish where bacteria acted with antibacterial stainless steels, but large amount of bacterial in the dish where bacteria acted with the contrast stainless steel. This indicates that the bacteria cultivated on the surface of antibacterial stainless steels have almost been extinguished.

3.2 EDTA complexing

The EDTA complexing experimental results are shown in Fig.2 and Table 2. It can be seen that after 1 mmol/L EDTA was added into the bacteria solution, the antibacterial rate only reached less than 50% and lots of bacteria still existed in the dish where they contacted the ferritic antibacterial stainless steel. However, when EDTA was not added into the solution, the antibacterial rate reached above 99.99%.

3.3 DNA smearing effect

The DNA image shown in Fig.3 displays that the DNA is intact. There is no dispersed distribution from the front of electrophoresis strip for the DNA of **E. coli** acted with both the contrast stainless steel and the antibacterial stainless steels, indicating that the way that antibacterial stainless steel leads to death of bacteria is not through damaging the DNA.

3.4 AFM observation

The morphology changes of **E. coli** acted with ferritic antibacterial stainless steel and contrast stainless steel for 24 h were observed under AFM, as shown in Fig.4. The normal **E. coli** should look short-stick and edge-trim, and the cell wall is compacted and intact. After acting with ferritic antibacterial stainless steel for 24 h, it can be seen from Fig.4(a) that the cell walls of **E. coli** were changed too much and lots of contents in the cell were leaked out. Following that, the whole cells turned thin and shriveled, which should lead to death of **E. coli**. While acting with the contrast stainless steel, the morphology of **E. coli** was not changed very much, as shown in Fig.4(b).

4. Discussion

The results in this study show that Cu ions can kill...
Table 1: Antibacterial spectrum of antibacterial stainless steels (E. coli, 10^6 cfu/ml)

<table>
<thead>
<tr>
<th></th>
<th>Austenitic antibacterial stainless steel</th>
<th>Ferritic antibacterial stainless steel</th>
<th>Contrast stainless steel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;99.99%</td>
<td>&gt;99.99%</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig.1: Photos showing the results after action of E. coli with different steels: (a) austenitic antibacterial stainless steel, (b) ferritic antibacterial stainless steel, (c) contrast stainless steel.

Table 2: Effect of EDTA complexing on the antibacterial rate of different samples on E. coli.

<table>
<thead>
<tr>
<th></th>
<th>Contrast steel</th>
<th>Antibacterial steel</th>
<th>Antibacterial steel+EDTA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>&gt;99.9%</td>
<td>46.9%</td>
</tr>
</tbody>
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Fig.2: Effect of EDTA complexing on action of ferritic antibacterial stainless steel with E. coli: (a) with EDTA complexing, (b) without EDTA.

bacteria by damaging their cell walls and cell membranes, because Cu-ions that have strong reduction ability can extract the electrons from bacteria, causing their cytoplasm to run off and oxidizing their cell nucleus. According to the work by Hong and Koo [16], as shown in Fig.5, the precipitation of ε-Cu phases on the surface of stainless steel enables the dissolution of Cu-ions from it, thus, coming into contact the bacteria on the surface of steel [17, 18]. EDTA complexing experimental result indicates that there should exist some metal ions that led to death of the bacteria on the surface of antibacterial stainless steels, which is different only in Cu addition compared with the contrast stainless steel. For the contrast stainless steel, with and without EDTA addition, there was almost no difference in the number of bacteria colonies. This can be explained that a certain concentration of EDTA in the bacteria solution complexed the Cu ions that dissolved from the surface of antibacterial stainless steels.

Large amount of ε-Cu precipitates on the passivated film should be contributed to producing more Cu-ions that can be dissolved on the surface of antibacterial stainless steels, resulting excellent antibacterial capability. In the present work, since the main difference between antibacterial stainless steel and the contrast stainless steel is the existence of ε-Cu phases in the steel, it should be the effect of copper ions that leads to death of the bacteria. The experimental result of EDTA complexing in this work proved the antibacterial effect of Cu ions from the surface of antibacterial stainless steels.

Recent studies indicate that metal ions play an important role in the mechanism of action, suppressing cell growth by inhibiting activity of DNA gyrase, an essential bacterial enzyme that maintains superhelical twists in DNA [19]. While in this study, the DNA smearing phenomenon of the bacteria did not appear,
Morphology changes of E.coli after action with antibacterial steel and contrast steel by AFM: (a) ferritic antibacterial steel, (b) contrast steel

5. Conclusions

(1) Copper-bearing antibacterial stainless steels have excellent antibacterial functions with antibacterial rate to E.coli over 99.99%.

(2) Copper ions play the dominant role in the antibacterial effect of antibacterial stainless steels acted with E.coli.

(3) DNA of E.coli is not obviously damaged after contacting antibacterial stainless steels.

(4) Morphology of E.coli is much changed after contacting antibacterial stainless steels, cell walls being damaged and lots of contents in the cells being leaked.

Acknowledgement

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REFERENCES